Cerebral Neurotrophins and Behavioral Aspects of a Neurodevelopmental Model of Schizophrenia

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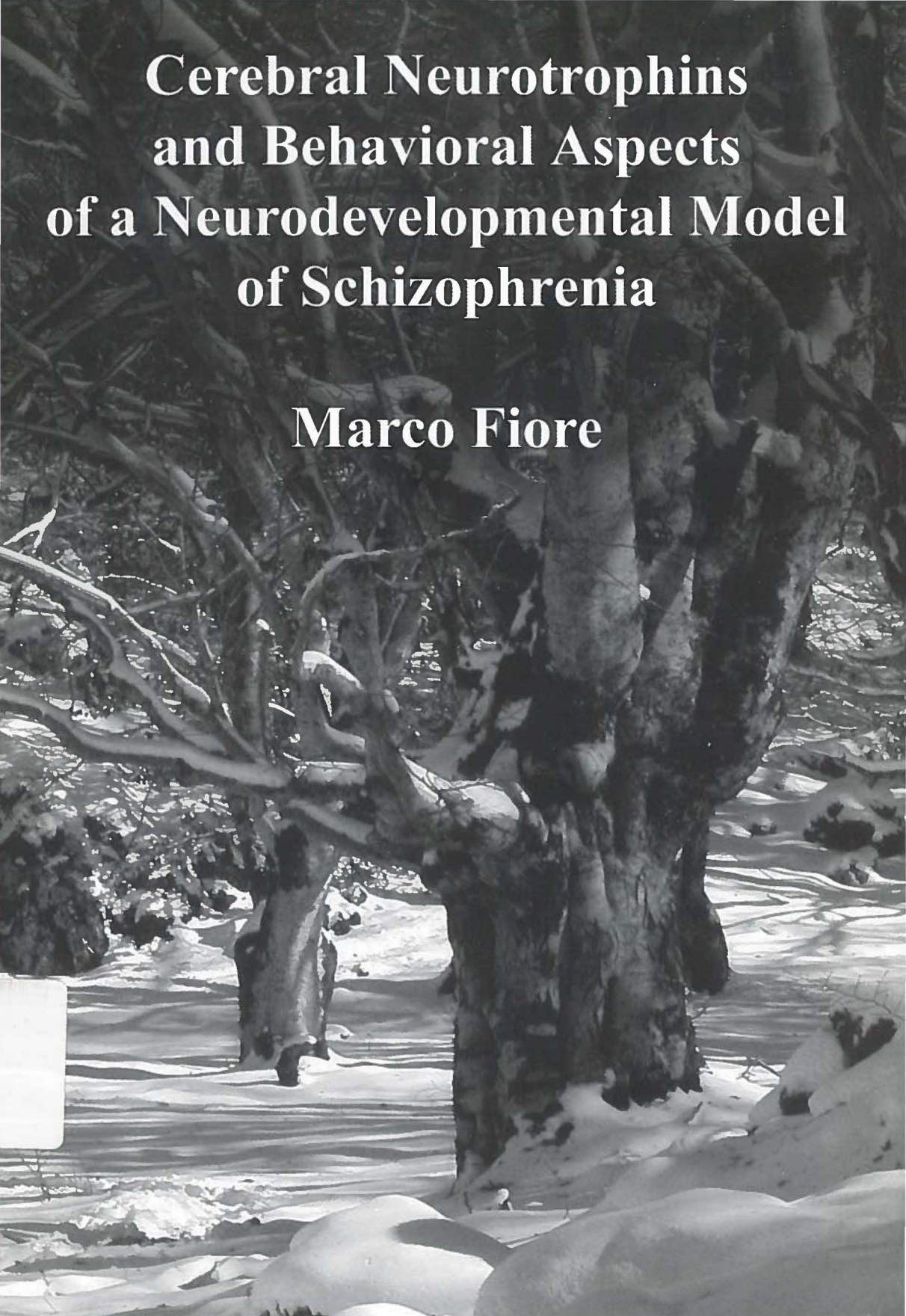

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A Vision of "Branches in the Brain"
Cover based on a photo by the author (2002)

The studies of the Present Thesis were based on the programs of the School of Behavioral and Cognitive Neurosciences, BCN, of the University of Groningen.

Stellingen

Cerebral Neurotrophins and Behavioral Aspects of a Neurodevelopmental Model of Schizophrenia

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1. The maldevelopmental model of schizophrenia postulates pathological alterations in embryonal neurogenesis involving several brain areas as the etiopathogenetic basis of schizophrenic psychosis; the neurotrophic factor hypothesis explains these changes as the result of disturbances of processes involving the trophic factors (J Neural Transm. 1998-105:85-100).

2. Effects during brain development lead to defective neural connectivity and altered biochemical functioning resulting in any dysfunction later in life, including cognition, emotions and attentional abilities.

3. Different kinds of acute and chronic stress induce changes in the content of neurotrophins in the brain.

4. Schizophrenics show alteration in the levels of neurotrophic factors in both brain and serum.

5. Social behavior and learning and memory processes are finely controlled by endogenous neurotrophins' presence.

6. The reliability of an animal model depends on the comparability with aspects of some human processes, the limit that should be taken into consideration is that animals are not humans.

7. The difference between science and opinion is that science begets knowledge whereas opinion generates ignorance (Hippocrates).

8. The primary lesion(s) in schizophrenia does not necessarily involve dopamine, glutamate, and GABA directly but could deal with a more general defect, such as a faulty connectivity of developmental origin (Annu Rev Pharmacol Toxicol. 2001-41:237-260).

9. Therapeutic efficacy of drugs blocking dopamine receptors does not prove that cerebral dopamine-containing neurons function abnormally in schizophrenia (Arch Gen Psychiatry. 2003 60:974-977).

10. The schizophrenia model proposed by Lipska and coworkers has no full heuristic values because it is based on the assumption of a discrete and single brain lesion being the main cause of the disorder (Neuropsychopharmacology. 1993-9:67-75).

11. Gene polymorphism of neurotrophins are not and cannot be associated with the vulnerability to develop schizophrenia since the brain damage has already occurred so the recovery of the neuronal circuitry is hard to carry out.

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Cerebral Neurotrophins and Behavioral Aspects of a Neurodevelopmental Model of Schizophrenia

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PART I: GENERAL INTRODUCTION

Schizophrenia

Schizophrenia is a brain disease that usually induces a lifetime of disability and emotional distress for affected subjects (Lewis and Lieberman 2000). The diagnostic clinical features of the disease typically appear in the late second to third decade of life, with the average age of onset generally about five years earlier in males than in females. Although schizophrenia affects approximately 1% of the population throughout the world, the specific factors producing the disorder remain not clear. A number of studies have tried to investigate the genetic and environmental components that separately, or cooperatively, may be causative of the disorder. The background of the question is particularly complex, including original observations and subsequent studies that fail to replicate findings of genetic linkage or environmental risk factors. These apparent discrepancies may, in fact, be due to the complex nature of schizophrenia, where subtle brain abnormalities induce a disease that has its onset in early adulthood and then proceeds with apparently scarce degenerative changes over the lifetime of the affected subjects.

As for other human diseases, the clinical syndrome recognized as schizophrenia may represent the termination of multiple different pathogenetic pathways. Attempts to produce a unifying concept of the etiology of schizophrenia include biological mechanisms that have their origins in developmental processes appearing before the beginning of clinical symptoms. Although agreement has not yet been achieved regarding the specific factors leading to schizophrenia and the time window during which they may act, the neurodevelopmental nature of schizophrenia appears to be a particularly attractive issue.

Environmental Contributions and Genetic Risk

The risk of developing schizophrenia is directly associated with the degree of biological relatedness to an affected subject (Gottesman 1991); indeed first degree relatives of an affected individual have a higher risk of manifesting the disease than do second-degree relatives, and a monozygotic twin of people with schizophrenia is at greater risk than a dizygotic twin. When the biological children of persons with schizophrenia are adopted, their risk of developing schizophrenia remains high, as expected for first-degree relatives (Kety et al. 1971; Ingraham and Kety 2000). Furthermore, the sons of identical twins discordant for schizophrenia have elevated rates of the disease, independent of whether the parent was affected or unaffected (Gottesman and Bertelsen 1989). The genetic origin of schizophrenia appears to be in a polygenic, non-Mendelian fashion (Risch and Baron 1984; Risch 2000). A number of loci shown associations with schizophrenia, including 22q11–

13, 6p, 13q, and 1q21–22 (Pulver 2000; Brzustowicz et al. 2000). Interestingly, some of these loci have genes that contain well-delineated neurobiological functions, though these data have not yet been converted into reliable single gene findings through the use of positional cloning techniques. However, linkage studies have proven difficult to replicate in subsequent cohorts and this phenomenon may highlight the genetic diversity of schizophrenia, with different subtypes derived from different important molecular defects. Although the origin of schizophrenia clearly involves genetic factors, with the heritability of the disease ranging from 70 to 85%, about 60% of people with schizophrenia have neither a first- nor second-degree relative with the disorder (Gottesman and Erlenmeyer-Kimling 2001). Furthermore, given that the degree of concordance for schizophrenia among monozygotic twins only approaches 50% (Gottesman 1991), genetic origin alone is not sufficient for the manifestation of the disorder. These findings suggest both that the genetic predisposition to schizophrenia is complex and that rare forms of the illness may exist. Thus considerations of the etiology of the disease include also the role of environmental factors. Indeed, it has been hypothesized recently that the entire genome may contain genes leading to schizophrenia, so the entire "envirome" must be examined for environmental risk factors (Tsuang et al. 2001). Consequently, some models of the etiology of schizophrenia propose multiple effects between genes and environmental events. Of the environmental factors that have been associated with an increased risk of schizophrenia, many occur during the prenatal or perinatal periods of life, long before the typical beginning, in late adolescence or early adulthood, of the psychotic symptoms required for the diagnosis of schizophrenia. This delay between environmental factors and the appearance of clinical illness has played an important role in the idea that schizophrenia may be a disease of neural development. The idea of a developmental etiology has also been supported by negative data. Specifically, the majority of postmortem studies failed to find evidence of gliosis, if examined by Nissl staining or immunoreactivity for glial fibrillary acidic factor, in the brains of schizophrenics (Roberts and Harrison 2000). Furthermore, glial membrane turnover does not seem to be potentiated, as assessed by magnetic resonance spectroscopy, at illness onset or in people with chronic schizophrenia (Bertolino et al. 1998). This apparent absence of gliosis has been discussed to exclude typical neurodegenerative processes. Given the epidemiological data for perinatal brain change as a precursor of the disease (discussed below), the absence of gliosis may also be surprising from a neurodevelopmental point of view because the brain may produce a gliotic response as early as the twentieth week of gestation. However, neuronal loss or other types of damage can certainly occur in either the developing or adult brain without a glial reaction (Milligan et al. 1991), so the absence of gliosis may only be informative about the nature of brain changes in schizophrenia,

rather than their timing.

The Hypothesis of Schizophrenia as a Neurodevelopmental Disorder

The view of schizophrenia as a neurodevelopmental disorder indicates that pathogenetic biological events or characteristics are present much earlier in life than the onset of the clinical manifestations of the disease (e.g. psychosis) required for diagnosis (Chua and Murray 1996). Indeed it has been proposed that schizophrenia occurs at puberty in individuals who are "slightly retarded" in both physical and mental development (see Takei and Murray 1998); it has been also observed that premorbid signs could be detected in early childhood (see Marenco and Weinberger 2000) and it has been noted that many subjects who developed schizophrenia exhibited "a tendency to seclusion, withdrawal together with moderate or severe degrees of irritability" (as quoted in Malmberg et al. 1998). Specific discussion on a neurodevelopmental origin of schizophrenia began to appear in the 1980s. Weinberger (1987) proposed that schizophrenia is a "neurodevelopmental disorder in which a selected brain lesion from early in life interacts with certain normal events that occur much later". This hypothesis was based on the idea that a "brain lesion can be maintained clinically silent until the normal developmental processes may lead the structures affected by the lesion to induce schizophrenia" (Marenco and Weinberger 2000). Other investigators, suggested that maldevelopment was applicable to only a subset of subjects with schizophrenia. For example, it has been proposed that (Murray et al. 1992) the age of appearance of the first behavioral changes may be used to delineate the following three types of schizophrenia: "congenital" adult onset, and late onset. The congenital or neurodevelopmental form of schizophrenia is of those individuals with brain abnormalities from pre- or perinatal life inducing subtle behavioral disturbances prior to late adolescence, when the full disease appears. Congenital schizophrenia has been thought to be more common in males, more severe, and associated with soft neurological signs and cognitive impairments (Pilowsky et al. 1993). It has been also postulated (Feinberg, 1982) that sleep architecture and cortical synaptic density that occur during normal adolescence, indicated that schizophrenia may be the result of a disturbance of late developmental events. Specifically, it has been proposed that altered cortical synaptic pruning during adolescence was the main pathogenetic process.

Data Leading to a the Prenatal Development of Schizophrenia

Maternal Exposure to Pathogens

A series of studies (Mednick et al. 1988) reported an incidence of schizophrenia in the offspring of women who had influenza during gestation. However, other studies failed to find a link between the timing of pregnancy and influenza

(Morgan et al. 1997; Selten et al. 1998; Jablensky 2000) and a number of methodological concerns were raised about the reports with positive findings (Crow 1994; McGrath and Castle 1995). Other findings, such as an increased risk of schizophrenia in offspring following maternal rubella infections (Brown et al. 2000) and elevated serum levels of IgG and IgM class immunoglobulins found before parturition in mothers of subjects who subsequently developed schizophrenia (Buka et al. 2001), provide interesting hypotheses, but no conclusive evidence of utero infectious etiology of schizophrenia.

Maternal Malnutrition

Only few data are available about maternal malnutrition. Individuals conceived during the second world war showed a twofold increase in the risk for schizophrenia in both male and female offspring (Susser et al. 1996). In contrast, maternal malnutrition during early or severe famine in later pregnancy were not associated with increased risk. Furthermore, the increased risk associated with malnutrition during pregnancy appeared to be specific to schizophrenia and to other psychiatric disorders. However, it is unclear whether other factors participated in the predisposition to schizophrenia. In addition, the unique circumstances that made this study possible rendered independent replications difficult.

Small Head Size

A few studies have reported reduced head circumference at birth, compared to controls, in subjects who developed schizophrenia (see McNeil et al. 2000a; Kunugi et al. 2001 for reviews) though some studies failed to find differences in total brain volume in adults with schizophrenia (Shenton et al. 2001). However, given the general variability in head/brain size among humans and the host of potential covariates influencing these measures, the possibilities that decreased brain size is possible (i.e. leading to small effect sizes) in schizophrenia, or present in only a subset of affected subjects, cannot yet be excluded.

Urban Birth

A significant positive link between the size of urban places of birth and the incidence of schizophrenia and other psychoses has been proposed (Marcelis et al. 1998; Mortensen et al. 1999). A recent study also suggests that, independent of place of birth, urban residence during postnatal development is associated with an increased risk of schizophrenia (Pedersen and Mortensen 2001). However, it is not clear whether this link with schizophrenia reflects higher exposure to other factors, such as infection, toxins, or malnutrition that may be more common in urban populations.

Minor Physical Abnormalities

Slight deviations in external physical features (e.g. low set ears, furrowed tongue, high arched palate, curved fingers, adherent earlobes) are supported to be

the consequence of disturbed prenatal development of the ectoderm. Because the CNS also develops from the ectodermal germ layer, minor physical abnormalities are thought to be associated with impaired development of the brain. These changes have been found with increased prevalence in subjects with schizophrenia and other psychiatric disorders (Lane et al. 1996), and also in individuals with other developmental disabilities such as Down syndrome and pervasive mental retardation. However, many of the studies that focused on schizophrenia were limited by a scorer bias because it is difficult to investigate minor physical abnormalities in adults in a way not including psychiatric diagnosis. It has been found that subjects with increased genetic risk for schizophrenia by virtue of having an affected relative, but assessed for minor physical changes before the clinical onset of symptoms, high scores in these changes were associated with a significantly greater probability of developing schizophrenia-like disorders (Schiffman et al. 2001). Moreover these subtle changes do not appear to be associated with genetic liability for schizophrenia in either high-risk offspring or unaffected siblings of schizophrenics, suggesting that the appearance of minor physical abnormalities may be independent of genetic risk. Thus these changes may be a marker of a "second" prenatal hit that increases vulnerability to schizophrenia (Green et al. 1994). However, the extent to which these minor changes actually represent the neurodevelopmental basis for schizophrenia is not fully clear because the high frequency of these subtle abnormalities in normal subjects has raised the question of whether they can truly be considered abnormalities (Marenco and Weinberger 2000; McNeil et al. 2000a).

Data Leading to a the Perinatal Development of Schizophrenia

Obstetrical Complications

Schizophrenia has been associated with obstetrical complications as reported in the medical histories of 20% or more of subjects affected by schizophrenia (Cannon 1997; McNeil et al. 2000a). It has been shown (Geddes and Lawrie 1995) that individuals with obstetrical complications are twice as likely to develop schizophrenia. However, the discussion of this finding is limited by the fact that the definition of obstetrical complications differs across studies, that is events during pregnancy, during delivery, and during the first postnatal month.

It has been speculated that delivery complications appear to be risk factors in a higher proportion of subjects with schizophrenia than do pregnancy complications (including viral exposure) or fetal maldevelopment (Cannon 1997). Family history and gender studies (Verdoux et al. 1997) showed that delivery complications were associated with increased risks of early-onset schizophrenia. However, in population-based studies 97% of people with delivery problems do not develop schizophrenia (Done et al. 1991; Buka et al. 1993), indicating that delivery

complications are not sufficient for the onset of the illness.

The impact of obstetric complications has also been tested in twin studies where differences between monozygotic twins discordant for schizophrenia must be taken into account to reflect the disease process and/or environmental factors, rather than genetic predisposition. Interestingly changes during delivery but not events during pregnancy, characterized monozygotic twins of which one or both was affected with schizophrenia but not twins of which neither was affected (McNeil et al. 2000b). Furthermore, for discordant twin pairs, when the twin affected with schizophrenia was born second, there were very high rates of prolonged labor and lower rates of complications earlier during pregnancy, whereas the opposite was true of twins in which the individual with schizophrenia was born first. Although these findings are interesting, they are limited by the small sample size.

Through what mechanisms might delivery problems act to increase the risk of schizophrenia? Low birth weight alone and combined with premature birth (Jones et al. 1998) were more common among schizophrenic subjects, findings suggestive of abnormalities in prenatal growth. This type of observation raises the possibility that delivery changes reflect abnormal fetal development rather than representing an independent risk factor for schizophrenia; however, other lines of investigation have failed to provide support for this interpretation (McNeil et al. 2000a).

Data leading to an Onset of Schizophrenia during Childhood and Early Adolescence

Movement Deficits

It has been shown (Walker et al.1994) that clinicians identified children who subsequently developed schizophrenia on the basis of abnormal movements, especially choreoathetoid movements and posturing of the upper limbs, and poorer motor skills and these defects were related to higher ventricular size in adulthood (Walker et al. 1996). Similarly, the high risk offspring of subjects with schizophrenia were reported to exhibit delayed development of certain motor skills, such as posture control standing, and walking (Fish et al. 1992; Marcus et al. 1993). Finally, in a large cohort of subjects born during a single week in 1946, the individuals who subsequently developed schizophrenia achieved milestones of motor development, especially walking, later than expected (Jones et al. 1994).

Thus, at least some subjects who later developed schizophrenia exhibited motor abnormalities during childhood, especially during the first 2 years of life. Though these findings are nonspecific and seem to be related to other psychiatric conditions, they suggest that disturbances in brain function are present at an early age in individuals with schizophrenia. However, whether these observations represent early signs of the disease, indicative of its neurodevelopmental pathogenesis, or risk factors that may contribute to translate a

genetic/environmental predisposition into the illness, remains to be shown.

Changes in IQ Scores and School Performances

Both IQ scores, assessed as early as age 8, and educational achievements have been shown to be lower in children who subsequently manifest the clinical manifestations of schizophrenia than in comparison subjects, such as individuals who later develop mood disorders (reviewed in Chua and Murray 1996). The offspring of subjects with schizophrenia had lower IQ and reduced general cognitive functioning than children with normal parents or with parents who had an affective disorder (Ott et al. 1998). Interestingly, preschizophrenic individuals achieve lower educational qualifications than individuals who later develop mood disorders (Isohanni et al. 1998). The highest premorbid occupational level of subjects with schizophrenia looks to be lower than their fathers whereas the occupational performance of individuals with a mood psychosis does not differ from their fathers (Jones et al. 1993). In Israeli army conscripts IQ was lower at age 16–17 in subjects who subsequently developed schizophrenia than in controls who attended the same high schools (Davidson et al. 1999). The risk of schizophrenia in Swedish army conscripts increased linearly with decrement in IQ, although lower IQ was also present in those who developed a nonschizophrenic psychosis (David et al. 1997).

All together these findings indicate that intellectual performance is impaired many years before the onset of psychosis, suggesting that a decrease in cognition does not simply represent the prodromal phase of schizophrenia. Lower intellectual function may reflect a causal factor for the psychotic manifestations of schizophrenia, predisposing an individual to the development of false beliefs and perceptions. For example, the decrease in cognitive function in Alzheimer's disease is associated with an increased risk of psychosis. However, IQ does not seem to be outside the normal range in preschizophrenic individuals; the differences from comparison subjects are small; and, in isolation, the positive predictive value of low IQ is very modest, predicting only 3% of cases of schizophrenia (David et al. 1997).

Considerations for Neurodevelopmental Models of Schizophrenia

The studies reviewed above lead on several conclusions: *i,* individuals who developed schizophrenia seem to be more likely than control subjects to have experienced one or a combination of a variety of potentially adverse events during pre- or perinatal life. Although an abundant literature, including replicated findings, supports these data, these events generally have low predictive value; indeed most individuals who experienced such an event do not manifest the clinical manifestations of schizophrenia later in life, and the absence of such an event does not preclude the later development of schizophrenia. *ii*, The increased risk

associated with problems during delivery may be the most robust finding. *iii,* Changes (usually subtle) in a variety of types of behaviors (e.g. motor, cognitive, or social) may be evident in individuals with schizophrenia years or decades before the diagnostic manifestations of the disease. Whether these findings represent (*a*) epiphenomena associated with other risk factors for schizophrenia, (*b*) independent risk factors that participate to the causality of the disease, or (*c*) different aspects of a spectrum of age-specific manifestations of schizophrenia remain critical issues. In any case the nature and number of these observations strongly suggest that changes in brain function are present very early in life in at least some individuals who subsequently developed schizophrenia.

As noted earlier, one view of schizophrenia as a neurodevelopmental disorder shows that (*a*) the primary pathogenetic event is a disturbance in brain development during pre- or perinatal life, (*b*) the action of the causative agent is relatively short in time, though the resulting brain lesion are static, and (*c*) the behavioral consequences remain in large part latent for a long time after the onset of the primary cause. The findings summarized above on pre- and perinatal abnormalities and insults, and recent experimental data in animal models, are consistent with an early beginning of the pathophysiological process, although clearly the process needs not be limited to a selected developmental time period. This kind of view of schizophrenia as an early-onset neurodevelopmental disorder, however, is difficult to reconcile with several other findings. For example, it has been noted (Woods 1999) that one of the structural brain features of schizophrenia, an excess of extraventricular cerebral spinal fluid (CSF), reflecting decreased brain volume, may not be explained by an early, static lesion. Because brain growth induces intracranial cavity growth, the brain grows outward from the ventricles and intracranial cavity size is not reducible after skull sutures fuse, any loss of brain tissue after brain growth reaches its maximum will produce increased CSF equivalently in both ventricles and extracerebral areas. However, loss of brain tissue in the pre- or perinatal period should produce a smaller cranial cavity and an increase in ventricular size, but not an increase in extracerebral CSF. Thus, the facts of smaller head size at birth in schizophrenia and larger extracerebral CSF would seem to require both an early lesion and later volume loss. However, it remains unclear whether the increased extracerebral CSF, which may be evident at the beginning of clinical illness, represents a permanent loss of brain tissue, the effect of physiological factors (e.g. hydration, nutritional status, medications) that can reversibly affect brain tissue volume (Marenco and Weinberger 2000), or genetically controlled disruptions in the expression of molecules regulating cellular components able to induce changes in brain volume.

A second potential problem with the early, static lesion model has been the absence of convincing data from postmortem studies of a brain abnormality in

schizophrenia that could only be explained by an early lesion. In this regard reports of cytoarchitectural disturbances in the entorhinal cortex of schizophrenic subjects (Jakob and Beckmann 1986; Arnold et al. 1991b) attracted a great deal of attention because the reported findings were strongly suggestive of an abnormality in neuronal migration (Weinberger 1999). Subsequent studies have confirmed these reports and provided other explanations for the initial findings (Beckmann 1999; Joyal et al. 2002) whereas other authors found different results (Heinsen et al. 1996; Akil and Lewis 1997; Krimer et al. 1997).

Other models of schizophrenia investigating late developmental changes have focused on maturational events happening during adolescence. This developmental period has been interpreted as critical for the onset of the clinical manifestations of schizophrenia for a number of reasons, such as the considerable "environmental and psychological adventure" and social stress that occurs during these years (Weinberger 1987) and the maturation of selected brain regions and functions that are crucial to reach a normal adult life. Indeed, the number of excitatory synapses in the cerebral cortex decreases markedly in the peri-adolescent period (Huttenlocher 1979; Rakic et al. 1986) with concomitant changes in gray and white matter volumes (i.e. gray matter volume normally decreases from childhood, beginning at around 5 years of age, whereas white matter volume increases throughout childhood and adolescence (Bartzokis et al. 2001). These biological events raises the possibility that changes in these processes may contribute to the pathogenesis of schizophrenia. The issue in schizophrenia of progressive increases in ventricular volume and decreases in whole or regional brain volumes might be discussed as a late or at least an ongoing pathophysiological process in schizophrenia. It has been shown an accelerated reductions in cortical gray matter in childhood-onset schizophrenia (Thompson et al. 2001). Furthermore, studies of brain volume sizes in adults with schizophrenia have revealed progressive changes, especially in the frontal lobes (see Shenton et al. 2001 for review). It should also be noted that the lack of structural changes after the beginning of the disease does not preclude the possibility of progressive disruptions prior to the clinical appearance of the illness. Thus, observable progression, suggestive of abnormalities in late developmental processes, may be limited to the initial phase of disease and/or to those with an early-onset form of schizophrenia.

Brain Changes in Schizophrenics Following the First-Episodes of Psychosis

Findings from postmortem studies suggest reduced cortical or hippocampal thickness in schizophrenics. However, only few data are available in first-episode schizophrenia using magnetic resonance imaging (MRI). Wiegand and co-workers (2004) showed that prefrontal cortical thickness was not significantly different among groups. But prefrontal gray matter volume was positively correlated in both

schizophrenia and controls. The product of boundary complexity and thickness, an alternative measure of volume, was also positively correlated with volume. Finally, age and age at first medication were negatively correlated with prefrontal cortical thickness only in first-episode schizophrenia. Correlations between cortical thickness and age and between cortical thickness and age at first medication suggest that the longer the schizophrenic process has been operative, the thinner the prefrontal cortex.

By contrast studies on the amygdala or hippocampus in first-episode schizophrenia found that persons with schizophrenia presented significantly lower amygdaloid volumes bilaterally or smaller anterior hippocampal formation volume in the early phases of schizophrenia without any significant correlations between the amygdaloid/hippocampal volumes and either the duration of the disease or the symptom severity (Joyal et al. 2003; Szeszko et al. 2003).

Other findings evidenced progressive structural brain abnormalities associated with clinical outcome in schizophrenics. Indeed, many studies have shown that structural brain abnormalities in schizophrenia are already present by the time of index evaluation of first-episode patients and progressively worsen during the subsequent course of the disorder (Lieberman et al. 2001; Ho et al. 2003). It has been found accelerated enlargement in cortical sulcal cerebrospinal fluid spaces early in the course of schizophrenia. Instead of the usual trajectory of volume enlargement, patients showed progressive reduction in frontal lobe white matter volume. A reciprocal increase in frontal lobe cerebrospinal fluid volume also occurred at a more rapid rate in patients than in controls. In addition, patients with poor outcome had greater lateral ventricular enlargement over time than patients with good outcome. Progressive decrement in frontal lobe white matter volume and enlargement in frontal lobe cerebrospinal fluid volume were associated with greater negative symptom severity. Reductions in frontal lobe gray and white matter volumes correlated with poorer executive functioning. These progressive changes seem to be most evident in the frontal lobes and to correlate with functional impairment (Ho et al. 2003). Disruptions in neurodevelopment or neural plasticity may act alone or in combination to bring about these progressive brain deficits in schizophrenia suggestive also of an involvement of neurodegenerative processes (Lieberman et al. 2001). In the work of Lieberman et al. (2001) ventricular enlargement and anterior hippocampal volume reductions in first episode schizophrenia patients were found. In addition, changes in selected structures over time in relation to treatment outcome, including increases in ventricular volume that were associated with poor outcome patients were shown. The finding of progressive ventricular enlargement in patients with poor outcome schizophrenia is consistent with the hypothesis that persistent positive and negative symptoms result in progressive brain changes in the form of ventricular enlargement, possibly due

to neurodegeneration rather than the confounding effects of treatment. The limit of this paper is that there were no significant reductions in cortical and hippocampal volumes over time indicating that neurodegeneration appears to be only a partial aspect of the question (Lieberman et al. 2001).

The Entorhinal and Hippocampal Region in the Development of Schizophrenia

The entorhinal cortex, subiculum, and hippocampus dealt great interest in the investigators both clinical and neuropathological features of schizophrenia. Postmortem studies (reviewed in Arnold 2000) have shown numerous changes, although many are still controversial or unconfirmed. The main cellular, molecular and neuropathological findings are (1) abnormalities in the cytoarchitecture of the entorhinal cortex appearing poorly formed in the layer II neuron clusters and with strong laminar disorganization; (2) normal neuron density but smaller neuron size in the superficial lamina of the entorhinal cortex and subiculum; (3) aberrant expression of the microtubule-associated protein MAP2 in the entorhinal cortex and subiculum; (4) altered glutamatergic and catecholaminergic innervation of the entorhinal cortex; (5) disrupted mRNA expression of several transcription factors, ion channels, and neurosecretory pathway-related proteins in stellate neurons of the entorhinal cortex; and (6) an evident absence of any neurodegeneration or gliosis.

Altogether, these findings suggest that abnormal neurodevelopmental processes play a crucial role in the pathobiology of schizophrenia that may provide a neuroanatomic basis for understanding many of the clinical and neuropsychological changes of the disease.

Schizophrenia is a severe multifactorial mental disorder affecting approximately 1% of humans characterized by deterioration of personality, increased hallucinations and delusions, and changes in cognition. As previously reported the hippocampus and parahippocampal regions dealt special interest in the study of schizophrenia and ventromedial temporal lobe has been frequently analyzed in both clinical and postmortem neurobiological studies (Arnold and Trojanowski 1996; Arnold 1997; Harrison 1999). Clinicians have long noted similarities between the features of schizophrenia and those of the schizophrenia-like disorders associated with temporal lobe changes such as epileptic foci, temporal lobe tumors and cerebrovascular lesions, herpes encephalitis, and neurodegenerative diseases (e.g. Alzheimer's disease) (Davison 1983).

Neuropsychiatric studies showed that associated with cognitive impairments there also are differential deficits in both verbal and visual declarative memory which have been attributed to hippocampal dysfunction (Saykin et al. 1991; Gruzelier et al. 1988) and other researches demonstrated that these changes persist even after an efficient antipsychotic treatment (Gur et al. 1998). This stability of

memory deficit leads to a static neuroanatomically-established abnormality in the ventromedial temporal lobe.

Structural neuroimaging findings with magnetic resonance imaging have reported selective volume deficits in the amygdalo-hippocampal region and parahippocampal gyrus in schizophrenics (Kelsoe et al. 1988; Becker 1996), although these findings are still debated (Suddath et al. 1989; 1990; Bogerts et al. 1990; Breier et al. 1992). It is also noteworthy mentioning that while magnetic resonance imaging studies strongly report smaller hippocampal and parahippocampal volumes, the postmortem data are less consistent. Indeed it has been reported decreased cross-sectional area as well as volume of the hippocampus and decreased parahippocampal area, volume and parahippocampal cortical thickness though other studies failed to find differences in hippocampus size in schizophrenics and a stereological study did not find differences in either hippocampus of parahippocampal gyrus size (reviewed in Arnold 2000). However it should be stressed that a number of methodological variables might explain these inconsistencies such as the limited postmortem studies sample sizes, the methods of sampling cross-sectional profiles for area determination, or varying methods for analyzing the boundaries of the hippocampus.

It has been also investigated the importance of the neuroanatomic abnormalities of the hippocampal formation in schizophrenia by correlating volumetric measures with the disease's symptoms and signs. Poor performance on tests of verbal memory, abstraction, and categorization as well as some positive symptoms correlate with reduced size of ventromedial temporal lobe structures (Shenton 1992) and functional neuroimaging studies using positron emission tomography and magnetic resonance imaging reported abnormal blood flow or metabolic activity in the hippocampal region that also correlate with psychiatric measures (Saykin et al. 1999; Schroeder et al. 1995) though findings have been diverse and controversial (reviewed in Arnold 2000). Other data revealed smaller entorhinal volumes bilaterally in early schizophrenic patients. This volume loss did not correlate with items on the Positive and Negative Syndrome Scale suggesting an early involvement of the entorhinal cortex in schizophrenia (Joyal et al. 2002) and a decrement in entorhinal cortex size has been confirmed also by a recent work which tested more than 50 schizophrenics (Turetsky et al. 2003).

The possibility of brain neurodegeneration has been supported only by a few longitudinal volumetric magnetic resonance imaging studies which evidenced progressive brain atrophy in some patients (Delisi 1996). In addition, other data on schizophrenia in late life focused a high frequency and severity of cognitive and functional disruptions among some elderly and chronic patients (Arnold et al. 1995) with a neuropsychological profile similar to that of people affected by Alzheimer (Kurtz et al. 2001). In these elderly patients the frequency of various

abnormal neuropathological findings (e.g. lacunar infarcts, Alzheimer's disease, Parkinson's disease, meningiomas, etc.) is only slightly greater than that found among a general community-based elderly population (Purohit et al. 1998) and the brain lesions are highly miscellaneous and most patients, even with severe dementia, still are without apparent abnormalities. Then, to verify the possibility of even subtle neurodegeneration or neural injury in schizophrenia, there have been several investigations which used different histopathological markers in schizophrenia. These include specific disease-related lesions such as neurofibrillary tangles, amyloid plaques, and Lewy bodies, as well as inflammation factors in degenerative, infectious, traumatic, or other causes, including astrocytosis, microglial proliferation, and excessive ubiquitin expression. Beyond its supported role in schizophrenia, the hippocampal formation has been also of special interest for its selective vulnerability in a number of neurodegenerative disorders (Braak and Braak 1991; Arnold et al. 1991a). The results of these quantitative neuropathological studies did not find evidence of ongoing neurodegeneration and neural injury in schizophrenia, even among the most severely ill elderly, and deteriorated patients. No abnormalities have been shown in the neurofibrillary tangles and senile plaques (Purohit et al. 1998), nor for other disease-specific neurodegeneration markers such as Lewy bodies, Pick bodies, or protease-resistant prions (Arnold et al. 1998; 1999). Likewise, it has not identified any change in astrocytosis using either traditional histological staining or methods examining glial fibrillary acidic protein (GFAP) expression (Falkai et al. 1992). A paper dealt the issue of neurodegeneration in schizophrenia in a wide-ranging manner by analyzing markers of neurodegeneration and neural injury as neurofibrillary tangles, senile plaques, Lewy bodies, GFAP-positive astrocytes, resting and reactive microglia and dystrophic neurites (identified by their immunoreactivity for ubiquitin) in the parahippocampal region, entorhinal cortex, subiculum, and CA1 of the hippocampal formation, as well as mid-frontal cortex, orbitofrontal cortex, and calcarine cortex (Arnold et al. 1998). The patients had cognitive and functional impairment to warrant an additional diagnosis of dementia associated with advanced age and severe and chronic schizophrenia. Authors did not find significant differences between the schizophrenics and controls for the densities of any of the neurodegenerative markers whereas both groups exhibited far fewer lesions than an Alzheimer's disease "positive" control group. Authors speculated that there is not correlation in neurodegeneration or ongoing neural injury in the cerebral cortex in schizophrenia beyond that observed in a normal control group and that degenerative lesions due to aging could not account for the dementia observed.

Without evidence of conventional neurodegenerative pathology in schizophrenia, other factors have to be taken into account in order to explain the

deterioration and dementia that occurs in at least some patients. It should be also possible that the effects of other normal age-related changes are amplified in the setting of presumably disrupted neural circuitry in schizophrenia. Thus, brain maldevelopment may represent a state of decreased cerebral reserve with commensurately increased vulnerability to the cognitive toxicity of even small amounts of neural injury or neurodegenerative lesions that accompany aging. Accordingly, schizophrenics may demonstrate neuropsychological deficits prior to their first psychotic episode. In considering this decreased cerebral reserve hypothesis, it is especially interesting to investigate the topographical similarities between the brain regions with reported cytoarchitectural and neuronal morphometric abnormalities in schizophrenia and those that are mainly susceptible in aging and the common neurodegenerative diseases, such as the entorhinal cortex.

Neurotrophins and the Central Nervous System

There are considerable experimental data indicating that neurotrophic factors promote the functional activities of brain neurons, both under normal and pathological conditions (Thoenen et al. 1987; Barde 1989; Aloe and Tirassa 1992). Indeed, the constitutive level of neurotrophic factors of patients affected by environmental stressors, aggressiveness, ECT, anxiety-like behaviors, depression and schizophrenic-like disorders undergoes significant changes (Bersani et al. 2000, Aloe et al. 2000). NGF is the first and best characterized member of the neurotrophin family including also Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4/5 (NT-4/5), and Neurotrophin-6 (NT-6) (Barde 1990). Neurotrophins are produced by a variety of non-neuronal cells, including cells localized in the central nervous system (CNS), though with regional and developmental-time differences and distribution. These molecules interact with two different types of receptors on the surface of neurons. One type, the high-affinity receptor belongs to the Trk family of tyrosine kinase receptors (TrkA, TrkB and TrkC) and binds to specific neurotrophins. The other type of receptor, p75, is a glicoprotein lacking of tyrosine domain and binds to neurotrophins with low affinity (Chao and Hempstead 1995).

It has been shown that neurotrophins of the NGF family can be implicated in some *maldevelopmental* aspects of neuropsychiatric disorders and the constitutive levels of NGF and BDNF are affected in pathological changes induced by surgical and chemical insults. Neurotrophins prevent behavioral and biochemical deficits associated with aging (Connor and Dragunow 1998). Changes in NGF were also observed following entorhinal cortex lesions (Connor et al. 1994; Shimode et al. 2003). Neurotrophins play also a role in neuropsychiatric diseases as Alzheimer's, Parkinson's and depression (Altar 1999; Dawbarn and Allen 2003). NGF is also

involved in mechanisms regulating learning abilities, stress and stress-related events both in animal models and humans (Alleva and Aloe 1989; Connor and Dragunow 1998). Specifically, intraspecific aggressiveness induced in mice by 6-8 weeks of isolation causes an increase in blood and brain NGF levels that is directly correlated to the frequency of fighting episodes carried out by the mice. Aggressive behavior also enhances NGF and NGF-mRNA presence in the CNS. Interestingly, animals characterized by submissive behavior showed high hypothalamic levels of NGF (Aloe et al. 1986; Spillantini et al. 1989). Within the brain, the major increase in NGF following fighting was observed in the hypothalamus suggestive of a NGF role in coping and neuroendocrine mechanisms (Levi-Montalcini et al. 1990). This brain area represents the most rostral part of the reticular formation and is involved in the activation of certain behavioral parameters and in physiological modifications inducing changes in the bodily homeostasis. Since arousal is probably associated with high NGF levels (Aloe et al. 1994; Mack et al. 1995), it has been hypothesized that sedative states are characterized by a central reduction of NGF levels. Findings indicating that stress-related behavior and altered homeostasis lead to significant changes in the basal NGF levels were reported in recent years by several groups. These studies showed that delivery and lactation (Luppi et al. 1993), anxiogenous-like behaviors (Maestripieri et al. 1990), environmental changes (Mohammed et al. 1990; Mohammed et al. 1993) and running (Matsuda et al. 1991) significantly influence the basal NGF levels both in the bloodstream and in the CNS. In addition, in humans it has been shown that the level of NGF increases in the blood of young soldiers who experienced their first parachute jump. These studies showed that the release of NGF in the bloodstream is triggered not only by jumping, but also by the anxiogenic-like behavior induced by the waiting for the novel experience. Because the release of NGF precedes the release of the classical stress hormone cortisol and ACTH, it was hypothesized a correlative event between released NGF and anxiogenic status (Aloe et al. 1994). These latter observations clearly indicate that NGF release in bloodstream, as well as the central level of NGF, is not correlated only to aggressive behavior but also to submission and anxiety conditions (Maestripieri et al. 1990).

Changes in NGF and BDNF levels also occur following physical and mental stress (Larsson et al. 1989; Neeper et al. 1996), seizure, ECT and depression (Altar 1999). In 1989, Gall and Isackson discovered a link between NGF and convulsions by demonstrating that there is an increase in the mRNA for NGF soon after a limbic seizure (Gall and Isackson 1989). Intraperitoneally injected phenytilenetetrazolium (Watson and Mildbrand 1989; Mack et al. 1995) or kainic acid, injected into the lateral ventricle (Gall et al. 1991), or bicuculline, injected with a microinjection into the prepiriform cortex (Riva et al. 1992) or NMDA, injected into the ventricles, or into the hippocampus (Gwag et al. 1993) or

quinolonic acid, injected into the left dorsal hippocampus (Rocamora et al. 1994) induced an increase in NGF and its mRNA in various brain areas. Similarly, unilateral electrolytic lesions of the dentate gyrus hilum (Gall and Isackson 1989; Isackson et al. 1991; Rocamora et al. 1992; Lauterborn et al. 1994), by installing steel electrodes into the CA1-CA3 areas of the ventral hippocampus (Ernfors et al. 1991; Bengzon et al. 1993), in the basal-lateral amygdala (Bengzon et al. 1993; Sato et al. 1996), or low (50-70 mA) and high (150 mA) intensity electroshock using keratic electrodes (Follesa et al. 1994) also result in modification of brain NGF levels.

Evidence Suggesting a Role for NGF and BDNF in Schizophrenia

NGF is highly expressed in the CNS where proliferation, migration and differentiation of neuronal precursors take place, and a variety of brain neurons are able to produce and respond to the action of both NGF and BDNF during early and late post-natal life (Ebendal 1989). Recent findings also indicated that NGF is produced and released not only in the hippocampus and frontal cortex, but also in the entorhinal cortex (Pham et al. 1999) and other studies have provided supportive evidence that NGF either alone or cooperatively with other neurotrophins, particularly BDNF, plays a crucial role in the development and physiopathological activity of the adult CNS (Sofroniew et al. 2001). Because of its important role in neurogenesis, it has been hypothesized that significant changes in NGF synthesis during a critical developmental time could alter the functional activity of specific sets of brain neurons in pre and post-natal life that may lead to schizophrenia-like deficits. There are anatomical and epidemiological evidences indicating that schizophrenia is a disorder due to cortical-hippocampal *maldevelopment* and that one of the major target area affected is the entorhinal cortex (Arnold et al. 2000).

The entorhinal cortex is among the first cortical plate areas to develop in rodents as well as in human fetus (Bayer and Altman 1987). The proliferation of this brain structure is thought to occur halfway gestation, just preceding the large burst of neurogenesis (Witter et al. 1989). Prenatal neuronal loss and/or neuronal disconnection during the adult life in the entorhinal cortex seems to be one of the prominent causes of schizophrenic-like deficits in humans. Infact, alteration in entorhinal cortex neurogenesis seems also to be one of the maternal risk factors in the development of the behavioral deficits leading to neurobiological disorders associated with schizophrenia-like deficits (Ibanez et al. 1995; Weinberger 1995).

Neurotrophins play a key role in entorhinal cortex neurogenesis which suggests that lack of an appropriate NGF trophic support during a critical period of entorhinal cortex neurogenesis might result in a damage to this brain region as observed in schizophrenia. There is also evidence that other neurotrophins might be implicated in the pathogenesis of schizophrenic-like disorders. For example, NT-3

gene polymorphism is associated with some forms of schizophrenia (Nanko et al. 1994) and brain tissues of schizophrenic patients show profound abnormalities in survival and growth compared with controls (Weinberger 1995) and schizophrenic subjects are characterized by low circulating levels of NGF protein (Bersani et al. 1999). Other studies showed that NGF and BDNF are abnormally regulated in the CNS of animal models of schizophrenia (Aloe et al. 2000; Bersani et al. 2000; Fiore et al. 1999; 2000; 2001; 2002), by evidence that schizophrenic patients have low circulating levels of NGF (Bersani et al. 1999) and by the fact that schizophrenics are characterized by reduced BDNF in the serum (Tojooka et al. 2002). NGF levels were also found decreased in never-medicated first-episode psychotic schizophrenic patients (Parikh et al. 2003). Findings obtained with studies on human post-mortem tissues have also evidenced a significant increase in BDNF concentrations in cortical areas and a decrease in this neurotrophin in the hippocampus of patients when compared with controls whereas other authors have found that BDNF protein is elevated in the anterior cingulate cortex and hippocampus of schizophrenic patients (Takahashi et al. 2000).

The Animal Model of Schizophrenia Based on the Maldevelopment of the Hippocampal-Entorhinal Axis

It has been shown (Talamini et al. 1998;1999) that interference with neurogenesis in the mediotemporal allocortex of rat embryos, during the earliest stages of cortical proliferation, results not only in a thickness reduction of the adult entorhinal cortex and hippocampus (Fiore et al. 2000; Talamini et al. 1999) but also in other morphological characteristics that may be compared with the changes observed in the hippocampal-entorhinal axis of patients with schizophrenia (Braak and Braak 1992). An animal model displaying these structural brain deficits was obtained administering a single injection of methylazoxymethanol acetate (MAM) in pregnant rats on gestational day 11 or 12 when the entorhinal-hippocampal axis was supposed to be in major cell proliferation (Bayer and Altman 1987).

MAM (Reviewed in Cattabeni et al. 1997) is a short-acting alkylating agent that methylates nucleic acids, leading to the death of cells that are actively replicating DNA. In rodents, 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.

Analysis of the forebrain in adult animals showed reduction in the entorhinal cortex size and this effect shifts from lateral to medial divisions of the entorhinal cortex with later administration of MAM, following a known developmental gradient. Morphological consequences of MAM administration appear to be largely confined to the hippocampal-entorhinal axis although slight reductions of the

frontal and occipital neocortex were also observed. MAM treatment on gestational day 12 revealed relatively more widespread damage, as reflected among others in a small decrease in brain weight. However, it should be noted that some behavioral characterizations of the model were discussed in a work (Jorgen-Relo et al. 2004) showing no changes, when compared to controls, in prepulse inhibition, latent inhibition with the two-way active avoidance, and in the freezing paradigms. Another time point that could be of particular interest is the impact of MAM on GD17, given studies showing that MAM administration at this time point will lead to disruption of brain circuits of known relevance to schizophrenia (Grace and Moore 1998; Moore et al. 2001). 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 display behaviors similar to rats with frontal lesions.

Why did we use MAM rather then other compounds or X-rays that are well known to inhibit DNA proliferation or cell development? To overcome the strong unspecific effects induced by X-rays, a number of compounds inducing inhibition of DNA synthesis and displaying antiproliferative activity such as hydroxyurea, 5-azacytidine, nitrosoureas, and MAM have been widely tested. Inhibition of DNA synthesis by these chemicals is due to different mechanisms: hydroxyurea inhibits the conversion of ribonucleotides to deoxyribonucleotides; 5azacytidine inhibits DNA methylation, whereas nitrosoureas and MAM induce aberrant DNA alkylation and methylation, respectively. All these agents have been shown to induce cellular ablations when given at different times during embryogenesis or in the early postnatal phase. A detailed account of all these studies can be found in the review by Rodier (1996), and what emerges from these studies is that although the general pattern of damage to the CNS is similar for these agents and X-irradiation, when given at the same gestational period, only MAM seems to selectively affect developing CNS and not other embryo's organs undergoing intensive cell proliferation at the time of its administration. This is further supported by data suggesting that a single treatment with MAM does not affect gestational parameters of the dams and has no teratogenic effects on other organs of the offspring (Balduini et al. 1991). However, the specificity of MAM in inducing antiproliferative effects on neuroepithelial cells in vitro has been demonstrated only very recently. Neuroepithelial cells from striatum primordia rat embryos treated with MAM at gestational day 14 were exposed to increasing doses of MAM. After a brief exposure at the dose of 10 mg, cell proliferation was completely blocked for 5 days in culture, without inducing cellular toxicity. This effect was shown to be cell cycle dependent, since quiescent cells in serum-free medium were not affected (Cattaneo et al. 1995). Interestingly, the proliferation of mature astrocytes was not

influenced by MAM exposure, up to doses that were frankly toxic (100 mg) to both astrocytes and neuroepithelial cells. Cells from totally different lineages, like vascular smooth muscle cells, were only temporarily affected by MAM, since after its removal from the medium, cell proliferation resumed and treated cultures contained the same number of cells as those of control cultures (Cattaneo et al. 1995). These observations strongly support the idea that neuronal precursors that are undergoing their final mitosis at the time of MAM exposure are specifically ablated, and because pre- and postmitotic neurons are not affected by the toxin, MAM can be considered an ideal tool to induce specific cellular ablations at specific and well-defined neurogenetic times in experimental animals (Cattabeni et al. 1997).

Aims of the Thesis

As reported above schizophrenia is a disease involving genetic roots, environmental contributions, neurodegeneration and neurodevelopmental causes. We approached and investigated the way in which maldevelopment inducing brain changes may generate and affect human adult routine by studying an animal model of brain maldevelopment producing disruptions in the entorhinal-hippocampal axis. Thus the main aim of this project was to further test a rat model of developmental brain disorders in order to evaluate the finding of a role played by neurotrophins in the developmental neurobiology of schizophrenia by regulating also the baseline levels of behavioral performances. However, it should be underlined that is not a main aim of this thesis to analyze the supposed maldevelopmental origins of the disease since, as previously stated, they may be addressed to genetic or environmental factors, or highly possibly, to an interaction between different factors. In particular we used an antimitotic compound, to resemble a hypothetic environmental factor, to manipulate brain cells' development, mainly because of the relatively high reliability of the chemical to induce brain cell maldevelopment without determine gross secondary outcomes. Interestingly, the effects produced by the model look similar to the ones obtained by other more invasive and with secondary effects animal models used by researchers in the field.

The animal model addresses to disturbances of higher brain function, and in line with data from schizophrenia investigation, to developmental interferences during a specific period of the ontogeny where selected limbic regions takes place as cingulate cortex and hippocampal and parahippocampal regions. All together these regions play crucial roles in human investigations in the context of cognitive and attentional diseases because for their participation in multimodal and supramodal association. Indeed, this strongly results for the entorhinal region of the parahippocampal gyrus where there is the main in- and output station of the hippocampal formation, converging cortical inputs (information reviewed in Lopes

da Silva et al. 1990 and Bayer 1990a, 1990b). We also administered MAM at gestational day 17 following the indication given bu the Grace's group (Grace and Moore 1998; Moore et al. 2001) to reveal lesions in the thickness of limbic and paralimbic cortices.

In a battery of laboratory investigations the normal route of brain rat development has been manipulated by the administration of MAM during selected gestational time points in pregnant rats. As reported above MAM alkylates the DNA of cells undergoing division killing the mitotic cells (Cattabeni et al. 1997). After MAM administration the effects are visible for about 24 h with a maximum 12 h after injection. MAM was administered when brain cells belonging to the entorhinal-hippocampal regions were supposed to undergo extensive mitotic division, namely rat gestational day 11 and 12. According to the literature on gestational day 11 middle limbic/early neocortical regions develop whereas gestational day 12 coincides with the development of late limbic regions, inclusive of the hippocampus and some neocortical areas (Bayer 1990a; 1990b; Smart and Smart 1982). In the offspring of such administered dams brain morphology and some selected biomolecular and biochemical markers of cortical and monoaminergic neurotransmission have been extensively analyzed (Talamini et al. 1998; 1999). Scope of the present thesis was to evaluate at different ages of rat life whether or not changes in brain neurotrophic factors due to brain maldevelopment induced by MAM could be associated with short or long-lasting changes in the behavior, namely learning and memory processes, sociality, nociception, locomotion, displacement behaviors, exploratory capabilities etc. In addition we investigated different neuropeptides and neurotransmitters known to participate in the fine tuning of some behavioral parameters and also known to be regulated by neurotrophic factors as NGF and BDNF. All together these findings include specific parameters known to be disrupted in schizophrenic patients in a way to produce possible comparisons with clinical observations. We speculate that in view of the neurotrophic hypothesis of the origin of schizophrenia (Thome et al. 1998) and based also on the related data available in the literature (reviewed in Arnold 2000) a complex of neuromorphological neurochemical and behavioral changes, as observed in schizophrenia, could be the consequence of a prenatal insult. We also speculate that based on data on NGF and BDNF in humans affected by neuropsychiatric diseases and in animal models it has been proposed that the neurotrophins' modulation may affect therapeutic interventions indicating that this approach may be a new way to promote also limbic system neuroplasticity.

PART II: MATERIALS AND METHODS

Animals and Treatments

Gestating Wistar WI rats (*Rattus norvegicus*) were obtained from Charles River, Germany, or from Harlan, USA, which mated animals over a period of 4 hours, 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 an air conditioned room (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*. Pregnant rats were randomly divided in 6 groups GD9, GD10, GD11, GD12, saline and naive or in 2 groups, GD17 and saline. Pregnant rats for each GD group underwent a single intraperitoneal injection of MAM (20 mg/kg) on gestational day 9 (GD9), or GD10, or GD11 or GD12 or 22 mg/kg at GD17. Dams of the saline group received saline solution. Finally the naive animals did not receive anything. At birth all litters were reduced to four males and four females and fostered to the biological dams following behavioral procedures previously described (Vorhees et al. 1984; Cimino et al. 1996). All behavioral tests took place in an experimental room with the same light-dark cycle as the housing facility, and with a conditioned air system. Thus, environmental conditions as humidity and temperature levels inside the room were very similar to those of the housing facility. 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 (86/609/EEC).

Tissue Preparation

As for the dissection of fresh tissues to be used for ELISA or PCR animals were euthanasized with an overdose of Pentobarbital the brain quickly removed and selected brain areas dissected out (see also the indication given by Cuello 1983) and stored at -70°C until used. Tissues were then homogenized and centrifuged at 8500 rpm and the supernatant used for NGF and BDNF protein and mRNA analyses. Regarding the immunocytochemical analyses deeply anesthetized animals were perfused transcardially with 250 ml of 0.1M phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium nitrite, followed by 250 ml of 4% paraformaldehyde in PBS. Brains were then removed, post fixed in PBS buffered 4% paraformaldehyde at 4°C for 12-18 hours, and cryoprotected in PBS 20% sucrose for 24 hours. Each brain was then mounted on a stage of a freezing

microtome, serial coronal sections (20 μm thick) were cut and stained with toluidine blue for general histology, or for immunohistochemistry.

Behavioral Studies

Social Interaction Test (Papers 1, 2)

The social interaction test was developed 25 years ago (File and Hyde 1978) as the first animal test of anxiety that endeavoured to use ethologically relevant sources of anxiety, and to use a natural form of behavior as the dependent measure. It, therefore, avoided the use of food or water deprivation and electric shock, and did not require extensive training of the animal. The dependent variable is the time spent by pairs of male rats in social interaction (e.g. sniffing, following or grooming the partner). Because the behavior of one rat influences that of the other, it is important that the pair of rats is treated as a unit, and only one score for the pair is used. Thus, it is possible to use a total or mean score for the pair, or if only one rat is treated (as is often the case for central drug administration), then only the scores of the treated rat should be used. It is a false inflation of the *n* to use separate scores for each member of the pair, as if they were two independent individuals. An increase in social interaction, without a concomitant increase in motor activity, is indicative of an anxiolytic effect, whereas a specific decrease in social interaction indicates an anxiogenic effect. The test conditions are manipulated to generate different levels of anxiety and both the light level and the familiarity of the test arena are manipulated. Thus, there are four test conditions: low light, familiar arena (generating the lowest level of anxiety); high light, familiar arena and low light, unfamiliar arena (generating moderate levels of anxiety); high light, unfamiliar arena (generating the highest level of anxiety). Social interaction is highest when rats are tested in a familiar arena lit by low light, and it decreases as the test conditions become more aversive or anxiogenic. In general it is easier to see anxiolytic effects (i.e. increases in interaction) when the rats are tested in novel conditions, in which the untreated levels of social interaction are low. Conversely, it is easiest to see decreases in social interaction (i.e. anxiogenic effects) when rats are tested in familiar conditions, in which the untreated levels of social interaction are high. This was the first animal test that was able to detect both increases and decreases in anxiety. This opened the way to investigating anxiogenic compounds, and provided a new approach to the neurobiological mechanisms underlying anxiety disorders. From the beginning, attempts were made to validate this test behaviorally and physiologically, as well as pharmacologically, and it has proved sensitive to changes in anxiety generated by nonpharmacological means. In the original validation of this test (File and Hyde 1978), it was shown that the reduction in anxiety that resulted from a brightly lit or unfamiliar arena was not mediated by olfactory cues (anosmic rats showed the same pattern of decreases in

social interaction across the four test conditions). It was also shown that reduced social interaction was not the result of the rats spending more time in other activities, such as walking round or exploring the unfamiliar test arena. In fact, measures of locomotor activity, rearing and exploration showed the same pattern of decrease across the four test conditions as did social interaction, but were less sensitive to the changes. With the decrease in social interaction, there was an increase in other behavioral indices of emotionality, such as freezing, self-grooming and defecation. In the social interaction test, the rats are normally singly housed for a short period before the test. This is because social isolation reliably increases the time spent in social interaction. As originally standardized, the test used 5 days of individual housing, and Niesink and van Ree (1982) showed that the interaction was maximal after 4–7 days of individual housing. However, it is important to realize that social isolation may also change the response to drugs and, for example, it has been reported to modify the effects of diazepam, chlordiazepoxide and nicotine in the social interaction test (Wongwitdecha and Marsden 1996; Vale and Montgomery 1997; Cheeta et al. 2001a). When rats are group-housed and tested, sequential removal of the rats from the cage (cohort removal) may act as a rapid and potent stressor for the remaining rats. When rats were treated in triads, those removed last position had shorter social interaction time and higher body temperature than those removed first (Kask et al. 2001). However, in rats treated in groups of five, Cheeta et al. (2001a) did not find any difference in the time spent in social interaction or in locomotor scores between the rats removed first from each cage and those removed last. The test was validated for male, adult rats, and there are some important sex differences in female rats that do not increase social interaction as markedly in response to increasing familiarity with the test arena (Johnston and File 1991). It is, therefore, possible that social interaction serves a different function in male and female rats and caution should be exercised when interpreting results in females. However, the test has been used in female rats, and Kellogg et al. (1991) have shown that adult female rats exposed in utero to diazepam (2.5 mg/kg) demonstrated a significant effect of the novel environment on social interaction, thus, responding like un-manipulated adult male rats. Adult female rats displayed a pattern of environment-related social interaction similar to that in male rats castrated as juveniles and tested at 60 days. The results from this study indicate that pubertal secretions of gonadal androgen(s) are necessary for the development of environment-related social interaction in adult male rats (Primus and Kellogg 1990a). Furthermore, while castration of rats as juveniles (day 19) altered the effect of diazepam on social interaction in adult rats, testosterone replacement during puberty (days 30–60) reinstated the ability of diazepam to modify social interaction. The results of these studies demonstrate that gonadal function during puberty is necessary for the development of specific neural

systems underlying social interaction in the adult rats (Primus and Kellogg 1990b). Adolescent male and female rats show the same levels of social interaction and both respond in the same way to manipulations of the test arena (File and Tucker 1984). The test can, therefore, be used in both sexes at this age, and it has been shown that there are important sex differences in response to nicotine, with female adolescents being more sensitive to the anxiolytic effects (Cheeta et al. 2001b).

In the experiments of the present thesis animals were tested for a single social interaction test. To this purpose, rats were transferred to the experimental room when they underwent (between 11 a.m. and 1 p.m.) a 7-min social encounter. The encounters took place in a test cage identical to the home cage supplied with clean sawdust bedding. Each animal was gently put in the Plexiglas box together with 5 animals of the other experimental groups. Immediately preceding the encounter, rats were marked for individual recognition with an atoxic, odorless, permanent marker. Separate behavioral scores for frequency and duration (in sec) were obtained for each individual in the box by running the tapes six times for each recording.

The choice of registered behavioral responses and their classification in two main groups (nonsocial and social) as described below are based principally on the ethological profile of rat behavior described by Grant and Macintosh (1963) and Meaney and Stewart (1981).

Nonsocial responses were: exploring (moving around the cage, exploring, scanning the air), selfgrooming (keeping the mouth and paws on the body or on the head), sniffing (olfactory investigation of walls and floors), digging (digging in the sawdust, pushing and kicking it around by using the snout and/or both forepaws and hindpaws, usually while moving around the cage), rearing and wall-rearing (vertical posture of the body with the forepaws raised or placed on the walls, respectively), freezing (in which the animal. stopped because of fear, is immobile in a crouched position with flattened ears), inactivity (absence of movements without signs of fear). Information about locomotor activity was obtained by counting, during tape running, the number of crossings of floor squares drawn on the TV screen with both forepaws.

Social responses were: anogenital sniffing (sniffing the anogenital region of a partner), social sniffing (sniffing the partner, except the anogenital region), chasing (following the partner around the cage, without any quick or sudden movement, often while maintaining a constant nose contact with its anogenital area), social grooming (licking, wiping the partner's body, in particular near the head and the dorsal region), social inactivity (lying flat, or standing still with the eyes open or closed, while maintaining close physical contact with the partners), aggressive grooming (similar to social grooming but carried out with violent movements).

Open-field Test (Papers 1, 2, 3)

Hall (1934) originally described the open field test for the study of emotionality in rats. The procedure consists of subjecting an animal usually a rodent, to an unknown environment from which escape is prevented by surrounding walls (Walsh and Cummins 1976). Hall's apparatus consisted of a brightly illuminated circular arena of about 1.2 m diameter closed by a wall 0.45 m high. He placed rats individually in the outer ring of the open field and observed the rat's behavior for 2 min, during daily repeated trials. Rats were sometimes tested after 24 or 48 h food deprivation. Hall observed that rats walked more when they were food deprived, but not all rats ate. Animals that did not eat were called emotional. When compared to non-emotional rats, they had fewer entries in the central part of the arena and higher levels of defecation.

The open field test is now one of the most popular procedure in animal psychology (see Belzung 1999 for a review). Different versions are available, differing in shape of the environment (circular, square or rectangular), lighting (lighting from above with a bulb above the open field or lighting from underneath with a bulb placed under a transparent floor, sometimes red light is used), presence of objects within the arena such as platforms, columns, tunnels (see for example Takahashi and Kalin 1989), etc. The procedure usually involves forced confrontation of a rodent with the situation. The animal is placed in the center or close to the walls of the apparatus and some behavioral items are recorded for a period ranging from 2 to 20 min (usually 5 min) as horizontal locomotion (number of crossings of the lines marked on the floor), frequency of rearing or wall-rearing (sometimes termed vertical activity), grooming (protracted washing of the coat). In such a situation, rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field. Indeed, mice and rats walk close to the walls, a behavior called thigmotaxis. Increase in time spent in the central part as well as of the ratio central/total locomotion or decrease in the latency to enter the central part are indications of anxiety. Some authors use a procedure in which the animals are allowed free access to the open field, from a familiar cage (see for example Kopp et al. 1997). In this case, the number of risk assessment postures directed to the open field may provide a good measure of the approach response toward novelty, that is, exploration. The open field has become so popular that its use has been extended to a great number of species, including calves, pigs, lambs, rabbits, pullets, primates, bush babies, honeybees and lobsters. In fact, it has become a convenient procedure to measure not only anxiety-like behaviors, but also sedation or activity. In fact, anxiety behavior in the open field is triggered by two factors: individual testing (the animal is separated from its social group) and agoraphobia (as the arena is very large relative to the animal's breeding or natural environment). It is clear that these two factors may trigger anxiety behavior only in

gregarious species and/or in species that show fear of open spaces into which they are forced. This is precisely the case with rodents that live in social groups and in small tunnels. This is of course not the case in species such as lambs or cows that live in large fields. For these reasons, in experiments involving rodents, observers are not measuring the effects of treatments on exploration, as is sometimes claimed, but the effects on the reaction of the subjects to a stressful event. Therefore, anxiolytic treatments do not themselves increase exploration in the open field but they decrease the stress-induced inhibition of exploration behavior. Behavior of rodents in the open field depends mainly on the tactile sensory factors. It should also be noted that exploration can be increased by some factors such as food or water deprivation: it is therefore very important to verify that a given treatment does not act on such variables, before concluding about possible effects on anxiety-like behaviors. Finally, open field behavior also depends on lighting conditions and the light– dark cycle, so that it may be relevant to ensure that a treatment does not modify internal clock-related behaviors and to test the treatment under different lighting conditions. The effects of many different drugs have been investigated in the open field, including compounds with effective or potential anxiolytic effects (benzodiazepines, serotonin ligands, neuropeptides) but also compounds with stimulant (amphetamine, cocaine), sedative (neuroleptic) or prostration- inducing (epileptogenic drugs) activity. An increase in central locomotion or in time spent in the central part of the device without modification of total locomotion and of vertical exploration can be interpreted as an anxiolytic-like effect while the contrary, that is a decrease of these variables, is associated with anxiogenic effects. Increased locomotion can be considered a stimulant effect while decreased vertical activity and locomotion are related to sedation. It should be said here that the decrease in vertical exploration appears at lower doses than does the decrease in rearing, so that this variable can be considered a more sensitive one.

In the experiments of the present thesis animals were tested in an open-field that consists of a Plexiglas box, with clean sawdust and with the floor subdivided in 18 equal squares, placed under white lights. Several behavioral events, selected among those previously described, were scored over a period of time of 10 min and expressed as frequencies and durations (in sec). These behaviors include exploring, grooming, sniffing, rearing and wall-rearing, freezing, inactivity and the number of fecal boluses emitted throughout the open-field test. Information about locomotor activity was gained by counting the number of crossings of floor squares with both forepaws.

To get more information about the potential effect of MAM administration on anxiety, animals were also tested for analyze baseline behavior in a further novel condition, the novel object investigation test (Fiore et al. 1995). It consists of a

stimulus object (a 35 mm black cartridge box) placed in the middle of the arena immediately following the 10 min open-field recording, and the behavioral responses were scored for 3 additional minutes. Care was taken to minimize the introduction of human or rodent odors along with the box. A number of elements were additionally recorded: the latency of both the initial movement and the first contact with the stimulus object, the number of contacts and approaches (sniffing the object). Behaviors were videotaped under white light and recordings were scored independently by an observer who was blind to the treatment received by the animals. Data were recorded using a keyboard event system feeding to a PC for analysis ("Observer", Event Recorder version 3.0, Noldus Information Technology b.v. Wageningen, The Netherlands, 1994).

Water Maze Test (Papers 3, 4)

More then two decades ago, a device was described to investigate spatial learning and memory in the laboratory rat (Morris 1981). In the meanwhile, it has become one of the most frequently used laboratory tools in behavioral neuroscience. The device consists of a large circular pool filled with opaque water in which a small escape platform is hidden. During a number of training trials, animals learn to find the platform and escape from the pool. Surely one of the reasons for its success is its relative simplicity. However, although the basic procedure is relatively simple, it has been used in some of the most sophisticated experiments in the study of the neurobiology and neuropharmacology of spatial learning and memory. As well it has been used in the validation of rodent models for neurocognitive disorders. In the process, water maze testing gained a position at the very core of contemporary neuroscience research. Throughout the years, the task has been given various names, such as Morris swimming pool, Morris maze, water maze (most notably by Morris and associates), swimming maze, spatial navigation task, etc.

Several characteristics of the experimental animals need to be controlled or taken into account when planning water maze experiments or analyzing results. Factors like body weight, physical development and age may influence swimming speed (Wenk 1998). Gender as well as strain/species of the animal subjects can affect water maze performance. Males tend to perform better than females, and this cannot be entirely reduced to differences in muscle strength or endurance. Important differences in performance not only exist between different species, but also between strains of rats and mice. In recent years, especially the interpretation of results obtained from transgenic mouse models has been complicated by these strain differences. Now, there is growing awareness that strain differences can significantly influence behavioral observations, and that these differences have to be taken into account. Finally, several studies demonstrated that spatial learning

can be severely impaired in stressed, sick, undernourished or aged animals.

As for the descriptions of the apparatus and the training procedures in the standard water maze briefly, a circular tank filled with water should be positioned in a room with a sufficient amount of external cues visible to the swimming animal. During training, the animals are repeatedly placed into the tank and must learn to escape by locating a platform hidden beneath the surface of the water. Exactly what may constitute a sufficient amount of extramaze cues is not entirely clear. Drawing a curtain around the pool will obviously slow the acquisition of the task (Morris 1984), whereas simplification of the extramaze environment tends to improve place learning (Lamberty and Gower 1991). Stewart and Morris (1993) have pointed out that it is difficult in this task to control precisely the external cues that determine the learning process. Nevertheless, reliability of the water maze task across laboratories appears to be good. Crabbe et al. (1999) reported that, contrary to many other behavioral test measures, escape latency during water maze training was not significantly affected by environmental variables, when elements like apparatus, training protocol, age of the animals, housing conditions, etc. were equated across testing sites. Mouse strain, on the other hand, did significantly influence water maze performance (see above). Basically, an animal can use three different strategies to reach the escape platform during a swimming trial (Brandeis et al. 1989). It can use a learned sequence of movements, which brings it to the platform (*praxis* strategy); it can approach the platform using proximal cues (*taxis* strategy); or it can navigate to the platform using information about the platform's location within the spatial configuration of distal cues (*mapping* or *spatial* strategy). Sprague–Dawley rats were able to use odor trails to locate the escape platform in a circular two-choice water maze (Means et al. 1992). The same could happen in a typical water maze experiment enabling animals to use an odor-based taxis strategy in the absence of visible intramaze cues. In addition, an animal may conceivably employ both praxis and mapping strategies to locate the hidden platform, even when random starting positions are applied (Dalm et al. 2000). Impaired performance could, thus, indicate a defect in either of these abilities, and further testing or the use of different performance parameters may be required to determine the underlying mechanism of a water maze deficit. Visible-platform acquisition training (e.g. by putting a flag on top of the platform) is sometimes used as a non-spatial control task, but even during visible-platform training, mice appear to use spatial information to locate the platform. Impaired visible-platform water maze performance will not necessarily mean, therefore, that the observed deficit solely relates to the non-spatial aspects of the task. On the other hand, normal visible-platform training coinciding with impaired hidden-platform performance will indicate that motivational/emotional or sensorimotor defects do not contribute significantly to the hidden-platform deficits. Apparatus as well as training

procedures have varied across studies. Various opacifiers were used including pure or powdered milk, nontoxic white paint or synthetic opacifiers. The requirement, especially in small rodents, to maintain water temperatures around or above 25°C may complicate the use of milk as an opacifier due to spoiling. Different sizes of pools were used varying in diameter from below 1 m to well above 2 m. Most authors using laboratory rats as experimental subjects have employed rather large-sized tanks. Morris (1984) originally described two tanks for use in rats: one 132 cm, and another 214 cm in diameter. Several authors have scaled down tank dimensions for use in mice. For example, it has been used a tank of 120 cm successfully, judging from the large amount of experimental work they have done with it, others used a smaller tank, 90 cm in diameter, or preferred a small tank of merely 61 cm in diameter for use in mice, whereas water tanks of 150 cm in diameter were employed in several recent work on transgenic mouse models (reviewed in D'Hooge and de Deyn 2001). Basic training protocols include hidden-platform acquisition training, probe trial testing and working memory testing (see, for example, Wenk 1998). However, many authors have described task variations consisting of alternative training protocols and tank/platform construction (see, for example Stewart and Morris 1993). These have included different pretraining procedures, on-demand or floating platforms, and mixed acquisition/probe protocols. For example, Markowska et al. (1993) developed an interesting procedure for rats, where they repeatedly performed probe trials after each training session. They obtained especially nice learning curves when using variable-interval probe trials, during which the escape platform was only available following a variable time interval. Standard hidden-platform training usually consists of blocks of four swimming trials starting randomly from four positions. After each successful trial the animal will have to remain on the platform for a short amount of time (e.g. 15 s). Especially in mice, extremely nervous or anxious animals will have to be gently restrained to avoid their jumping back to the water. Each trial will take a maximum of 60–120 s, after which time the animal is put on or guided to the platform by the experimenter. Data from the four starting positions are usually pooled to provide summed or averaged data per trial block. After a series of acquisition trial blocks, a (number of) probe or transfer trial(s) is usually performed, during which the platform is removed from the pool and the trained animal is allowed to swim freely for a fixed amount of time (e.g. 100 s). During such a probe trial the spatial accuracy of the animal is determined, represented by the time it spends looking for the platform in the quadrant where the platform used to be (target quadrant) or by the number of times it crosses the former platform area. A well-trained animal will show high preference for the target quadrant, and spend on average about 50% or more of its free swimming time scanning this quadrant. Alternatively, a series of standard acquisition trial blocks can also be

followed by a series of hidden-platform trials during which the position of the platform is changed once, at the beginning of the series (reversal training), or on a daily basis (working memory testing). Most authors now use video tracking systems to quantify water maze performance. In the experiments of the present thesis we used the EthoVision system by Noldus Information Technology (Wageningen, The Netherlands).

Data obtained with video tracking systems can, of course, be further analyzed off-line according to the needs of the experimenter. Standard performance measures during the acquisition phase of the water maze task usually include escape latency (time required to reach the platform) and length of the swimming path. Lindner (1997) argued that path length, though not a pure measure, might be the most appropriate index of cognitive performance in the water maze. It has been recommended the use of alternative measures, like path directionality or cumulative distance to platform, which may allow more refined distinction between swim patterns or search strategies. Performance measures, often pooled per trial block, can be graphed as means for each experimental group of animals to obtain a learning curve. Statistical analysis of between- group differences in the repeated measurement of such parameters can be performed using two-way analysis of variance (ANOVA) for repeated measures with experimental group and trial (block) as sources of variation. Subsequent multiple comparison testing will enable the identification of statistically significant differences between pairs of means. Probe trial performance can be represented as time spent in different areas of the pool or number of target area crossings, and ANOVA procedures will usually suffice here as well to assess the significance of differences between means.

The use of the Morris maze permitted to know what are the brain regions involved in water maze learning. Most studies have so far examined the role of a few well-defined brain regions in water maze learning. Specific deficits were found in animals with damage to hippocampus, striatum, basal forebrain, cerebellum and several neocortical areas, and specific roles in water maze performance have been proposed for these regions. Other regions have been implicated and may influence water maze performance in various ways, but research has been scanty in these areas, and often it is not known whether deficits are due to effects on memory proper or on other processes affecting performance. It has been well established that the integrity of the hippocampal formation is essential for spatial learning, but the neural mechanism of such hippocampal involvement is not entirely clear. The involvement of brain regions in spatial navigation may be considerably more complex and may comprise a larger number of regions and pathways than originally proposed (Cain and Saucier 1996). However, future findings might still change our current and sometimes highly fragmentary notions about the exact

contributions of different brain regions to water maze learning. Most importantly, spatial learning in general and water maze performance in particular appear to depend upon the coordinated action of different brain regions constituting a functionally integrated neural network. Rats with lesions of the thalamic lateral internal medullary lamina displayed impairments in hidden- and visible-platform learning (Savage et al. 1997). Spatial working memory was also impaired in rats with mammillary body lesions (Santin et al. 1999), and several studies have implicated the amygdala in different aspects of Morris maze performance (Roozendaal and McGaugh 1997; Spanis et al. 1999). Lesions to locus coeruleus and raphe nuclei were shown to affect Morris maze performance (Riekkinen et al. 1990). Raphe dorsalis lesions did not affect Morris maze learning as such, but they did aggravate the effects of nucleus basalis lesions (Riekkinen et al. 1990). Nucleus accumbens lesions did not impair Morris maze acquisition (Thifault et al. 1998), but the structure might play a role in the consolidation of spatial information (Setlow and McGaugh 1998).

It is important to note in this respect that disconnecting rather than destroying brain regions relevant for spatial learning may impair Morris maze performance as well. For example, lesions of fimbria and fornix interrupt the many fiber connections that pass in these structures. Hippocampal-subicular input to the nucleus accumbens and projections from the ascending raphe nuclei to the dorsal hippocampus are two of the pathways that run through the fimbria–fornix. Fimbria–fornix transection abolishes the hippocampal–subicular input to the nucleus accumbens as well as the cholinergic input to the hippocampus. Consequently, fimbria–fornix transection renders the hippocampus dysfunctional although fimbria–fornix fibers may mediate functions that are not attributable to the hippocampus. Eichenbaum et al. (1990) demonstrated that fimbria–fornix lesioned rats were indeed slower in acquiring the task than controls, but they were especially flexible when cues or starting positions were changed. Simplification of the task by restriction of distal cues enabled fimbria–fornix lesioned rats to reach the same level of proficiency as controls (Wortwein et al. 1995).

Ever since its introduction, the water maze task has been used in studies on the involvement of neurochemical systems in place learning and memory, and the effects of neuropharmacological manipulation on spatial functions. McNamara and Skelton (1993) reviewed the involvement of different neurotransmitter and modulator systems in spatial learning, and suggested that only the cholinergic, glutamatergic, and some peptidergic systems may really be required for this kind of learning, whereas systems using γ-aminobutyric acid (GABA), opioids or biogenic amines are either detrimental or unrelated to these functions. In the meanwhile, many studies have indicated that the effects of neuropharmacological agents on water maze performance as well as the involvement of the different neurochemical

systems in spatial learning may be more complex than initially thought.

The cholinergic system is undoubtedly the most important neurochemical system investigated in the water maze. Early on, Sutherland et al. (1982) found that rats receiving intra-peritoneal injection of atropine were unable to use a spatial mapping strategy to solve the hidden-platform water maze. The effects of many other agents on water maze learning have been investigated, but none as extensively as those affecting the glutamate and acetylcholine systems. As for other neurotransmitters Morris et al. (1986) were the first to show that intraventricular infusion of the competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, AP5, impairs hidden-platform acquisition and probe trial performance. This and ensuing reports have suggested that NMDA-type glutamate receptors play a crucial role in place learning, which seems restricted to the acquisition process, but does not involve processes of storage or recall (McNamara and Skelton 1993).

The use of animal models, which imitate pathological states occurring in a target species, has contributed and may continue to contribute significantly to medical progress. Animal models are research tools in the study of pathophysiological mechanisms, but also in the evaluation and development of therapeutic and diagnostic materials and methods as well as in education and training. The quality or utility of a model often depends upon its validity, and the assessment of cognitive abilities is an important aspect of the validation and experimental use of models for neurocognitive disorders or aging. Numerous authors have employed water maze testing in this subfield of neurocognitive research. In addition several authors have used water maze performance to detect behavioral teratogenicity or to characterize cognitive impairment in rodent models of developmental disorders like Down's or fragile X syndrome. These studies could help to identify environmental and genetic risk factors for these developmental disorders as well as their underlying mechanisms. In some applications, water maze was found to have the highest sensitivity to detect spatial learning deficits, but experimenters need to be aware that water maze may not always be the choice test. Akaike et al. (1994) reported increased escape latencies and path lengths during hidden-platform acquisition and reversal training in methylnitrosourea-induced microcephaly rats. Comparing the results of four different spatial learning tests, they found that the radial eight-arm maze might possess the highest sensitivity to detect spatial learning deficits in this model. Behavioral teratogenicity and developmental defects were demonstrated following different physical or chemical exposures and deficits in water maze performance were often used as indicators of developmental cognitive impairment.

For the behavioral studies of the experiments of the present thesis we used as apparatus a black Plexigas circular pool 150 cm in diameter and 50 cm in height designed according to Morris, placed in the middle of an experimental room

(dimension 5 x 4 x 3 m). The pool was filled with tap water kept at a temperature of 24-26°C. A plastic black platform (20 cm in diameter) was placed 0.5 cm below the water surface and 15 cm from the edge of the pool.

Male rats were tested. All tests were conducted between 9:00 hr and 14:00 hr under red light condition. The day before the first day of the acquisition phase rats were trained by placing them on the platform till they stayed, three times consecutively, 10 sec without leaving the platform. The entire procedure took 5 days. The position of the hidden platform remained fixed for the first 3 days (acquisition phase). Each subject was allowed 6 individual trials for the first two days and 5 trials the third day. In addition, a 6^{th} trial without platform during the 3^{rd} day was considered as "probe" test. Rats were also tested for the reversal phase of the Morris maze during the 4^{th} and 5^{th} day, in which the platform was subsequently moved to a location symmetrically opposite with respect to the center of the pool for 6 individual trials for day.

To avoid visual orientation prior the release, rats were transferred from their cages into the pool in a non-transparent plastic cup, from which they glided into the water facing the pool wall. Release points were balanced across 4 symmetrical positions on the pool perimeter. Animals were left swimming either until they found the platform or until 60 sec had elapsed. Platform finding was defined as staying for at least 3 sec on it. If rats crossed the platform without stopping (jumping immediately into the water), they were left swimming until they met the above criteria. After staying for about 10 sec on the platform, the rats were given the opportunity to climb on a wire-mesh grid attached to a stick which was also used to pick up the animals from the water after the cut off time. Between trials the animals were placed under infrared lamps and allowed to warm up and dry for about 3 min. Inter-trial times varied between 40-50 min.

The length of the swim path (total distance moved), the time to reach the platform (escape latency) and mean swimming speed of the rats were recorded (as previously mentioned) by means of a computer-based video-tracking system Ethovision (Noldus, Wageningen 6700 AG, the Netherlands). In the probe test, the time to reach the platform was not considered, while was also analyzed the time spent in each quadrant.

Passive Avoidance Test (Paper 1)

Both learning and memory are inferred from changes in performance, with learning generally being defined as "a change in performance that occurs as a result of experience" (Walker 1968) and memory being the persistence of the learning (Jarvik 1964). Changes in performance can also arise from a number of other factors including growth, fatigue, nutrition and changes in the stimulus situation, perception or motivation (Gorelock et al. 1975). Before a change in performance

can be attributed to learning and memory processes, therefore, other factors which can change performance must be ruled out. Fear-motivated avoidance tests are usually based on electric current as source of punishment. In many tests, the floor of the apparatus is made up by a grid that can be electrified. In so-called consummatory conflict tests, the animal receives an electric shock when touching food or water. Avoidance tests are divided into two categories: passive avoidance and active avoidance. In passive avoidance, the animal has to refrain from executing a previously response, e.g. touch food or water, step down from an elevated position (to a grid floor) or step into a narrow and apparently safer place (with a grid floor). Indeed step-down or step-through tests are most frequently used to measure passive avoidance behavior. The latency to refrain from performing the punished act expresses the ability to avoid. At some later time the animal is tested to see if it has learnt to suppress the response. Three types of passive avoidance task are mainly used, step-through, step-down and a variation of step-through which is referred as an "exploratory" task. In all cases the animal is punished for making a normal locomotor response, such as moving from a light to a dark chamber (in most step-through tasks) or stepping down from a platform. At a later test trial. the latency to make the response is measured and used as an indicator of the ability to suppress the response. In an "exploratory" task, however, the measurement of interest is not the latency to step-through from one chamber to another, but rather the relative amounts of time spent in the safe and "shock" chambers during a 2 or 3 minute (usually) exposure to the passive avoidance equipment.

Four neurotransmitter systems are considered to play a key role in rodent passive avoidance response, the cholinergic, glutamatergic, GABAergic, noradrenergic, dopaminergic and serotoninergic systems. The most influential neurotransmitter on passive avoidance is dopamine according to the present findings. The second most influential transmitter is glutamate. Drugs with effects on GABAergic or cholinergic activity seem to have somewhat weaker impact. Half of the agents tested on serotonin-based neurotransmission were effective, whereas noradrenergic agents have rather weak influence on passive avoidance behavior. Serotonergic activity has been linked to emotional aspects of behavior. It has been shown that high anxiety in rats has been related to decreased levels of serotonin in the ventral striatum (Schwarting et al. 1998). Furthermore, rats exposed to conditioned fear stress show reduced freezing behavior following administration of citalopram, a selective serotonergic reuptake inhibitor. This finding strongly suggests that facilitation of serotonergic neurotransmission decreases fear (Hashimoto et al. 1999). Rats in the fear-provoking passive avoidance situation may have reduced levels of inhibitory acting serotonin. This assumption may explain why depletion of serotonin has no effect upon passive avoidance behavior,

whereas increased release has effect.

In the present thesis we used methods for rat step-through passive avoidance previously described. Briefly the passive avoidance apparatus consisted of a starting Plexiglas platform brightly illuminated by a 80 W bulb connected via a guillotine door to a Plexiglas chamber. This chamber (50x50x50 cm) had black walls, the ceiling was not illuminated and the floor consisted of 70 bars of stainless steel (0.1 cm diameter), connected to a source of scrambled shock. Some rats were subjected to a multi-trial passive avoidance acquisition session (reinforcement condition) followed 24 h later by a single-trial retention session in extinction condition. The remaining animals underwent a similar procedure, but without shock in the acquisition session (non-reinforcement condition). In the reinforcement condition, the acquisition session consisted of a maximum of ten trials, all initiated by gently placing the experimental subject in the starting platform. A trial ended when the rat stepped (step-through) with all four paws into the black chamber, which produced the closure of the guillotine door, or after 120 s had elapsed without such a response. Each step-through response was punished by a 3 s footshock of nominal intensity set at 0.5 mA (reinforcement condition). At the end of each trial. rats were removed from the apparatus and left undisturbed for a 60 s intertrial interval into the home cage. The session ended when a subject had either remained in the starting platform for 120 s in three consecutive trials, or completed 10 trials without reaching the criterion just mentioned. When a subject reached the criterion before the 10th trial. a 120 s latency score was assigned to each of the omitted trials. In the non-reinforcement condition, rats were subjected to the same procedure, but without punishment of step-through responses and with a fixed number of 10 trials. For all animals, the retention session consisted of a single trial without foot shock (extinction trial).

Hot-plate Testing (Paper 1)

Nociception indicates the study of pain sensitivity. Many behavioral tests have been used to measure nociception in rodents that can even determine the outcome with opposing alterations in pain reactivity (i.e. hypo and hyperalgesia). Among the various analgesic tests using thermal nociceptive stimulation, the most popular are the tail-flick (D'Amour and Smith 1941) and the hot plate test (Woolfe and McDonald 1944). In the hot plate test, animals are exposed only once to the heat stimulus, resulting in minimal tissue injury. The assay may be performed without any previous habituation and offers good reliability and reproducibility (Cochin 1968). In the hot plate test, in addition to analgesic responses, high hyperalgesic responses can also be detected, usually by summarizing several withdrawal behaviors (licking feet, jumping or rapidly stamping paws) that are computed as a whole (Nishihara et al. 1995; Richardson et al. 1997; 1998).

In the experiments of the present thesis pain reactivity was measured using a hot-plate apparatus. Temperature was set at 50 ± 0.2°C, cut-off time was 60 sec. Pain reactivity was measured by scoring latency to the first episode of nociceptive heat sensitivity (jumping, forepaw or hind paw licking). Latency time was determined using a digital stopwatch. The hot-plate was carried out in MAM rats and controls at post-natal day 43. Two days later, animals were tested on the hot-plate again to verify the presence of habituation profiles.

Biochemical Analyses

NGF Determination by ELISA (Papers 1, 2, 3, 4)

NGF evaluation was carried out in selected tissues of the rat brain. NGF was measured using a highly sensitive two-site immunoenzymatic assay which recognized both mouse and human NGF as previously described (Weskamp and Otten 1987; Bracci-Laudiero et al. 1992). Briefly, polystyrene 96-well microtubes immunoplates were coated with affinity purified monoclonal mouse anti-NGF antibody (Boehringer, Germany) which does not cross react with brain derived neurotrophic factor diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA) for evaluation of the non-specific signal. After an overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris-HCl (pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatin, 0.1% Triton X-100). After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl. pH 7.2, 400 mM NaCl. 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-ß-NGF-galactosidase (Boehringer Mannheim, Germany) for 2 hours at 37 °C and, after further washing, 100 µl of substrate solution (4 mg/ml of chlorophenol red, Boehringer Mannheim, Germany, substrate buffer: 100mM HEPES, 150 mM NaCl. 2mM $MgCl_2$, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 hours at 37° C, the optical density was measured at 575 nm using an ELISA reader (Dynatech, USA), and the values of standards and samples were corrected by taking into consideration the non-specific binding. NGF concentrations were determined, from the regression line for the NGF standard (ranging from 15 to 1000 pg/ml purified mouse NGF) incubated under similar conditions in each assay. The recovery of NGF during assay procedure was estimated by adding a known amount of highly purified NGF to the samples or to the homogenization buffer, as internal control. The yield of the exogenous NGF was calculated by subtracting the amount of endogenous NGF

from the value of endogenous plus exogenous values. Under these conditions, the recovery of NGF in our assay ranged from 80 to 90%. The sensitivity of the assay was about 5 pg/g of wet tissue and cross-reactivity with other related neurotrophic factors (BDNF, Neurotrophin-3 and Neurotrophin-4) was less than 3%. Data are represented as pg/g wet tissue and all assays were performed in triplicate.

BDNF Determination by ELISA (Papers 2, 3, 4)

The concentrations of BDNF in selected tissues of the rat brain were measured with an ELISA kit "BDNF Emaxtm ImmunoAssay System number G6891" by Promega, (Madison, WI, USA) following the instructions suggested by the manufacturer. Tissues were homogenated 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 per well of monoclonal anti-mouse-BDNF antibody. After an overnight incubation at 4°C, the plates were washed three times with wash 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 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 1M (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). BDNF concentrations were determined, from the regression line for the BDNF standard (ranging from 7.8 to 500 pg/ml purified mouse BDNF) incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/g of BDNF and cross-reactivity with other related neurotrophic factors (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.

NGF Determination by ELISA (Paper 5)

NGF evaluation was carried out in the hippocampus, entorhinal cortex, striatum, hypothalamus, frontal cortex and parietal cortex of the rat brain with an ELISA kit "NGF Emaxtm ImmunoAssay System number G7631" by Promega, (Madison, WI, USA) following the instructions provided by the manufacturer. Tissues were homogenated 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 per well of monoclonal anti-mouse-NGF antibody. After an overnight incubation at 4°C, the plates were washed three times with wash 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 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 1M (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 concentrations were determined, from the regression line for the NGF standard (ranging from 7.8 to 500 pg/ml purified mouse NGF) 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 of wet tissue and cross-reactivity with other related neurotrophic factors (BDNF, Neurotrophin-3 and Neurotrophin-4) was less than 3%. Data are represented as pg/g wet tissue and all assays were performed in triplicate.

BDNF Determination by ELISA (Paper 5)

The concentrations of BDNF in the same brain areas of the NGF assay of the rat brain were measured with an ELISA kit "BDNF Emaxtm ImmunoAssay System number G6891" by Promega, (Madison, WI, USA) according to the instructions of the manufacturer. Tissues were homogenated 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 per well of monoclonal anti-mouse-BDNF antibody. After an overnight incubation at 4°C, the plates were washed three times with wash 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 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 1M (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). BDNF concentrations were determined, from the regression line for the BDNF standard (ranging from 7.8 to 500 pg/ml purified mouse BDNF) incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/g of BDNF and cross-reactivity with other related neurotrophic factors (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.

RT-PCR ELISA for NGF and BDNF(Paper 4)

RT-PCR ELISA for NGF and BDNF was carried out following methods recently described (Tirassa et al. 2001). Briefly, total RNA was extracted from selected tissues of rat brain by using the method of Chomczynsky and Sacchi (1987) as modified in the TRIzol Kit (Gibco). RNA was analyzed by gel electrophoresis and its concentration was measured by spectrophotometer reading at 260 and 280 nm (1 A_{260} adsorbance unit equal to 40 mg/ml).

Complementary DNA (cDNA) was synthesized from 1μg of total RNA using 200 Units of M-MLV reverse transcriptase (Promega Italia, Milano, Italy) in 20μl of total volume reaction. To optimize reproducibility of cDNA synthesis, a master mix solution containing 250 ng Oligo $(dT)_{12-18}$ primer, 0.5 Units RNasin ribonuclease inhibitor and 0.5mM dNTP in 5X Reaction Buffer (250 mM Tris-Cl pH 8.3; 375 mM KCl; 15 mM $MGCl_2$; 50mM DTT) was used. The mixture was incubated at 42°C for 1h and the reaction was terminated with a further incubation at 95°C for 5 min. One to ten dilutions in H_2O DEPC of the synthesized cDNAs were aliquoted and stored at -20°C until use.

PCR amplification is carried out using 5' biotinylated primers to generate biotinylated PCR products detectable by digoxygenin labeled probes in an immunoenzymatic assay (ELISA). Briefly, an aliquot of cDNA was mixed with 5μl 10x buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 Units of Taq DNA polymerase (Promega), 12.5 pmol of NGF ($^{5'}$TCCACCCACCCAGTCTTCCA$^{3'}$; $^{5'}$GCCTTCCTGCTGAGCACACA$^{3'}$), 7.5 pmol of BDNF ($^{5'}$AGCTGAGCGTGTGTGACAGT$^{3'}$ $^{5'}$TCCATAGTAAGGGCCCGAAC$^{3'}$) and 3.12 pmol of GAPDH ($^{5'}$CACCACCATGGAGAAGGCC$^{3'}$; $^{5'}$CACCACCATGGAGAAGGCC$^{3'}$) primers in a final volume of 50 μl. A sample containing all reaction reagents except cDNA was used as PCR negative control in any amplification. Ten microlitres of 1:10 RT mixture without enzyme was used as further PCR negative control. The mixes were incubated for the indicated cycles (denaturation 1 min at 95°C; annealing 1 min at 55°C; extension 2min at 72°C) in a GeneAmp PCR System 9600 (Perkin-Elmer). The correct size of all PCR products was confirmed by comparing with a DNA standard on agarose gel and the identity was confirmed by Southern blotting (data not shown).

Biotinylated PCR products diluted in PBS containing 3% Bovine Serum Albumine (PBSB) were distributed in triplicates (100 μl/well) onto avidin-coated microplates and incubated 1 hr at room temperature. After incubation the microplates were washed three times with PBS containing 0.02% Tween 20 (washing buffer). DNA was denatured using 0.25 M NaOH at room temperature for 10 min. Following the washing, 100 μl/well of 4 pmol/ml digoxygenin-labeled probes (for NGF $^{5'}$TCCTGTTGAGAGTGGTGCCGGGGCATCGA$^{3'}$; for BDNF $^{5'}$TAACCCATGGGATTACACTTG-GTCACGTAG$^{3'}$ and for GAPDH

5'ACAATCTTGAGTGAGTTGTCATATTTCTCG3') in DIG Easy Hybridisation buffer (Boheringer Mannheim) were added and incubated at 42°C for two h. After washes, anti-digoxygenin POD-coupled antibody (Boheringer Mannheim) was added (1:1000 in PBSB) and incubated 1hr at 37°C. The reaction was developed by TMB (3,3',5,5'-tetramethylbenzidine; 0.6 mg in citrate buffer, pH 5.0) and blocked after 30 min with 2 M HCl. The amount of amplified products is measured as optical density at 450/690 nm ($OD_{450/690}$) using a Dynatech ELISA Reader 5000. GAPDH $OD_{450/690}$ level is used to normalize for the relative differences in sample size, integrity of the individual RNA and variations in reverse transcription efficiency.

Immunocytochemistry for ChAT, p75, NPY (papers 2, 3, 4)

Deeply anesthetized animals were perfused transcardially with 100 ml of 0.1M phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium nitrite, followed by 200 ml of 4% paraformaldehyde in PBS. Brains were then removed, post fixed in PBS buffered 4% paraformaldehyde at 4°C for 12-18 hours, and cryoprotected in PBS 20% sucrose for 24 hours. Each brain was then mounted on a stage of a freezing microtome, serial coronal sections (20 μm thick) were cut and stained with toluidine blue for general histology, or for immunohistochemical analysis. Polyclonal antibody against NPY was purchased from Peninsula Laboratories, UK. Monoclonal ChAT antibody was obtained following methods previously described (Angelucci et al. 1999). A monoclonal mouse p75 antibody was kindly donated by prof. E.J. Johnson, Washington University, St. Louis, MO, USA.

For immunolocalization brain sections containing the entorhinal cortex, the hippocampus, the septum and the Meynert's nuclei were used. We utilized anti-NPY diluted 1:100, anti-ChAT (2 μg/ml) and anti p75 diluted 1:250. Free-floating brain sections were first incubated in 0.1 M PBS (pH 7.4) containing 10% normal goat serum, 5% BSA and 0.1% Triton X-100 for 1 h at room temperature and then overnight at 4°C with NPY or ChAT antibodies (2μg/ml) in PBS containing 0.1% Triton X-100. Sections were washed three times in diluent (0.1 M PBS containing 0.1% BSA and 0.1% Triton X-100), followed by incubation with biotinylated secondary IgG anti-rabbit (1:300) for NPY or anti-mouse (1:300) for ChAT and p75 and then with avidin biotin complex kit conjugated to horseradish peroxidase as suggested (Vectastain Elite ABC kit, Vector Laboratories), for 1 h at room temperature. Antibody staining was visualized using 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxide with 0.025% cobalt chloride and 0.02% nickel ammonium sulfate as chromogens. Stained sections were mounted onto gelatin-coated slides, dried and covered for immunostained cells identification. Control experiments were carried out to ensure the specificity of the immunostaining by omission of the primary antibodies or by

preadsorbtion with an excess of antigen. No staining was observed under these conditions. Cell count was carried out by a Zeiss Axiophot microscope connected to a pc and an image analysis program (IAS 2000, Delta Sistemi, Rome, Italy or Zeiss Vidas System, Germany) by an observer who was blinded on the group assignment of the animals. To avoid errors, measurements were standardized between the experimental groups by using the same calibration system, and only cells with the range area between 100 and 200 μm^2 were considered. We selected for comparisons corresponding coronal sections exhibiting the same cytoarchitectonic features determined by using the rat brain atlas. This allowed cells to be counted in coronal section at comparable rostrocaudal positions.

Substance P Analysis (Paper 1)

A highly specific radioimmunoassay (RIA) was used (detection limit 1.5 fmol = 2 pg per incubate; detectable concentration 15 fmol/l = 20 pg per ml). Paw tissue samples were extracted with acetic acid 2N. After centrifugation, samples were lyophilized and taken up with 300 ml of assay buffer (0.1 M phosphate buffer, pH 7.4, containing 0.1% w/v BSA, 0.01% sodium azide, 0.09% sodium chloride and 1 mM EDTA) and diluted as needed to perform the radioimmuno assay using 125I-SP. A volume of standard SP, or the unknown was incubated with a volume of antiserum and a volume of tracer (5000 cpm) and the tubes were further incubated for 20 h at 4°C. The antigen-antibody complex was separated from free antigen by adding a peg/gar per nrs solution. After centrifugation the supernatant was discarded and the radioactivity of the bound fraction measured in a gamma-counter for 60 s. Recovery of SP was approximately 60% by using this procedure. Unknown values were calculated by comparing the mean counts per minutes of duplicate samples with a calibration curve of standard samples ranging from 20 to 1200 pg/ml. 125I-(p-HP)-Arg-SP was used as a tracer, with a specific activity of 60 to 65 Tbq/mol per 1 with 50% to 60% binding to the antibody in a dilution of 1:240000. The antibody (M27 from guinea pig) was directed against the C-terminal as well as the N-terminal sequence of SP. Results were expressed as pg/mg of SP and all assays were performed in duplicate.

Statistical Analyses

Data were analyzed by ANOVA considering the prenatal MAM treatment as between subject variable. For the behavioral data this required the use of nested type design (Winer 1971), in order to account for the random litter effects which could be confounded with the prenatal treatment effects (Chiarotti et al. 1987). Furthermore, for the open-field, the hot-plate tests, and the water-maze test ANOVAs for repeated measures were also applied for analyzing the 10-min session of the open-field (two successive within-session blocks, 5-min each), the

two hot-plate trials (two levels, first and second hot-plate test) and the 5 days of the test in Morris maze. As for the passive avoidance test, data were analyzed by ANOVA for the 10 trials of the acquisition phase considering the prenatal MAM treatment and the reinforcement condition as between subject variables, and the 10 trials as repeated measures. An ANOVA considering prenatal MAM treatment and reinforcement condition was also used to evaluate the number of trials needed to reach learning criterion (in the acquisition phase), and latency to step-through response (in the retention phase). In addition, the repeated latency measures were condensed into one score (RLS, repeated latency scores: number of trials needed to reach learning criterion/sum of latencies to step-through response of all repeated measures). Post-hoc comparisons within logical sets of means were performed using the Tukey's HSD test, the use of which is permissible or even recommended also in the absence of significant main or interaction effects in the ANOVA, in order to minimize frequency errors of both type I and II following the indications given by Wilcox (1987, pp 187-189).

PART III: STUDIES ON THE ANIMAL MODEL

PERGAMON

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Prenatal methylazoxymethanol acetate alters behavior and brain NGF levels in young rats: a possible correlation with the development of schizophrenia-like deficits

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Abstract

It has been hypothesized that a deleterious key contribution to schizophrenia (SZ) development is a failure of migration and setting of young neurons into their appropriate cortical target sites, particularly in the entorhinal cortex (EC). To test this hypothesis in an animal model, we injected, in pregnant rats, on gestational day (GD) 9, or 10, or 11, or 12, the antimitotic compound methylazoxymethanolacetate (MAM) known to cause EC neuronal loss. We investigated whether or not EC disruption during prenatal development is able to affect behavior, including memory and learning, and brain nerve growth factor (NGF). Prenatally MAM treated young rats didn't display gross behavioral changes in social interaction, open-field and novel object investigation tests. By contrast, GD11 and GD12 MAM treated rats had a retardation in passive avoidance acquisition, while, in GD12 animals, pain sensitivity was reduced. GD12 animals also showed increased NGF in the EC and remaining cortex. MAM treated animals showed no changes in paw NGF or substance P levels suggesting that the altered nociceptive response is not related to local downregulation of these two molecules. The possibility that these behavioral and biochemical alterations might be associated with the onset of SZ is discussed.

Keywords: MAM; NGF; Entorhinal cortex; Development; Behavior; Animal model; Schizophrenia

1. Introduction

Mounting evidence indicates that non-heritable factors in the pathogenesis of schizophrenia (SZ) are associated with abnormalities during prenatal development. Epidemiological researches have shown increased incidence of SZ in humans prenatally exposed to virus (Sham et al., 1992), malnutrition and other gestational complications (DeLisi et al., 1988; Davis et al., 1995). Post mortem studies have revealed that certain, predominantly limbic, or associative areas of SZ subjects, are characterized by decreased volume and cell numbers (Brown et al., 1986; Falkai and Bogerts, 1986), reduced content of certain microtubule associated proteins and disorganized cytoarchitecture (Arnold et al., 1991). These findings have suggested a failure of both migration and setting of young neurons into their appropriate cortical target sites, particularly in the entorhinal cortex (EC) (Braak and Braak, 1992). The EC is a brain structure contained in the limbic allocortex of the medial temporal lobe playing a key role in processing associational information (Cho and Kesner, 1996) and is linked to the hippocampus and other subcortical structures (Witter et al., 1989). It has also been assumed that memory and learning processing as well as memory consolidation involves interactions between the hippocampus and parahippocampal regions, including the EC (Eichenbaum et al., 1994). An involvement of the latter structure in human learning capabilities is suggested by a recent PET-study (Klingberg et al., 1994) and by the fact that EC damage in several neuropathologies (i.e. schizophrenia) is associated with

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cognitive impairments (Gold and Weinberger, 1995; Weinberger and Lipska, 1995).

Animal models of disease, particularly those resembling analogous defects in cortical development, could be useful to investigate how such defects translate into a disorder with cognitive, and behavioral characteristics of SZ. Such models might also offer an opportunity to verify the hypotheses that specific etiologic factors, such as changes in growth factor levels or drugs are capable of affecting the cortical development producing SZ-like abnormalities. Indeed, the administration of drugs producing or exacerbating humans' SZ symptoms or brain lesion models have been widely conducted (Port et al., 1991; Beauregard and Bachevalier, 1996; Joel et al., 1997; Sams-Dodd et al., 1997).

It has been recently shown (Talamini et al., 1998) that interference with neurogenesis in the mediotemporal allocortex of rat embryos, during the earliest stages of cortical proliferation, results in a thickness reduction of the adult EC as well as other morphological characteristics resembling those observed in patients with SZ. This animal model was obtained administering a single injection of methylazoxymethanol acetate (MAM) in pregnant rats on gestational day 9, or 10, or 11 or 12 (see Cattabeni and Di Luca, 1997) when the EC is thought to undergo major cell proliferation (Bayer and Altman, 1987).

According to previous studies, alteration in the levels of growth factors might be associated with neuropathological diseases (Hoffer and Olson, 1997). Nerve growth factor (NGF) is the best characterized member of the neurotrophin family (Levi-Montalcini, 1987), it plays an important role not only in the development and maintenance of the sensory and sympathetic nervous system, but is also present in the central nervous system (CNS), where it exerts a trophic action on the forebrain cholinergic system (Rylett and Williams, 1994). Centrally NGF is mainly produced in the hippocampus and cortex (Korsching et al., 1985) and from these structures transported to NGF-receptive neurons located in basal forebrain cholinergic neurons (Thoenen et al., 1987). NGF has been shown to be implicated in alterations of cognitive functions either following selective brain damage or aging processes (Hefti, 1986; Fisher et al., 1994) while studies carried out on young and adult mammals demonstrated that NGF plays a crucial role in the neuroplasticity of certain brain neurons (Garofalo et al., 1992). Administration of NGF can prevent neuronal cell death that would otherwise occur following injury (Kromer, 1987). Nerve growth factor also increases both in the brain and bloodstream following stressful events and anxiety-associated behaviors (Alleva et al., 1993; Aloe et al., 1994). According to a recent finding, NGF seems to be also involved in regulation of trophism and innervation of the EC (Conner et al., 1994; Van Praag et al., 1996) suggesting that deficits in the synthesis and/or release of NGF during a critical period of EC neurogenesis might result in neuronal loss in this brain region.

The main aim of the present work was to investigate, in young rats, the behavioral effects and the changes in brain NGF levels of prenatal MAM treatments aimed to disrupt EC development. The possible role of NGF in this developmental model was considered interesting, particularly, in view of the overlap of brain NGF producing areas with the regions specifically affected by MAM treatments. In order to address these issues, we tested the animals at different juvenile age points in mildly stressful experimental conditions using the following standardized methodologies: a social interaction test at postnatal day (PD) 26 aimed at detecting any subtle alterations in social and play behaviors at a critical developmental stage; an open-field and a novel object investigation test at PD 31 for evaluating locomotor activity and exploratory and displacement behaviors; a passive avoidance learning task at PD 37-38 for testing rat learning and memory capabilities; hot-plate tests at PD 43 and PD 45 to check the basal level of pain sensitivity. We also investigated the endogenous levels of NGF in the EC, in the remaining cortex and in the hippocampus. Since brain NGF has been shown to be involved in the regulation of peripheral pain sensitivity, we additionally analyzed the levels of NGF in the paws of MAM treated animals. Finally, we investigated the peripheral level of the neuropeptide substance P (SP), which is known to be both involved in pain sensitivity and regulated by NGF (Lewin et al., 1994).

2. Materials and methods

2.1. Subject housing and MAM treatment

Gestating Wistar WI rats (*Rattus norvegicus*) were obtained from an animal farm (Charles River, Germany), 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 an air conditioned room (temperature 21 ± 1°C, relative humidity 60 ± 10%), with white lights on from 07:00 to 19:00 h, in Plexiglas boxes with a metal top and sawdust as bedding. Regular rat pellet food (Hope farms, Woerden, The Netherlands) and water was available ad libitum. Pregnant rats were randomly divided into six groups GD9, GD10, GD11, GD12, saline and naive. Five pregnant rats for each GD group underwent a single intraperitoneal injection of MAM (20 mg/kg) on gestational day 9 (GD9), or GD10, or GD11 or GD12. Dams of the saline group (5 rats) received 20 mg/kg of saline solution on one of the gestational days 9 to 12.

Finally the naive animals (5 rats) did not receive anything. At birth all litters were reduced to four males and four females and fostered to the biological dams following behavioral procedures previously described (Vorhees et al., 1984; Cimino et al., 1996). Post weaning 120 prenatally MAM treated male young rats were used in the behavioral studies and 18 females for biochemical studies. As for the use of females in the latter studies, according to previous findings, we did not expect important gender differences in the SP or NGF levels of developing rats. In fact, it has been reported that NGF-mRNA expression does not differ in males and females during development (Kornack et al., 1991) and no differences were found in NGF protein either (Nishikuza et al., 1991). Moreover, the male animals of this study were destined for further behavioral and biochemical testing at the adult age. All behavioral tests took place in an experimental room with the same light–dark cycle as the housing facility, and with a conditioned air system. Thus, environmental conditions such as humidity and temperature levels inside the room were very similar to those of the housing facility. All efforts were made to minimize and reduce animal suffering and to limit the number of animals used. All animal experiments were carried out following the procedure described by the guidelines of the European Community Council Directive (86/609/EEC).

2.2. Social interaction test

Upon weaning on PD 26, nine subjects of each experimental group were randomly chosen for a single social interaction test. For this purpose, rats were transferred to the experimental room when they underwent (between 11:00 and 13:00 h) a 7-min social encounter. The encounters took place in a test cage identical to the home cage supplied with clean sawdust bedding. Each animal was gently put in the Plexiglas box together with five animals of the other experimental groups. Immediately preceding the encounter, rats were marked for individual recognition with an atoxic, odorless, permanent marker. Behaviors were videotaped under white light using a Sony VO-5360 apparatus equipped with a CH-1400CE videocamera. Recordings were scored independently by an observer who was blind to the treatment received by the animals. Data were recorded using a keyboard event system feeding to a PC for analysis ('Observer', Event Recorder version 3.0, Noldus Information Technology b.v., Wageningen, The Netherlands, 1994). Separate behavioral scores for frequency and duration (in s) were obtained for each individual in the box by running the tapes six times for each recording.

The choice of registered behavioral responses and their classification into two main groups (nonsocial and social) as described below are based principally on the ethological profile of rat behavior described by Grant and Mackintosh (1963) and Meaney and Stewart (1981).

Nonsocial responses were: exploring (moving around the cage, exploring, scanning the air), selfgrooming (keeping the mouth and paws on the body or on the head), sniffing (olfactory investigation of walls and floors), digging (digging in the sawdust, pushing and kicking it around by using the snout and/or both forepaws and hindpaws, usually while moving around the cage), rearing and wall-rearing (vertical posture of the body with the forepaws raised or placed on the walls, respectively), freezing (in which the animal, stopped because of fear, is immobile in a crouched position with flattened ears), inactivity (absence of movements without signs of fear). Information about locomotor activity was obtained by counting the number of crossings of floor squares with both forepaws during tape recording.

Social responses were: anogenital sniffing (sniffing the anogenital region of a partner), social sniffing (sniffing the partner, except the anogenital region), chasing (following the partner around the cage, without any quick or sudden movement often while maintaining a constant nose contact with its anogenital area), social grooming (licking, wiping the partner's body, in particular near the head and the dorsal region), social inactivity (lying flat, or standing still with the eyes open or closed, while maintaining close physical contact with the partners), aggressive grooming (similar to social grooming but carried out with violent movements).

2.3. Open-field and novel investigation tests

For this behavioral investigation we used 56 rats (*n*; 9 GD9, 12 GD10, 10 GD11, 8 GD12, 9 saline, 8 naive), which were not used in the social interaction test. The open-field consisted of a Plexiglas box with clean sawdust, and with the floor subdivided into 18 equal squares, placed under white lights. Several behavioral events, selected among those previously described, were scored over a period of time of 10 min and expressed as frequencies and durations (in s). These behaviors include exploring, grooming, sniffing, rearing and wall-rearing, freezing, inactivity and the number of fecal boluses emitted throughout the open-field test. Information about locomotor activity was gained by counting the number of crossings of floor squares with both forepaws.

As for the novel object investigation test, a stimulus object (a 35 mm black cartridge box) was placed in the middle of the arena immediately following the 10 min open-field recording, and the behavioral responses were scored for 3 additional min. Care was taken to minimize the introduction of human, or rodent odors along with the box. A number of elements were additionally

recorded: the latency of both the initial movement and the first contact with the stimulus object, the number of contacts and approaches (sniffing the object).

2.4. Passive avoidance

The passive avoidance apparatus consisted of a starting Plexiglas platform brightly illuminated by a 80 W bulb connected via a guillotine door to a Plexiglas chamber. This chamber (50 × 50 × 50 cm) had black walls, the ceiling was not illuminated and the floor consisted of 70 bars of stainless steel (0.1 cm diameter), connected to a source of scrambled shock. Some rats were subjected to a multi-trial passive avoidance acquisition session (reinforcement condition, n; 9 GD9, 10 GD10, 9 GD11, 8 GD12, 11 saline, 10 naive), followed 24 h later by a single-trial retention session in extinction condition. The remaining animals underwent a similar procedure, but without shock in the acquisition session (non-reinforcement condition, n; 9 GD9, 10 GD10, 10 GD11, 8 GD12, 9 saline, 9 naive).

In the reinforcement condition, the acquisition session consisted of a maximum of ten trials, all initiated by gently placing the experimental subject in the starting platform. A trial ended when the rat stepped (step-through) with all four paws into the black chamber, which produced the closure of the guillotine door, or after 120 s had elapsed without such a response. Each step-through response was punished by a 3 s footshock of nominal intensity set at 0.5 mA (reinforcement condition). At the end of each trial, rats were removed from the apparatus and left undisturbed for a 60 s intertrial interval into the home cage. The session ended when a subject had either remained in the starting platform for 120 s in three consecutive trials, or completed 10 trials without reaching the criterion just mentioned. When a subject reached the criterion before the 10th trial, a 120 s latency score was assigned to each of the omitted trials. In the non-reinforcement condition, rats were subjected to the same procedure, but without punishment of step-through responses and with a fixed number of 10 trials. For all animals, the retention session consisted of a single trial without foot shock (extinction trial).

2.5. Hot-plate response

Pain reactivity was measured using a hot-plate apparatus. Temperature was set at 50 ± 0.2°C, cut-off time was 60 s. Pain reactivity was measured by scoring the latency to the first episode of nociceptive heat sensitivity (jumping, forepaw or hind paw licking; n: 18 GD9, 20 GD10, 20 GD11, 16 GD12, 20 saline, 19 naive). Latency time was determined using a digital stopwatch. The hot-plate response was carried out at PD 43. Two days later, animals were tested on the hot-plate again to verify the presence of habituation profiles.

2.6. NGF determination

NGF evaluation was carried out in the EC, in the remaining cortex, hippocampus and in the paws, of 45-day-old female rats (3 animals for each prenatal MAM treatment group). After an overdose of Nembutal, paws and brain regions were quickly removed and the levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay (Weskamp and Otten, 1987) which recognizes human and murine NGF and does not cross react with brain derived neurotrophic factor. Briefly, polystyrene 96-well microtube immunoplates (Nunc) were coated with affinity purified, polyclonal goat anti-NGF antibody, diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA), for evaluation of the non-specific signal. Following an overnight incubation at room temperature and a 2 h incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris–HCl, pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatin, 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris–HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-β-NGF-galactosidase (Boehringer Mannheim, Germany) for 2 h at 37°C and, after further washing, 100 μl of substrate solution (4 mg/ml of chlorophenol red, Boehringer Mannheim, Germany, substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 h at 37°C, the optical density was measured at 575 nm, using an ELISA reader (Dynatech), and the values of standards and samples were corrected by taking into consideration the non-specific binding. Under these conditions the sensitivity was 3 pg/ml and the recovery of NGF in our assay ranged from 80 to 90%. The recovery was estimated by adding to the hippocampus and cortex extracts a known amount of purified NGF and the yield of the exogenous NGF was calculated by subtracting the amount of this NGF from the endogenous NGF. Data were represented as pg/g wet wt. and all assays were performed in triplicate.

2.7. Substance P analysis

A highly specific radioimmunoassay (RIA) was used (detection limit 1.5 fmol = 2 pg per incubate; detectable concentration 15 fmol/l = 20 pg per ml). Paw tissue samples were extracted with acetic acid 2N from 50-day-old female rats (3 animals for each prenatal MAM treatment group). After centrifugation, samples were lyophilized and taken up with 300 μl of assay buffer (0.1 M phosphate buffer, pH 7.4, containing 0.1% w/v BSA, 0.01% sodium azide, 0.09% sodium chloride and 1 mM EDTA) and diluted as needed to perform the radioimmuno assay using ^{125}I-SP. A volume of standard SP, or the unknown was incubated with a volume of antiserum and a volume of tracer (5000 cpm) and the tubes were further incubated for 20 h at 4°C. The antigen-antibody complex was separated from free antigen by adding a peg/gar per nrs solution. After centrifugation the supernatant was discarded and the radioactivity of the bound fraction measured in a gamma-counter for 60 s. Recovery of SP was approximately 60% by using this procedure. Unknown values were calculated by comparing the mean counts per minutes of duplicate samples with a calibration curve of standard samples ranging from 20 to 1200 pg/ml. ^{125}I-(p-HP)-Arg-SP was used as a tracer, with a specific activity of 60 to 65 TBq/mol per l with 50% to 60% binding to the antibody in a dilution of 1:240 000. The antibody (M27 from guinea pig) was directed against the C-terminal as well as the N-terminal sequence of SP. Results were expressed as pg/mg of SP and all assays were performed in duplicate.

2.8. Statistical analysis

Data were analyzed by ANOVA considering the prenatal MAM treatment as the between subject variable. For the behavioral data this required the use of nested type design (Winer, 1971), in order to account for the random litter effects which could be confounded with the prenatal treatment effects (Chiarotti et al., 1987). Furthermore, for the open-field and the hot-plate tests, ANOVAs for repeated measures were also applied for analyzing the 10-min session of the open-field (two successive within-session blocks, 5-min each) and the two hot-plate trials (two levels, first and second hot-plate test). For the passive avoidance test, data were analyzed by ANOVA for the 10 trials of the acquisition phase considering the prenatal MAM treatment and the reinforcement condition as between subject variables, and the 10 trials as repeated measures. An ANOVA considering prenatal MAM treatment and reinforcement condition was also used to evaluate the number of trials needed to reach learning criterion (in the acquisition phase), and the latency to the step-through response (in the retention phase). In addition, the repeated latency measures were condensed into one score (RLS, repeated latency scores: number of trials needed to reach learning criterion/sum of latencies to step-through response of all repeated measures). Post-hoc comparisons within logical sets of means were performed using the Tukey's HSD test, the use of which is permissible or even recommended in the absence of significant main or interaction effects in ANOVA, in order to minimize frequency errors of both type I and II following the indications given by Wilcox (1987), pp. 187–189).

3. Results

The prenatal MAM treatment, as adopted in this study, induces a number of alterations in adult brain morphology, described in detail by Talamini et al., (1998). Briefly, analysis of the forebrain indicated abnormal development of the EC in all treatment groups (GD9, GD10, GD11 and GD12). This was apparent from reduced cortical thickness, disorganized cytoarchitecture, and abnormal temporal asymmetry. Abnormalities shifted from lateral to medial divisions of the EC, following a known developmental gradient, and were progressively more severe, with later administration of MAM. The morphological effects of MAM were predominantly found in the EC in GD9, GD10 and GD11 treated rats. By contrast, GD12 treated rats showed somewhat more widespread damage, involving more general cortical thinning and reductions in the regions of the ventral hippocampus and amygdala.

3.1. Social interaction test

Table 1 shows the effects of prenatal MAM treatment on 26-day-old rats. ANOVA showed that MAM did not affect significantly social behaviors of the animals whereas it induced only slight changes in non-social behaviors. A main effect of MAM was revealed on locomotor activity [$F(5,18) = 4.58$, $P = 0.01$]. Post-hoc comparisons show that GD12 rats displayed an increased number of crossings compared to the GD10 group ($P < 0.05$ in post-hocs) missing significance with respect to saline and naive animals ($P = 0.06$ in post-hoes).

3.2. Open-field and novel investigation tests

Fig. 1 shows the behavioral effects of MAM except for responses with little informative value due to low baseline rates that were not enhanced by MAM (freezing, inactivity). Conventional frequency data are reported for the behaviors, because duration data are similar, but less illustrative of MAM effects. A main effect of prenatal MAM treatment was revealed by

ANOVA for both rearing frequency and duration [$Fs(5,18) = 6.57$, 5.48, respectively, $Ps < 0.01$]. Specifically, post-hocs revealed that GD12 rats had increased vertical movements of the body more evident when compared to the saline group ($Ps < 0.05$).

During the 10-min test rats displayed significant habituation profiles for locomotor activity, exploring (both frequency and duration), sniffing (frequency and duration) and wall-rearing bouts [$Fs(1,18) = 16.88$, 17.97, 24.89, 12.77, 5.20, 16.61, respectively, $Ps < 0.05$ in the repeated measures]. In contrast, grooming (frequency and duration) and time spent in sniffing increased during the observation period [$Fs(1,18) = 12.73$, 15.65, 5.20, respectively, $Ps < 0.05$ in the repeated measures].

An interaction between prenatal MAM treatment and repeated measures was shown by ANOVA for locomotor activity, time spent in grooming (for both in absence of any litter effect) [$Fs(5,50) = 2.34$, 2.69 respectively, $Ps < 0.05$] and rearing (frequency and duration) [$Fs(5,18) = 2.43$, $P < 0.05$]. According to post-hoc comparison, GD12 animals had a stronger decrease in locomotion and rearing during the test when compared to the other groups ($P < 0.05$). GD9 and GD10 rats during the test showed a higher potentiation of this grooming behavior with respect to saline or naive animals ($P < 0.05$).

Prenatal MAM treatment did not produce changes in the behavior during the novel object investigation test (Table 2) where rats spent more time in fear related behaviors (freezing) than in exploring the object.

3.3. Passive avoidance

No major effect of MAM administration was found during the ten trials of the acquisition session. Overall, the step-through latency increased over successive trials of the acquisition session (Figs. 2 and 3A) in both the reinforcement and non-reinforcement groups [repeated measures, $F(9,216) = 110.80$, $P < 0.01$]. This increase

Table 1
Selected elements of the non-social/social behavioral repertoire of prenatally MAM treated rats during a 7-min social interaction test

	GD9	GD10	GD11	GD12	Saline	Naive
Non-social behaviors						
Exploring						
F	114.44 ± 4.47	117 ± 8.90	113.44 ± 4.90	128.00 ± 6.88	115.22 ± 5.97	123.55 ± 7.30
D	150.33 ± 6.23	170.66 ± 28.66	141.88 ± 8.71	144.00 ± 10.24	151.66 ± 6.37	145.00 ± 8.94
Selfgrooming						
F	6.11 ± 1.11	5.44 ± 1.26	4.55 ± 0.86	7.00 ± 1.14	4.33 ± 1.00	6.33 ± 2.90
D	34.77 ± 7.98	24.00 ± 5.58	24.77 ± 5.88	31.77 ± 5.32	18.77 ± 4.41	42.33 ± 13.93
Sniffing						
F	88.11 ± 4.41	94.22 ± 6.81	88.66 ± 5.06	97.55 ± 4.35	88.22 ± 5.66	90.66 ± 6.67
D	94.66 ± 7.49	95.44 ± 6.85	103.33 ± 8.32	108.22 ± 6.42	111.88 ± 10.86	90.55 ± 8.37
Rearing						
F	9.88 ± 2.26	7.11 ± 1.32	15.55 ± 3.07	13.22 ± 1.90	11.66 ± 3.87	15.55 ± 2.68
D	9.88 ± 2.60	8.66 ± 1.90	19.00 ± 5.02	16.77 ± 2.62	14.00 ± 4.82	18.22 ± 3.16
Wall-rearing						
F	30.00 ± 2.34	30.66 ± 4.59	33.66 ± 1.81	34.77 ± 1.52	26.44 ± 2.31	30.88 ± 4.51
D	59.33 ± 6.21	63.44 ± 8.98	57.33 ± 4.19	62.66 ± 5.64	51.55 ± 7.82	52.44 ± 7.59
Crossings	152.66 ± 8.29	120.55 ± 12.81	145.66 ± 7.27	171.88 ± 5.40*,**	138.55 ± 8.26	141.55 ± 6.32
Social behaviors						
Anogenital sniffing						
F[a]	9.88 ± 1.21	7.44 ± 2.25	14.44 ± 3.36	10.00 ± 2.04	12.44 ± 3.44	9.77 ± 1.97
D[a]	24.53 ± 6.14	20.33 ± 6.44	28.44 ± 6.04	19.88 ± 4.73	28.44 ± 6.18	18.33 ± 3.55
Chase						
F	3.11 ± 0.94	2.77 ± 0.77	4.55 ± 0.89	2.88 ± 0.94	1.66 ± 0.33	2.77 ± 0.57
D	2.77 ± 0.81	2.44 ± 0.68	3.33 ± 0.64	2.33 ± 0.74	1.33 ± 0.23	2.22 ± 0.59
Social grooming						
F	5.22 ± 0.74	4.44 ± 0.93	7.11 ± 1.24	7.66 ± 1.89	6.77 ± 1.79	7.55 ± 2.42
D	9.11 ± 2.16	11.66 ± 4.16	16.44 ± 3.88	14.66 ± 3.16	19.33 ± 6.11	15.22 ± 4.97
Aggressive groom						
F	0.11 ± 0.11	0.44 ± 0.242	1.66 ± 0.73	0.77 ± 0.46	0.44 ± 0.242	0.22 ± 0.14
D	0.11 ± 0.11	1.55 ± 1.10	2.44 ± 1.14	0.88 ± 0.56	0.44 ± 0.242	0.77 ± 0.66
Social sniffing						
F	13.00 ± 2.23	11.55 ± 2.23	18.22 ± 3.96	17.66 ± 1.50	18.44 ± 3.89	14.88 ± 1.72
D	17.55 ± 3.66	17.00 ± 5.63	20.33 ± 4.26	18.22 ± 1.29	21.00 ± 4.50	16.66 ± 2.47

[a] F = frequency; D = duration (s).
* $P < 0.05$;
** $P = 0.06$, see results for post-hoc explanation.

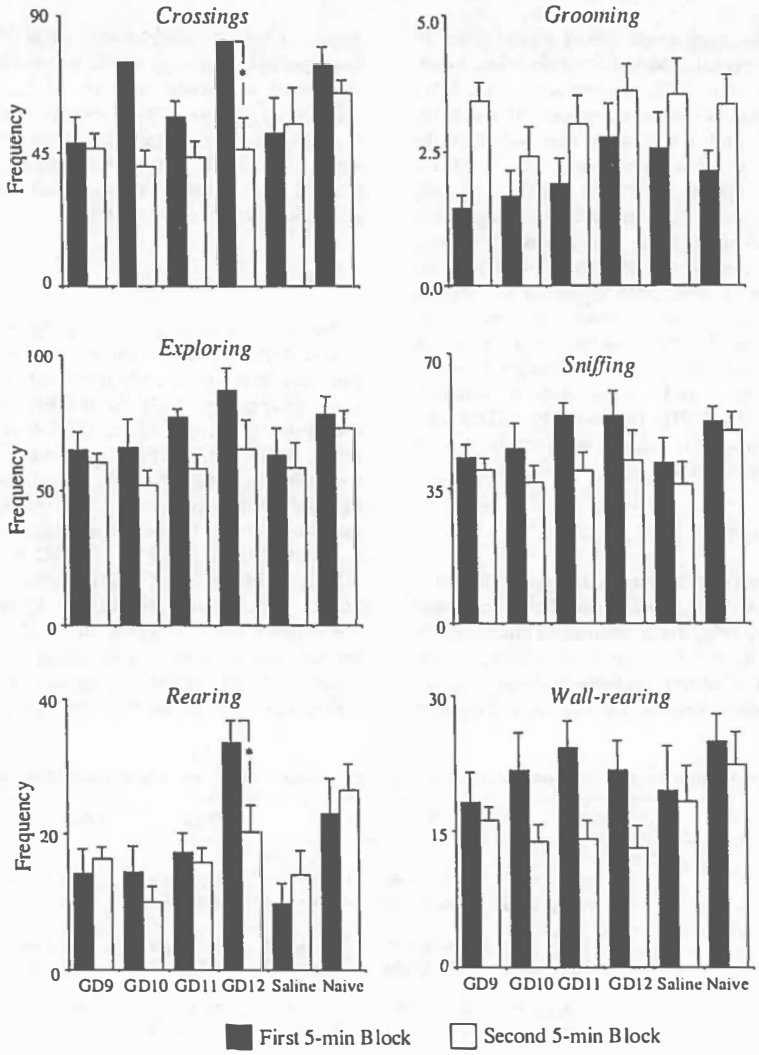

Fig. 1. Mean locomotor activity (number of crossings) and mean frequencies of behaviors recorded during a 10 min session (two successive within-session blocks, 5-min each) in an open-field of prenatally MAM treated rats and their respective controls. Data represent mean levels ($\pm$SEM). Asterisks indicate significant between-group differences (* $P < 0.05$).

was much more marked in the reinforcement condition that in the non-reinforcement condition, providing strong evidence of effective passive avoidance learning [reinforcement condition × repeated measures, $F(9,216) = 107.36$, $P < 0.01$]. In addition, this effect was also confirmed by the significant effect of the reinforcement condition during the ten passive avoidance trials of the acquisition phase [reinforcement condition, $F(1,24) = 2456.80$, $P < 0.01$].

The analysis of the number of trials needed to reach the learning criterion and RLS scores (Fig. 3B, Fig. 3C) showed that both GD11 and GD12 rats showed a retardation ($P < 0.05$ in post-hocs) in passive avoidance acquisition when compared to saline or naive animals [main effect of MAM treatment, $Fs(5,24) = 8.16, 4.93$, respectively, $Ps < 0.01$]. Although the ANOVA did not reveal a significant interaction between MAM treatment, reinforcement condition and repeated measures, statistical analysis also showed significant differences between reinforced GD11/GD12 rats and reinforced saline/naive

animals during the acquisition phase [main effect of MAM treatment; prenatal MAM × reinforcement condition, $Fs(5,24) = 7.81$, 3.72, respectively, $Ps < 0.05$]. ANOVA of the number of trials needed to reach the learning criterion and RLS score revealed also the expected effect of the reinforcement condition [$Fs(1,24) = 577.87$; 806.40, $Ps < 0.01$]. Furthermore, the fact that the impairment was limited to the speed of the acquisition process more than acquisition per se was confirmed by the absence of any MAM effect in the retention trial latency (Fig. 3D) suggestive of changes only in short-term attentional levels and acquisition capabilities. During the retest, ANOVA showed the expected marked difference in step-through latencies between reinforced and no-reinforced animals [$F(1,24) = 328.88$, $P < 0.01$]. Interestingly, GD11 and GD12 rats had a latency to step-through similar to that of saline and naive animals.

3.4. Hot-plate response

Statistical analysis of the data showed that rats prenatally treated with MAM at gestational day 12 exhibited significant analgesia (Fig. 4) suggestive of a change in the baseline level of pain sensitivity [$F(5,18) = 7.89$ $P < 0.01$ for the main effect of MAM treatment]. During the two hot-plate tests, animals showed a significant habituation profile in licking behavior [$F(1,18) = 177.95$ $P < 0.01$ in the repeated measures]. Furthermore ANOVA showed a significant interaction between treatment and repeated measures [$F(5,18) = 5.90$, $P < 0.01$]. Also this interaction is due to the higher baseline levels of pain sensitivity showed by GD12 rats during the two hot-plate tests ($Ps < 0.05$ in post-hocs comparison) and by the absence of habituation in GD9 animals.

3.5. NGF determination

Fig. 5 shows the NGF levels in the EC and hippocampus of MAM treated animals. ANOVA revealed that this treatment significantly increased ($Ps < 0.01$ in post-hocs) endogenous NGF in the EC in GD12 animals compared to naive, saline, GD9 and GD10 animals ($P < 0.01$ for the main effect of MAM). In the remaining cortex both GD12 and GD11 animals had higher values of NGF compared to the other groups ($Ps < 0.05$ in post-hocs) ($P < 0.01$ for the main effect of MAM treatment: GD9, 782.91 ± 13.85; GD10, 810.15 ± 28.42; GD11, 981.02 ± 43.64; GD12, 983.47 ± 20.83; saline, 864.32 ± 56.31; naive, 842.48 ± 37.32 pg/g wet tissue). In the hippocampus, changes in NGF levels in MAM treated animals were not significant ($P = 0.069$ for the main effect of MAM treatment). In the paws, no differences were found between groups ($P = 0.064$ for

Table 2
Selected elements of the behavioral repertoire of prenatally MAM treated rats during a 3-min novel object investigation test

	GD9	GD10	GD11	GD12	Saline	Naive
Exploring						
F[a]	13.00 ± 3.74	17.75 ± 4.09	15.80 ± 2.57	26.00 ± 4.63	16.44 ± 3.96	23.87 ± 6.32
D[a]	64.00 ± 13.12	54.08 ± 10.31	68.00 ± 13.14	54.37 ± 9.96	61.44 ± 11.55	75.37 ± 13.48
Grooming						
F	0.55 ± 0.44	0.41 ± 0.22	0.20 ± 0.20	0.87 ± 0.47	0.22 ± 0.14	0.12 ± 0.12
D	4.00 ± 3.87	0.91 ± 0.66	8.70 ± 8.70	7.12 ± 6.16	0.33 ± 0.23	0.12 ± 0.12
Sniffing						
F	8.00 ± 2.30	10.33 ± 2.77	10.30 ± 2.37	17.25 ± 3.38	9.66 ± 2.55	16.25 ± 5.10
D	7.77 ± 2.43	9.50 ± 2.58	9.20 ± 2.37	14.00 ± 2.84	8.44 ± 2.82	13.62 ± 3.90
Rearing						
F	0.44 ± 0.24	1.33 ± 0.66	0.70 ± 0.59	1.87 ± 1.06	0.66 ± 0.44	1.50 ± 1.22
D	0.88 ± 0.48	2.41 ± 0.29	0.60 ± 0.49	2.12 ± 1.27	0.66 ± 0.44	2.62 ± 2.34
Wall-rearing						
F	0.88 ± 0.61	3.00 ± 0.95	0.70 ± 0.39	3.00 ± 1.21	2.00 ± 1.26	2.62 ± 1.01
D	4.88 ± 3.33	7.83 ± 3.06	2.10 ± 1.77	4.37 ± 2.49	4.11 ± 2.77	6.87 ± 3.08
Crossings	7.66 ± 6.05	14.66 ± 4.09	6.10 ± 1.98	32.00 ± 8.33	16.66 ± 5.51	17.37 ± 6.64
Freezing						
F	2.11 ± 0.42	2.33 ± 0.48	2.30 ± 0.44	2.50 ± 0.92	2.22 ± 0.49	2.87 ± 0.81
D	90.22 ± 21.31	69.33 ± 20.64	71.80 ± 18.67	70.00 ± 17.00	85.00 ± 19.30	66.12 ± 22.61
Novel object contact						
F	1.33 ± 0.74	7.33 ± 2.30	5.70 ± 2.28	10.50 ± 2.90	5.88 ± 2.15	6.12 ± 2.56
D	1.22 ± 0.66	16.91 ± 7.89	10.50 ± 5.25	14.50 ± 4.74	7.11 ± 2.67	6.50 ± 3.01
Approaching the novel object						
F	2.88 ± 1.89	9.66 ± 2.61	7.00 ± 2.53	13.37 ± 3.28	8.22 ± 2.97	8.37 ± 3.20
D	6.77 ± 5.57	13.91 ± 3.98	8.70 ± 3.09	12.50 ± 2.92	12.11 ± 5.46	8.62 ± 2.91
Latency to novel object contact(s)	135.44 ± 23.86	91.66 ± 20.67	99.20 ± 23.05	91.25 ± 21.49	110.44 ± 24.63	72.12 ± 31.65

[a] F = frequency; D = duration (s).

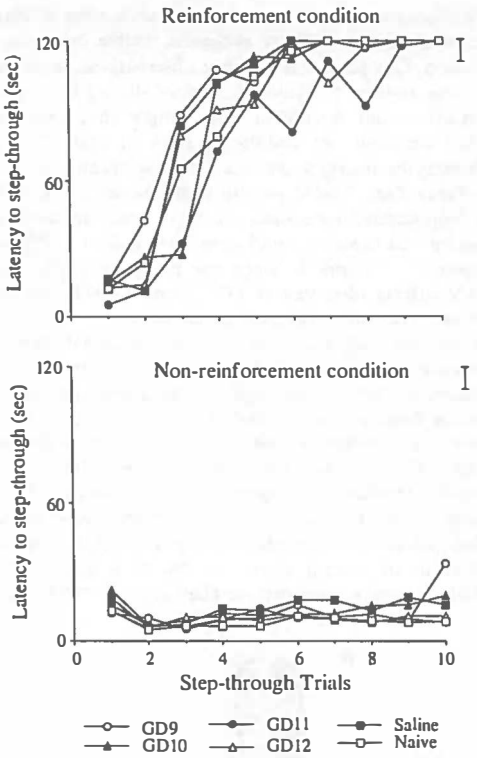

Fig. 2. Acquisition of step-through passive avoidance during 10 successive trials in prenatal MAM treated animals and control rats. Data represent mean levels. The vertical lines in the figure indicate pooled SEM's derived from appropriate error mean square in the ANOVA.

the main effect of MAM treatment; GD9, 27.75 ± 6.50; GD10, 31.43 ± 8.95; GD11, 32.55 ± 7.91; GD12, 46.25 ± 4.95; saline, 43.95 ± 7.42; naive, 51.70 ± 5.65 pg/g wet tissue), suggesting that paw NGF seems not to be implicated in pain sensitivity changes discovered in GD12 rats.

3.6. Substance P analysis

Similarly to paw NGF, the analysis of peripheral SP did not reveal differences between groups ($P = 0.88$ for the main effect of MAM treatment; GD9, 3.97 ± 0.74; GD10, 3.67 ± 0.44; GD11, 3.45 ± 0.34; GD12, 3.80 ± 0.40; saline, 3.96 ± 0.48; naive, 3.35 ± 0.32 pg/mg wet tissue).

4. Discussion

Our results show that prenatal MAM treatment can induce changes, in both behavior and brain NGF levels in young rats. Morphological data show that interference in cell division during prenatal development, from gestational day 9 to day 12 in rats, progressively reduces formation of the EC, in the posterior temporal region of the brain. Within this brain region, cortical reduction is more pronounced caudally than rostrally in all groups. Furthermore the lateral EC is predominantly affected by treatment on GD9, GD10, or GD11, whereas GD12 treated rats also show abnormalities of the medial EC. Moreover, in GD12 animals, MAM treatment also produced cortical and hippocampal thinning, disorganised cortical layering and abnormal temporal asymmetries (see Talamini et al., 1998 for further explanations). Brain abnormalities due to prenatal MAM failed to importantly affect social behavior of young rats. This finding suggests that, during development, slight disruptions of cortical proliferation are not sufficient to induce changes in social behavior. Because social and play behaviors are prominent in mammals, and mammals have a more elaborated cortical structure than non-mammalian species, it has been suggested that the cortex could play a crucial role in the regulation of these behaviors (see review in Vanderschuren et al., 1997). However, neuropharmacological investigations have shown that opioid, dopamine, cholinergic and noradrenergic systems play a role in the regulation of these behavioral activities, suggesting that the expression of a complex phenomenon such as social behavior involves a wide variety of neuronal systems. Thus, different aspects of social behavior are also regulated and/or modulated by different neuronal pathways, such as those of the olfactory bulb, thalamus, septum, caudate putamen and amygdala (see references in VanderSchuren et al., 1997). Our present findings suggest that slight disruptions of early cortical proliferation, and the EC changes this induces, apparently do not affect social behavior of young rats. This concords with the supposed role of the EC in processing complex contextual information (Klingberg et al., 1994), which probably is more important in adult, than in pre-adolescent social interaction.

Abnormalities in locomotion and rearing were observed in the open-field. Alterations in locomotion and exploratory activities in novel environmental situations (e.g. in social interaction/open-field) have been suggested to reflect an impaired capability to cope with stressful conditions (Belzung and Le Pape 1994). As previously reported (Talamini et al., 1998), prenatal MAM administration on gestational day 12 induces slight but widespread cortical changes associated with a small brain reduction in rats. These small brain abnormalities could be associated with the behavioral changes observed in the open-field test in the locomotor activity and exploration of GD12 rats, since the neural substrates of these behavioral patterns are widely distributed all over the brain.

Another effect of prenatal MAM administration was apparent in open-field behavior of GD9 and GD10-treated rats where grooming increased in these animals. In rodents, grooming can represent a common attitude of body care, a displacement behavior due to a stressful environmental situation and/or a behavioral pattern in which expression is affected by specific damage to its neural substrates (see for review Spruijt et al., 1992). Indeed, it has been reported that grooming is influenced by manipulation of the striatonigrocollicular pathway and by alterations in cholinergic neurons and muscarinic receptors (see references in Spruijt et al., 1992). However, forebrain morphological analysis (Talamini et al., 1998) did not provide evidence of disruptions or abnormalities due to MAM in the striatum of these rats. Hence, grooming as shown in GD9 and GD10 rats most likely represents a displacement reaction to a new environment.

GD11 and GD12 rats showed a retardation in passive avoidance acquisition. The alterations in learning and memory processes were limited to the acquisition phase, since no effects were found during the retention phase, which took place 1 day later. This is suggestive of a disruption in the associative mechanisms serving the learning and memory processes, active during acquisition. Our passive avoidance observations, together with the findings previously described during the social interaction and open/field tests, imply that prenatal MAM treatment on gestational days 11 and 12 may influence the neural systems underlying specific behavioral responses. The observation that both the EC and the hippocampal formation are brain areas involved in learning and memory capabilities (Vnek et al., 1995) is suggestive of a link between the brain morphological MAM effects observed in GD11 and GD12 animals and the reported behavioral impairments.

Hot-plate responses showed that prenatal MAM treatment on gestational day 9 induces an absence of habituation during two repeated tests whereas GD12 animals displayed an overall increased analgesia. Pain sensitivity in rodents seems to be regulated by different peripheral and central pathways (Kavaliers, 1988). For example, exposure to exogenous or endogenous TNF-α is able to induce changes in nociception (Fiore et al., 1996) and so does the administration of NGF, via the activation of several neuropeptides such as SP and calcitonin gene related peptide (Lewin et al., 1994). Our

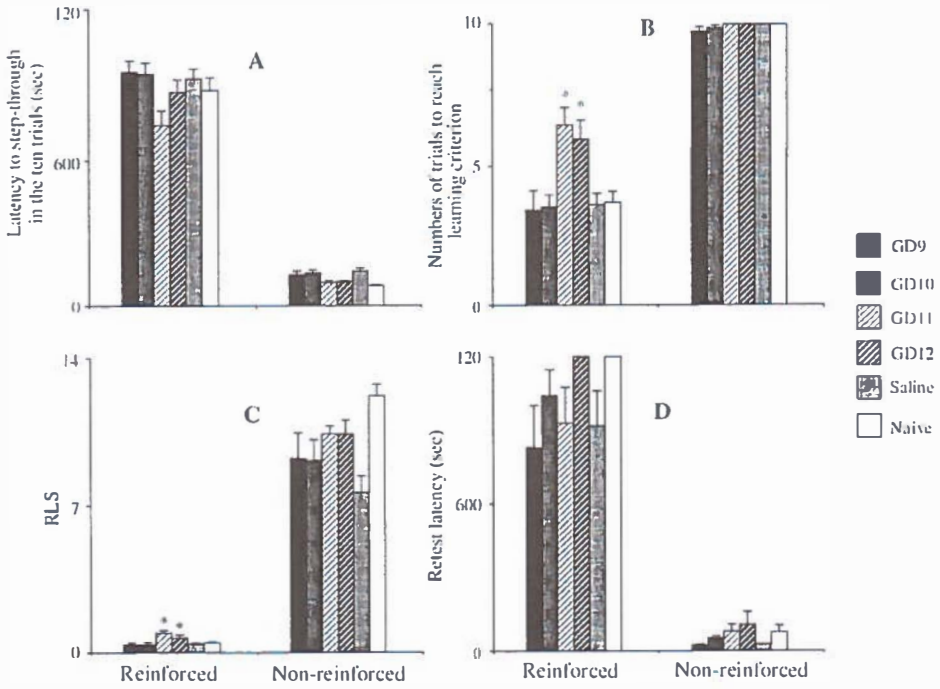

Fig. 3. Latency to step-through response throughout the 10 trial sessions of the acquisition phase (A), number of trials to reach criterion during the acquisition phase (B), repeated latency score (number of trials needed to reach learning criterion/sum of latencies to step-through response of all repeated measures) (C) and latency to step-through response in the retention phase (D) in prenatal MAM treated rats and control animals, both reinforced and non-reinforced. Data represent mean levels (± SEM). Asterisks indicate significant differences between groups (* $P < 0.05$).

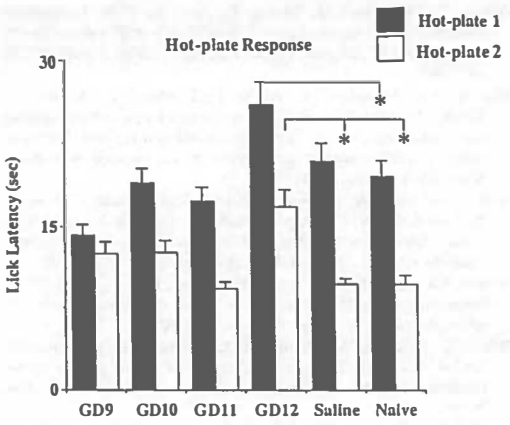

Fig. 4. Hot-plate responses of rats prenatally treated with MAM. The nociceptive thresholds was measured as latency to the first episode of heat sensitivity (jumping or paw licking). Data represent mean levels (± SEM). Asterisks indicate significant differences between groups (* $P < 0.05$).

preliminary immunoenzymatic finding show that both NGF and SP did not change in the paws of MAM treated rats, suggesting that this treatment and maldevelopment of the EC might alter other central pathways regulating pain sensitivity. Interestingly, it has been reported that humans affected by the SZ disorder show altered peripheral sensory responses leading to a strong analgesia (Guieu et al., 1994; Dworkin, 1994).

Nerve growth factor increased in GD12 animals in the EC and in the remaining cortex. The mechanisms underlying this increase in the EC and in the remaining cortex in GD12 rats is at present not known. One possibility is that the abnormal neuronal cytoarchitecture induced by MAM treatment disrupted the NGF pathways and altered the distribution of NGF-receptors on NGF-responsive cells leading to an accumulation and non-utilization of NGF. Accumulation of NGF in the cortex due to deficits in NGF-receptors' distribution and/or failure of brain neurons responding to the action of NGF has been previously reported in human subjects affected by other neurological diseases such as Alzheimer's disease (Scott and Crutcher, 1994). In addition, increased NGF-like activity in young rat brain has been also revealed after lesions (Scott et al., 1994). Alternatively, it is conceivable that neurons resistant to the antimitotical effects of MAM, for some unknown reason, produced elevated amounts of NGF or alternatively, glial cells, which under normal condition do not extensively contribute to NGF release, could produce this neurotrophin after neuronal damage (Bakhit et al., 1991). Interestingly, it has been found previously that SZ patients had decreased NGF levels in the bloodstream (Bersani et al., 1996) compared to healthy subjects while no data are available on the levels of this trophic factor in the brain of schizophrenics. Since NGF is a large molecule and it does not cross the blood-brain barrier, these differences might be associated with changes in the synthesis and/or uptake of NGF into the circulation or the brain. Altered levels of NGF were also found in SZ patients treated with haloperidol, a neuroleptic drug commonly used for psychiatric disorders (Aloe et al., 1997). Moreover, using an animal model, it has been shown that rodents treated with haloperidol are characterized by low NGF levels in the brain and in the bloodstream (Alleva et al., 1996). The hypothesis that neurotrophins might be implicated in the neurodevelopmental deficits of SZ is supported by other recent reports showing that Neurotrophin-3 gene polymorphism is associated with this disorder (Nanko et al., 1994) and by the fact that embryonic hippocampal tissue derived from SZ women and transplanted into rat hosts, displayed profound abnormalities in survival and growth compared to control tissue (Freedman et al., 1994).

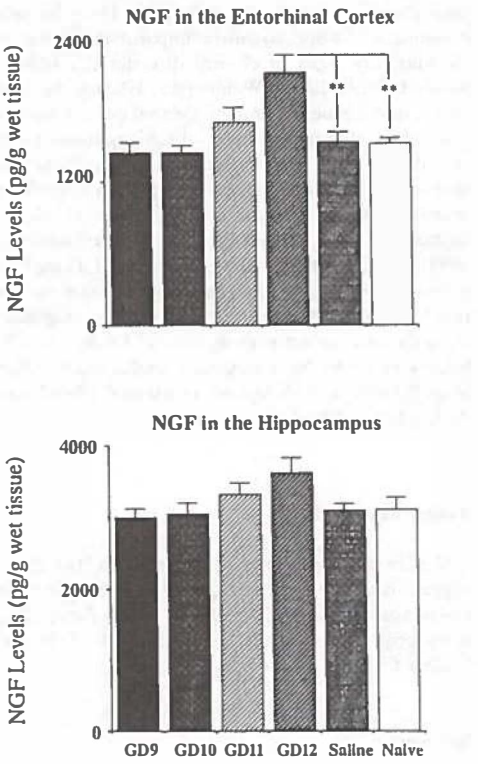

Fig. 5. Levels of endogenous NGF (expressed as pg/g of fresh tissue) in the entorhinal cortex and hippocampus of prenatal MAM treated rats and their respective controls (saline and naive). Data represent mean levels (± SEM). Asterisks indicate significant differences between groups (** $P < 0.01$).

The morphological changes due to the EC disruptions observed present some general similarities with post mortem studies on schizophrenic subjects. In addition, our combined behavioral observations suggest that prenatal MAM treatment on gestational days 11 and 12 can induce impairments in coping with mildly stressful stimuli and in the associative, or attentional aspects of learning. It should be also noted that the behavioral alterations observed in GD12 rats could suggest that these animals might be abnormally reactive to external stimuli (Ferguson et al., 1993). This interpretation is also supported by the supposed role of the EC in sensory integration. The slower acquisition rate found in GD12 MAM rats might then result from altered information processing during the learning task. Considering the possible relevance of this procedure in modeling neurodevelopmental aspects of SZ, it is noteworthy that the developmental behavioral abnormalities it induces are subtle, in line with most reports on SZ-like behavior. In fact, our findings on memory and learning capabilities of young rats also bare some likeness to observations carried out on humans affected by SZ. Many SZ patients demonstrate subtle cognitive impairments long before showing any signs of clinical disorder (Comblatt and Kellp, 1994; Gold and Weinberger, 1995), indeed, subtle social, developmental and intellectual deficits were noted in children who later developed schizophrenia (Jones et al., 1995). Since disturbances in transmitting signals through the EC and hippocampus plays a key role in the development of schizophrenia (Arnold et al., 1991; Beauregard and Bachevalier, 1996; Akil and Lewis, 1997), the morphological and behavioral animal model proposed here might offer an additional tool to analyze this hypothesis. Moreover, our data also suggests that learning and memory dysfunctions related to SZ-like behaviors could be a potential useful marker for the identification and definition of clinical illness liability during the onset of SZ disease.

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Prenatal exposure to methylazoxymethanol acetate in the rat alters neurotrophin levels and behavior: considerations for neurodevelopmental diseases

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Abstract

We did a single injection of methylazoxymethanol acetate (MAM) in pregnant rats on gestational day (GD) 11 or 12 to investigate the long-lasting effects of early entorhinal cortex (EC) and hippocampus maldevelopment on behavior, brain nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) levels, and the neurotrophin receptor p75 and choline acetyltransferase (ChAT) immunoreactivity. Adult animals treated with MAM had compromised EC development and showed changes in locomotion and displacement activities. In addition, rats treated on GD 12 had increased concentration of NGF and BDNF in the EC and hippocampus if compared to control rats. Prenatal MAM administration did not affect significantly p75 and ChAT distribution in the EC and septum. Results are discussed in reference to the neurodevelopmental hypothesis of psychiatric disorders. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: NGF; BDNF; Behavior; Schizophrenia; ChAT; p75

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are expressed in a variety of brain regions both during prenatal development and adult life [11]. High concentrations of NGF and BDNF are present in the developing central nervous system (CNS) [52] where they are known to play a crucial role in growth, plasticity, and function of brain neurons and in a variety of CNS disorders, including those associated with deficits in cognitive functions [31]. NGF is also a neurotrophin influencing the development and functioning of the forebrain cholinergic neurons [30,38,46,61] and participates in the neuroregulation and fine-tuning of behavior [2]. Moreover, it has also been shown that cholinergic pathways are implicated in the pathogenesis of neuropsychiatric diseases [10,60]. Both NGF and BDNF have been found in the cortical association areas as the entorhinal cortex (EC) and their expression undergoes significant changes following chemical or surgical insults [22,44]. The EC and the hippocampus are associational areas [40] where neurotrophins are expressed playing a key role in the regulation of the behavior, and entorhinal–hippocampal projections may be among the first cortical connections to be established in the human brain. Human studies on EC and hippocampus also revealed that changes in these brain areas are associated with a wide number of neuropsychiatric disorders [57] and recent studies have suggested that neurotrophic factors may also be implicated in neurodevelopmental disorders [14,53].

Thus, the aim of the present investigation was to characterize in an animal model of maldevelopment of both EC and hippocampus the long-lasting changes on behavior and neurochemical markers such as NGF, and BDNF implicated in cholinergic and non-cholinergic brain neurotransmission. This animal model [29,58,59] consists in disrupting the EC/hippocampal axis during the earliest stages of cortical proliferation in rats by administering a single injection of methylazoxymethanol acetate [MAM, [18,27]] in pregnant rats at gestational day (GD) 11 or 12

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when the EC is thought to undergo major cell proliferation [12]. We tested the adult animals in: a social interaction test aimed at detecting any subtle alterations in social behaviors; an open-field and a novel object investigation test for evaluating locomotor activity and exploratory and displacement behaviors such as grooming or olfactory investigation. We also measured the levels of NGF and BDNF in four brain regions using a sensitive enzyme-linked immunosorbent assay (ELISA) and the immunopositivity for p75 (the low affinity neurotrophin receptor) and choline acetyltransferase (ChAT) in the EC. p75 is a receptor that binds to both NGF and BDNF [11] whereas ChAT is known to be regulated by NGF [38,42].

1. Methods

1.1. Subject housing and MAM treatment

Gestating Wistar WI rats (*Rattus norvegicus*) were obtained from an animal farm (Charles River, Germany), 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 an air-conditioned room (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 (Hope Farms, Woerden, the Netherlands) and water were available ad libitum. According to procedures previously described [58,59], pregnant rats were divided in four groups GD11, GD12, saline and naive. Five pregnant rats for each GD group underwent a single intraperitoneal injection of MAM (20 mg/kg between GD11 and GD12. Dams of the saline group (five rats) received 20 mg/kg of saline solution on GD 11. The naive animals (five rats) did not receive anything. The choice of day 11 and 12 of pregnancy for administering MAM has been done following the indication given previously [29,58,59], which showed that major behavioral and EC impairments are present in animals that received prenatal MAM on GD 11 or 12.

At birth all litters were reduced to four males and four females and fostered to the biological dams following behavioral procedures previously described [21,65]. Post-weaning prenatally MAM-treated male adult rats (age 6 months) were used for the behavioral and biochemical studies. All behavioral tests took place in an experimental room with the same light–dark cycle of the housing facility, and with an air-conditioned system. Thus, environmental conditions such as humidity and temperature levels inside the room were very similar to those of the housing facility. All efforts were made to minimize and reduce animal suffering and to limit 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 number of animals used for neuropathological studies in each experimental group (for immunocytochemistry $n=3$; for the ELISA $n=5$) was reduced for the guidelines of our Intramural Ethical Committee.

1.2. Social interaction test

At 6 months of age, animals were tested for a single social interaction test (n; eight GD11, nine GD12, nine saline, nine naive). To this purpose, rats were transferred to the experimental room where they underwent (between 11 a.m. and 1 p.m.) a 7-min social encounter. The encounters took place in a test cage identical to the home cage supplied with clean sawdust bedding. Each animal was gently put in the Plexiglas box together with five animals of the other experimental groups [29]. Immediately preceding the encounter, rats were marked for individual recognition with an atoxic, odorless, permanent marker. Behaviors were videotaped under white light and recordings were scored independently by an observer who was blind to the treatment received by the animals. Data were recorded using a keyboard event system feeding to a PC for analysis ("Observer," Event Recorder version 3.0, Noldus Information Technology, Wageningen, the Netherlands, 1994). Separate behavioral scores for frequency and duration (in seconds) were obtained for each individual in the box by running the tapes six times for each recording.

The choice of registered behavioral responses and their classification in two main groups (non-social and social) as described below are based principally on the ethological profile of rat behavior described by Grant and Macintosh [36] and Meaney and Stewart [48].

Nonsocial responses were: exploring (moving around the cage, exploring and scanning the air), self-grooming (keeping the mouth and paws on the body or on the head), sniffing (olfactory investigation of walls and floors), digging (digging in the sawdust, pushing and kicking it around by using the snout and/or both forepaws and hindpaws, usually while moving around the cage), rearing and wall-rearing (vertical posture of the body with the forepaws raised or placed on the walls, respectively), freezing (in which the animal, stopped because of fear, is immobile in a crouched position with flattened ears), and inactivity (absence of movements without signs of fear). Information about locomotor activity was obtained by counting, during tape running, the number of crossings of floor squares drawn on the TV screen with both forepaws.

Social responses were: anogenital sniffing (sniffing the anogenital region of a partner), social sniffing (sniffing the partner, except the anogenital region), chasing (following the partner around the cage, without any quick or

sudden movement, often while maintaining a constant nose contact with its anogenital area), social grooming (licking, wiping the partner's body, in particular near the head and the dorsal region), social inactivity (lying flat, or standing still with the eyes open or closed, while maintaining close physical contact with the partners), and aggressive grooming (similar to social grooming but carried out with violent movements).

1.3. Open-field and novel object investigation tests

One week after the social interaction test, animals were tested in an open-field (n; eight GD11, nine GD12, nine saline, nine naive). The open-field consisted of a Plexiglas box, with clean sawdust and with the floor subdivided in 18 equal squares, placed under white lights. Several behavioral events, selected among those previously described, were scored over a period of time of 10 min and expressed as frequencies and durations (in seconds). These behaviors include exploring, grooming, sniffing, rearing and wall-rearing, freezing, inactivity and the number of fecal boluses emitted throughout the open-field test. Information about locomotor activity was gained by counting the number of crossings of floor squares with both forepaws.

As for the novel object investigation test, a stimulus object (a 35-mm black cartridge box) was placed in the middle of the arena immediately following the 10 min open-field recording, and the behavioral responses were scored for 3 additional minutes. Care was taken to minimize the introduction of human or rodent odors along with the box. A number of elements were additionally recorded: the latency of both the initial movement and the first contact with the stimulus object, the number of contacts and approaches (sniffing the object).

1.4. NGF determination

NGF evaluation was carried out in the EC, parietal cortex, hippocampus and striatum of five animals for each prenatal MAM treatment group 5 days after the open-field. After an overdose of Nembutal rats were killed, brains extracted and then brain regions inspected and quickly removed and the levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay [68] that recognizes human and murine NGF and does not cross-react with BDNF. Samples were collected and centrifuged at 8500 × g for 30 min. Briefly, polystyrene 96-well microtubes immunoplates (Nunc) were coated with affinity-purified, polyclonal goat anti-NGF antibody, diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA), for evaluation of the non-specific signal. Following an overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris–HCl, pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatin, 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris–HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-β-NGF-galactosidase (Boehringer Mannheim, Germany) for 2 h at 37°C and, after further washing, 100 μl of substrate solution (4 mg/ml of chlorophenol red, Boehringer Mannheim, substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 h at 37°C, optical density was measured at 575 nm, using an ELISA reader (Dynatech), and the values of standards and samples were corrected by taking into consideration the non-specific binding. Under these conditions the sensitivity was 3 pg/ml and the recovery of NGF in our assay ranged from 80% to 90%. The recovery was estimated by adding to the hippocampus and cortex extracts a known amount of purified NGF and the yield of the exogenous NGF was calculated by subtracting the amount of this NGF from the endogenous NGF. Data were represented as pg/g wet weight and all assays were performed in triplicate.

1.5. BDNF determination

The concentrations of BDNF in brain samples of MAM-treated rats and controls (n=5 for each group) were measured with an ELISA kit "BDNF Emaxtm ImmunoAssay System number G6891" by Promega, Madison, WI, following the instructions suggested by the manufacturer. After an overdose of Nembutal brain regions were inspected, quickly removed and dissected on ice and stored at −70°C until neurochemical analysis. EC, parietal cortex, hippocampus and striatum were homogenated in the kit calibration buffer and centrifuged at 10000 × g. The brain tissues were homogenized with ultrasonication in extraction buffer 0.2% triton. Briefly, 96-well immunoplates (Nunc) were coated with 100 μl per well of monoclonal anti-mouse-BDNF antibody. After an overnight incubation at 4°C, the plates were washed three times with wash 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 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). BDNF concentrations were determined from the regression line for the BDNF standard (ranging from 7.8 to 500 pg/ml purified mouse BDNF) incubated under similar conditions in each assay. The sensitivity of the assay was 15 pg/ml of BDNF and cross-reactivity with other related neurotrophic factors (NGF, NT-3 and NT-4) was less than 3%. Data were represented as pg/g wet weight and all assays were performed in duplicate.

1.6. Immunocytochemistry for p75 and ChAT

A week after the end of the behavioral studies, three animals per group were anaesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4. The brains were removed and placed in a cryoprotectant solution of 20% sucrose in PBS and sections were cut on a cryostat. In order to quench endogenous peroxidase activity, sucrose post-equilibrated coronal brain sections (30 μm in thickness) were incubated with phenylhydrazine (20 mg/ml in PBS, 0.1% Triton) in a 1:10 dilution of either normal horse or goat serum for 45 min depending on the species that the biotinylated antibody was raised in. Using an avidin–biotinylated horseradish peroxidase system (Vector Laboratories, Burlingame, CA), the following proteins were used as primary antibodies for overnight incubation at 4°C; a monoclonal mouse p75 antibody (kindly donated by E.J. Johnson from the Dept. of Pharmacology, Washington University, St. Louis, MO) and a mouse monoclonal anti-ChAT both raised against rat brain ChAT. This latter monoclonal antibody has been produced and characterized previously and for further details of the methods see Angelucci et al. [6]. Immunopositive EC neurons were evaluated in six comparable between groups consecutive sections starting from plate 38 to plate 42 of the rat brain atlas [51] using a Zeiss Axiophot microscope. Since NGF is produced in the hippocampus and retrogradely transported in the septum, neurons of six consecutive sections of the hippocampus (plates 29–35 of the atlas) and six consecutive sections of the medial septum (15–20 of the atlas) were also evaluated. Cell counts were carried out according to methodology previously described [7]. The results were analyzed using an image analysis morphometry program (Zeiss Vidas System, Germany).

1.7. Statistical analysis

Data were analyzed by ANOVA considering the prenatal MAM treatment as variable. For the behavioral data this required the use of nested type design [70], in order to account for the litter effects that could be confounded with the prenatal treatment effects [19]. Furthermore, for the open-field, ANOVAs for repeated measures were also applied for analyzing the 10-min session of the open-field (two successive within-session blocks, 5 min each). Post-hoc comparisons within logical sets of means were performed using Tukey's test, the use of which is permissible or even recommended also in the absence of significant main or interaction effects in the ANOVA, in order to minimize frequency errors of both type I and II following the indications given (Ref. [69], pp 187–189).

2. Results

2.1. Morphological observations

Prenatal MAM treatment, as used in this study, induces a number of changes in adult brain morphology previously described in detail by Talamini et al. [58,59]. Briefly, analysis of the forebrain indicated abnormal development of the EC in all treatment groups (GD11 and GD12). This was evident from reduced cortical thickness, disorganized cytoarchitecture and abnormal temporal asymmetry. Histological changes shifted from lateral to medial divisions of the EC, in line with a known developmental gradient. Minor effects of MAM outside the EC occurred in a few limbic affiliated regions. GD12 treated rats showed also more extensive cortical thinning and reduction in the regions of the ventral hippocampus. Consistent with the observation reported by Talamini et al. [58], the EC of rats treated with MAM displays a marked reduction of thickness, as a result of neuronal loss in all cortical layers (Figs. 1G and 2H,I).

2.2. Social interaction test

In the social interaction test animals did not show aggressive behaviors (which were not included in the analysis) but mainly exploratory activities. ANOVA showed that MAM did not affect significantly the social behaviors of the animals but induced only slight changes in non-social behaviors. A main effect of MAM was revealed for number of crossings [$F(3,15)=7.76$, $p<0.01$]. Post-hoc comparisons show that GD12 rats had increased locomotion compared to control animals ($p<0.05$ in post-hoc; GD11, 157.40 ± 11.57; GD12, 181.47 ± 6.38; saline, 146.47 ± 6.93; naive, 148.58 ± 9.56).

2.3. MAM effects on open-field behavior

MAM-treated rats did not show gross changes in the behavior in the open-field. During the 10-min test rats displayed significant habituation profiles for locomotory activity, exploring (both frequency and duration) and sniffing (frequency) [$F(1,15)=12.38$, 21.37, 19.34, 16.45, $p<0.01$, respectively]. By contrast, time spent in grooming

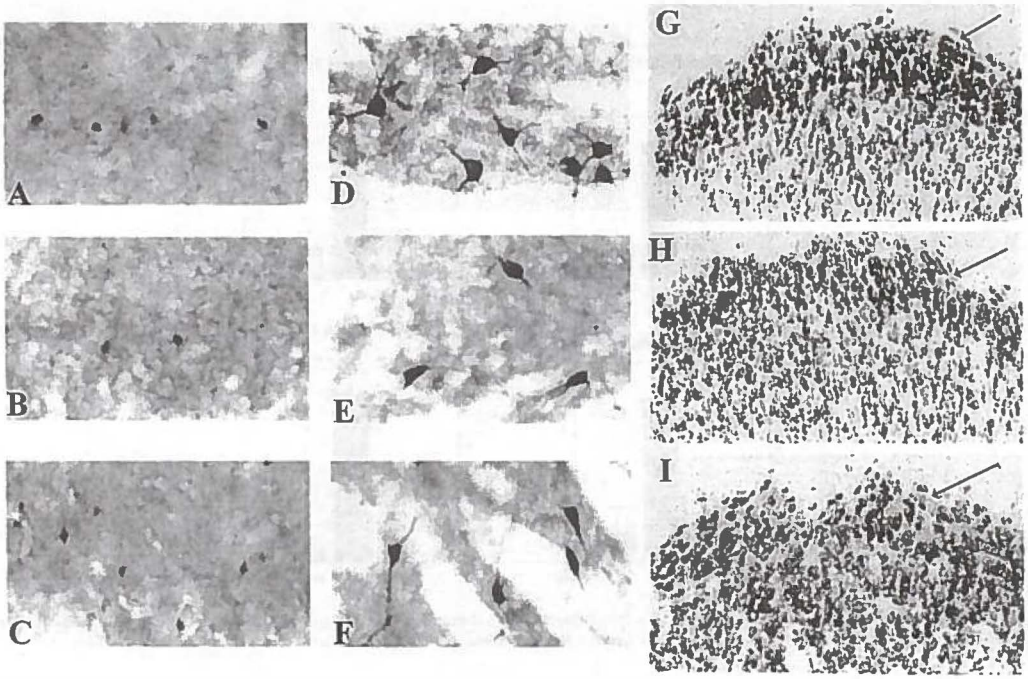

Fig. 1. (A,B,C) Photomicrographs of the EC of adult saline (A), GD11 (B) and GD12 rats (C) displaying NGF-receptor immunopositivity (× 300). (D,E,F) Photomicrographs of the EC of adult saline (D), GD11 (E) and GD12 rats (F) displaying ChAT immunopositivity. The number of ChAT-immunoreactive cells were more numerous and were larger than NGF-R-positive cells, suggesting that these markers are not co-expressed by the same cells (× 300). (G,H,I) Light toluidine blue microscopic evaluation illustrating the histological changes in the EC of MAM-treated rats. Note the layer II (arrow) disorganization in the EC of GD11 or GD12 MAM-treated rats (H and I, respectively) as compared to a Saline brain (G) (× 150).

and rearing (frequency and duration) increased during the observation period [$F(1,15)=5.02$, 6.63, 14.54, respectively, $p<0.05$ in the repeated measures]. Furthermore, no interaction between prenatal MAM treatment and repeated measures was shown by ANOVA.

Prenatal MAM treatment produced changes in displacement and escaping behaviors during the novel object investigation test in GD12 animals. ANOVA showed that these animals potentiated grooming (both frequency and duration) [$F(3,15)=4.00$, 4.01, respectively, $p<0.05$ for the main effect of the treatment] as compared to controls ($p<0.05$ in post-hoc, Fig. 2) and also increased wall-rearing duration [$F(3,15)=3.25$, $p<0.05$] if compared to saline ($p<0.05$ in post-hoc, Fig. 2).

2.4. MAM effects on basal brain NGF levels

MAM-treated rats showed changes in brain NGF concentration expressed in pg/g wet weight. As shown in Fig. 3, ANOVA revealed an increase in NGF in GD12 rats in the hippocampus and EC ($p<0.05$ for the main effect of the treatment) when compared to naive or saline-treated animals ($p<0.05$ in post-hoc comparisons). Since both hippocampus and EC in GD11 and GD12 rats showed significantly reduced wet weight [58], the total amount of NGF available is lower (40% decreasing in GD12 animals) though, for compensatory mechanisms or other, the hippocampal and EC tissue of MAM-treated rats produces more NGF protein per gram of tissue if compared to controls. No differences were found in the parietal cortex and in the striatum.

2.5. MAM influences on brain BDNF levels

ELISA for BDNF showed similar results to those observed for NGF (Fig. 4). ANOVA revealed that GD12 rats had increased levels of this neurotrophin in the hippocampus and EC ($p<0.05$ for the main effect of the treatment) when compared to naive or saline rats ($p<0.05$ in post-hoc). Similarly to NGF, for the reduced wet weight of EC and hippocampus, the total amount of BDNF available is lower (35% decreasing in GD12 animals).

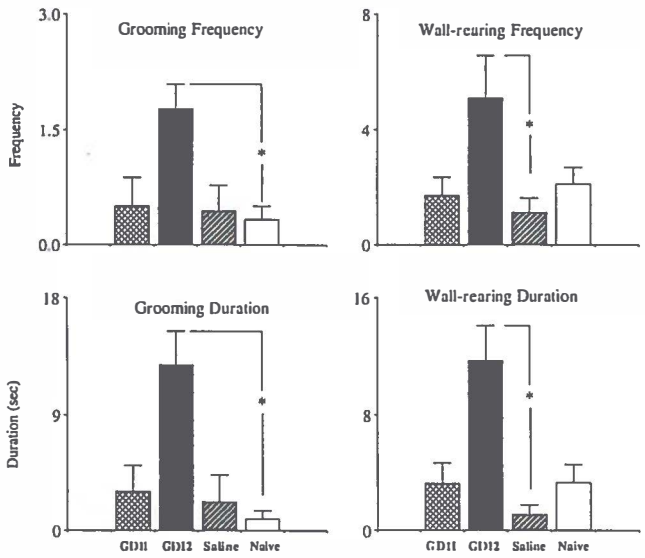

Fig. 2. Mean frequency and duration of grooming and wall-rearing behaviors of MAM-treated rats and their respective controls (saline and naive) measured in a 3-min novel object investigation test. Data represent mean levels (±SEM). Asterisks indicate significant between-group differences (* $p < 0.05$).

Striatum and the parietal cortex remained unaffected. The present study also revealed that the constitutive levels of BDNF in all brain regions tested, including EC, is higher than the amount of the respective NGF. This difference is highly evident and occurs both in controls and MAM treated rats.

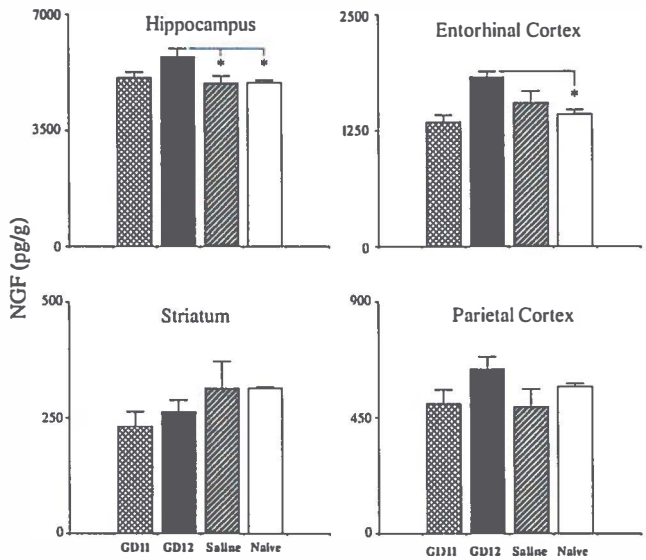

Fig. 3. Levels of endogenous NGF (expressed as pg/g of fresh tissue) in the brain of prenatal MAM-treated rats and their respective controls (saline and naive). Data represent mean levels (± SEM). Asterisks indicate significant differences between groups (* $p < 0.05$).

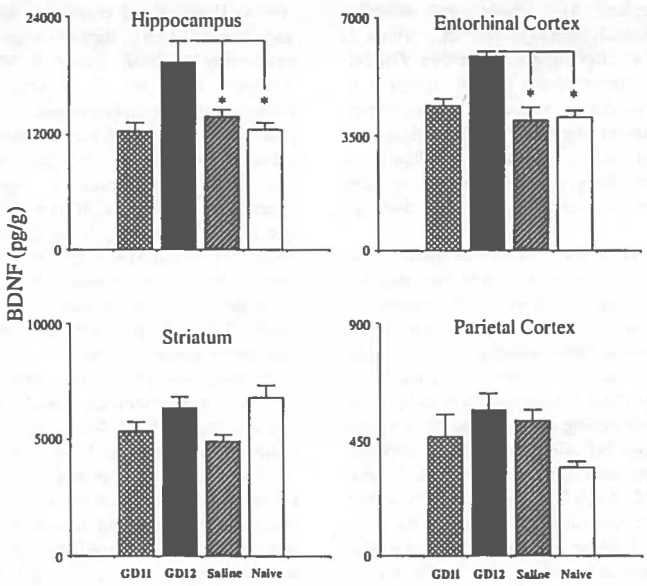

Fig. 4. Levels of endogenous BDNF (expressed as pg/g of fresh tissue) in the brain of prenatal MAM treated rats and their respective controls (saline and naive). Data represent mean levels (±SEM). Asterisks indicate significant differences between groups (* $p < 0.05$).

2.6. P75 and ChAT distribution

To gain additional information about neurotrophin alteration we studied the distribution of low affinity NGF receptor p75 and ChAT immunocytochemistry on serial brain section. As shown in Fig. 1, statistical analysis indicates that p75 (Fig. 1A–C) and ChAT (Fig. 1D–F) immunopositivity distribution and staining intensity in the EC were not affected by prenatal MAM administration. In the septum also statistical evaluation showed no significant changes in immunopositive cells for these markers though a tendency to numerical increase ($p = 0.08$) was observed.

3. Discussion

3.1. General consideration

The aim of our investigation was to gain potential useful information about the role of EC/hippocampal axis development in neurotrophin synthesis in rats treated prenatally with MAM. Our working hypothesis was that changes in neurotrophin synthesis and release or utilization during a prenatal developmental time may be a cause or contribute to EC maldevelopment. The results of this study show that prenatal MAM administration causes long-lasting structural alteration in adult rats. It was shown that the EC and hippocampus of GD12 rats are characterized by a significant increase in NGF and BDNF, two neurotrophins playing a critical role in neuronal growth and plasticity [11,34]. As for behavior, in the social interaction test we found that MAM adult animals displayed increased locomotion, while in the open-field treated rats displayed only mild changes in behavior compared to control rats. Locomotion and exploratory activity were unaffected while displacement behaviors were potentiated. Interestingly, animals showing increased NGF/BFDF levels had stronger changes in behavior.

3.2. Behavioral findings

Although elements of social behavior appeared unchanged, nonsocial behavior was affected in GD12 treated rats. These animals, in fact, displayed slightly increased locomotion during the social interactions. Alterations in locomotion and exploratory activities in novel environmental situations (e.g., in social interaction/open-field) have been suggested to reflect impaired capability to cope with stressful conditions [13]. In addition, it has been demonstrated that MAM microencephalic rats have notable hyperactivity compared to control rats, as measured by several behavioral parameters in an automated field apparatus [66]. It is known that the hippocampus is involved in motor control. Thus, both adult hippocampal

lesions and microencephaly with hippocampal reduction result in notable hyperactivity [17]. The EC, which is considered part of the hippocampal formation [20,24], might play a subtler role in motor control. It might to some extent be co-involved in motor regulation, which could explain the "context dependent" hyperactivity observed in the present study. Moreover, it should be mentioned that general hyperactivity could be a very unspecific effect that might result from several developmental insults.

MAM rats in a stressful situation as the novel object investigation potentiated some displacement and escaping behaviors. As for grooming, in rodents it can represent a repetitive/stereotyped behavior indicating a common attitude of body care, a displacement behavior due to stressing environmental situation and/or a behavioral pattern the expression of which is affected by specific damage to its neural substrates [56]. Grooming behavior can be observed in situations in which two behavioral systems are activated simultaneously or when the course of an activated behavioral routine is blocked. It can be clearly related to a state of indecisiveness or stressing situation of the animal (e.g., in response to a novel environment). It has been also reported that grooming is affected in rodents with lesions of nigro-striatal pathways [56] suggestive of an involvement of dopaminergic pathways. In fact, the substantia nigra pars reticulata seems to process internal information into behavior and the colliculus converts external inputs into behavior [15,62].

The behavioral observations observed here suggest that prenatal MAM treatment on GD 12 can induce impairments in coping with mildly stressful condition. It should be also noted that the behavioral alterations observed in GD12 rats could suggest that these animals might be abnormally reactive to external stimuli [26,27]. This hypothesis is also supported by the presumed role of EC in sensory integration [16,41]. In addition, the hippocampus and EC are involved in memory formation and are affected in neurodegenerative diseases with memory impairments such as Alzheimer's disease and prenatal MAM-treated rats show changes in passive avoidance acquisition with puberty [29] and in water maze response with aging (unpublished results).

3.3. Changes in neurotrophin levels

NGF and BDNF [52] regulate the naturally occurring neuronal death during neurogenesis and promote repair in several neurological central and peripheral insults [38,43,54]. Moreover, NGF and BDNF are both known to play a crucial role in brain neuropeptide and neurotransmitter synthesis and release [14,23,53], including those implicated in neuropsychiatric-like disorders. Neurotrophins and their receptors could be regulated by neuronal activity, by mechanisms involving the release of endogenous neurotransmitters and hormones [5] as well as stress-related events including aggressive behavior and anxiety [3,4], further suggesting a link between availability of NGF and/or BDNF and neurobehavioral disorders. Since the EC is structurally and functionally connected to the hippocampus [24,63,64], the increase in neurotrophin concentration in this latter brain structure seems to indicate that the structural EC disorganization can lead to an increase in these neurotrophins. The functional significance of this potentiation is at present not known. However, it is interesting to note that the effect of prenatal MAM administration in the brain of our rats occurs in the absence of gliosis, a finding that is consistent with that observed in neurodevelopmental discases [25,67]. Since neurotrophins are implicated in preventing neuronal damage [11,38], in protecting the cells from apoptosis [55], and counteracting the effects of chemical and pharmacological insults [28,43], an increased synthesis of NGF and BDNF in the brain of MAM-treated mice might be important in the regulation of neuronal survival. In a previous work [29] we found increased NGF levels in the brain of young rats prenatally treated with MAM and we speculated that this potentiation could represent a defense mechanism to protect the remaining neurons from potential damage or to compensate early neuronal disruptions. However, in the present work, since animals treated with MAM were analyzed at the adult stage, other hypotheses can be done. It might be possible that changes in NGF/BDNF concentration could be related with the observed changes in the behavior of GD12 rats. In fact, stress-related behaviors lead to significant changes in the basal NGF levels both in humans and animals. In humans, the level of NGF increases in the blood of young soldiers who experienced their first parachute jump and the release of NGF in the bloodstream is triggered not only by jumping, but also by the anxiogenic-like behavior induced by the novel experience [4]. Because the release of NGF precedes the release of cortisol and ACTH, it was hypothesized that a correlated event occurs between released NGF and anxiogenic status [4]. In rodents, highly stressing situations as such aggressive or submissive behaviors altered the basal levels of NGF [2] both in the brain and in the periphery. All these results are suggestive of a NGF role in coping and neuroendocrine mechanisms.

This study also indicates that in adult rats prenatally treated with MAM the expression of ChAT or p75 is unaffected (only in the septum did we find a non-significant increase in positive cell number in line with a previous work that demonstrated that ChAT specific activity increased following prenatal MAM administration at GD 13 in the neocortex of rats [39]). This observation suggests that these biological mediators are not directly implicated in the deficits due to MAM treatment or alternatively that other compensatory mechanisms for both the EC–hippocampal or EC–septum formations have been developed.

4. Conclusions

Human studies showed that neuronal disruptions at different stages of prenatal development can affect both behavior and brain morphology [45]. The MAM model presented here induces subtle changes in limbic structures resembling post-mortem observations carried out in schizophrenic patients showing a main EC prenatal role in the development of the disease [1,8,9,29]. These studies prospected that changes in EC development during prenatal life could be a cause of schizophrenia though this hypothesis is still debated [35,37,71]. At the behavioral level we presently show that MAM-treated rats had affected displacement behaviors suggestive of changes in coping mechanisms and patients affected by neuropsychiatric diseases such as schizophrenia may display multiple repetitive behaviors suggesting that these changes are varied manifestations of a single underlying biobehavioral disturbance [15,47,50]. We also found disrupted brain neurotrophin levels in MAM rats. Altered levels of NGF were found in humans affected by neuropsychiatric disorders and treated with haloperidol [5] and schizophrenic patients had decreased NGF levels in the bloodstream [14] compared to healthy subjects while no NGF reliable data are available on the brain of schizophrenics. The hypothesis that BDNF and NGF might participate in diseases characterized by brain maldevelopment such as schizophrenia is supported by other recent works [32,33,49], evidencing a major role played by neurotrophins in impaired CNS development.

In conclusion, in the present study we have provided evidence that two well-characterized neurotrophins, normally produced in the developing and adult brains of mammals, including humans, might play an important role in the development of EC abnormalities. Since both human recombinant NGF and BDNF are available, the possibility exists that these neurotrophins or their antibodies might be useful for preclinal or clinical assays as biological markers or potential therapeutic agents for studying neuropsychiatric disorders with a supposed developmental origin.

Acknowledgments

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Bromodeoxyuridine and methylazoxymethanol exposure during brain development affects behavior in rats: consideration for a role of nerve growth factor and brain derived neurotrophic factor

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Abstract

Rats prenatally exposed to the neurotoxins methylazoxymethanol (MAM) or 5-Bromo-2′-deoxyuridine (BrdU) are used as animal models of brain maldevelopment. We administered in rats MAM (20 mg/kg), or BrdU (100 mg/kg) or both at gestational day 11. Locomotion was not affected by any prenatal treatment whereas learning was delayed in the Morris maze in MAM animals. BrdU induced decreased NGF and BDNF levels in the hippocampus. In the parietal cortex prenatal BrdU administration induced NGF potentation associated with decreased BDNF. Animals treated with both MAM and BrdU showed also an increased immunopositivity for choline acetyltransferase (ChAT) and low affinity neurotrophins' receptor (p75) in the septum and Meynert's nuclei. These findings suggest that embryonic exposure to MAM and/or BrdU may be useful for studying mechanisms associated with neurodegenerative diseases affecting brain morphology and behavior.

Keywords: NGF; BDNF; Neurodevelopment; Bromodeoxyuridine; Methylazoxymethanol; Rat

A critical aspect of the mammalian brain development is characterized by neuronal growth, differentiation, migration and proper matching of pre-synaptic axon to specific post-synaptic target neurons. However, the understanding of the mechanisms of these processes is still largely unknown, although it has been shown that trophic factors such as NGF and BDNF play a crucial role in regulating brain development and behavior [1,13]. Animal models of disease, particularly those resembling defects in cortical development, are widely utilized as novel approach to investigate how such defects translate into neurological disorders and behavioral impairments including neuropsychiatric-like diseases. Prenatal administration of 5-Bromo-2′-deoxyuridine (BrdU), a halogenated pyrimidine analog, or methylazoxymethanol (MAM), a compound which has been successfully used to inhibit cortical proliferation, have been extensively adopted as teratogens [3,15]. Embryonic BrdU competes with thymidine for sites on replicating DNA strand inducing brain malformations and several behavioral changes [11]. MAM is a short-acting alkylating agent that methylates nucleic acids, leading to the death of cells that are actively replicating DNA and rats prenatally exposed to MAM display severe impairments in cognition, locomotion and mood which were associated with changes in brain neurotrophins and neuropeptides distribution [6,7,16,17].

Aim of the present study was to compare MAM and BrdU effects on the behavioral and molecular mechanisms associated with changes in brain neurotrophins levels and cognition. We administered in pregnant rats or MAM, or BrdU, or both MAM and BrdU or saline solution as controls. We analyzed also ChAT immunopositivity and p75 neurotrophins' receptor in selected brain regions [9,12]. According to previous studies [11,17], pregnant rats received, at gestational day 11 (GD11), an intraperitoneal injection of MAM 20 mg/kg or an injection of BrdU (100 mg/kg) or both. Dams of the saline group received saline solution on GD11. At birth all litters were reduced to four males and four females and fostered to the biological dams following behavioral procedures previously described [18]. Post weaning prenatally treated male adult rats (age 6 months) were used for the behavioral and biochemical studies.

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Experiments were carried out following the procedure described by the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC). Animals were tested in an open-field to check locomotion and in the Morris maze to investigate memory and learning (eight Saline, 11 MAM, ten MAM + BrdU, ten BrdU). The open-field consisted of a Plexiglas box, with clean sawdust and with the floor subdivided in 18 equal squares, placed under white lights. Locomotion was measured for 20 min. The Morris water maze test [14] consisted of a black Plexiglas circular pool 150 cm in diameter and 50 cm in height. The test consisted in a 3 days acquisition phase and in a 2 days reversal phase. The length of the swim path (total distance moved), the time to reach the platform (escape latency) and mean swimming speed of the rats were recorded by means of a computer-based video-tracking system Ethovision (Noldus, Wageningen 6700 AG, the Netherlands). At the end of behavioral testings animals were sacrificed with an overdose of Nembutal for NGF, BDNF, ChAT and p75 analyses. As for NGF and BDNF assays (four Saline, five MAM, five MAM + BrdU, five BrdU), brain areas were quickly removed using a mouse brain matrix (ASI Instruments, Inc. Co., USA). NGF evaluation was carried out in the hippocampus and parietal cortex by using a highly sensitive two-site immunoenzymatic assay [19]. BDNF in the parietal cortex and hippocampus of prenatal treated rats and controls were measured with an ELISA kit 'BDNF Emaxtm ImmunoAssay System number G6891' by Promega, Madison, WI, USA [6,7]. Comparable 20 μm brain sections of controls and treated rats (four Saline, six MAM, five MAM + BrdU, five BrdU) were immunostained for immunolocalization of low affinity NGF receptor (p75) and ChAT in the septum and Meynert's nuclei following methods previously described [2] and cell count was carried out by a Zeiss Axiophot microscope and an image analysis program (IAS 2000, Delta Sistemi, Rome, Italy). Statistics were performed using ANOVA with prenatal treatments as factors. For the behavioral data this required the use of a nested type design and of the repeated measure factor. Post-hocs were performed using the Tukey's test. According to a previous report [11] a few rats exposed to BrdU at GD11 developed kinked tails (which were not used in the tests). No gross changes in BrdU group were observed in brain morphology except for a slight reduced cortical thickness whereas animal exposed to MAM (both groups) had slight changes in limbic structures such as hippocampus and entorhinal cortex as previously described [17]. ANOVA showed no effects of the prenatal treatments in the open-field test (data not shown) in both distance moved or mean velocity. However, learning was slightly affected by prenatal MAM exposure during the acquisition phase of the Morris maze (Fig. 1, $F(3,11) = 3.52$, $P < 0.01$) but not in the reversal phase. Post-hocs evidenced an increased latency time in MAM rats if compared with controls during the first day of the Morris maze acquisition ($P < 0.05$). No changes in distance moved in the pool or in mean velocity were found during the maze test (data not shown). Brain NGF levels (Fig. 2) were affected by BrdU exposure. In the hippocampus NGF decreased in both BrdU groups ($P < 0.05$ in the ANOVA) whereas NGF was higher in the parietal cortex ($P < 0.05$ in the ANOVA, see post-hocs). Also hippocampal BDNF (Fig. 2) decreased in BrdU groups ($P < 0.05$). However, in the parietal cortex BDNF levels were reduced ($P < 0.05$ in the ANOVA) (see single post-hocs of Fig. 2). ChAT immunopositivity (Fig. 3) increased in both septum and Meynert's nuclei in MAM + BrdU rats ($P < 0.05$ in the ANOVAs; septum: Saline 28.00 ± 1.48, MAM 35.95 ± 2.98, MAM + BrdU 43.33 ± 2.87, BrdU 33.37 ± 3.64; Meynert's nuclei: Saline 18.56 ± 0.98, MAM 21.77 ± 1.32, MAM + BrdU 25.83 ± 1.49, BrdU 18.81 ± 1.74 mean cell count per section). Analogously, p75 immunopositivity (Fig. 3) increased in both septum and Meynert's nuclei following MAM + BrdU exposure in rats ($P < 0.05$ in the ANOVAs; septum: Saline 19.36 ± 2.02, MAM 25.75 ± 2.24, MAM + BrdU 33.81 ± 1.66, BrdU 23.53 ± 1.17; Meynert's nuclei: Saline 13.83 ± 1.12, MAM 18.69 ± 0.97, MAM + BrdU 22.25 ± 1.28, BrdU 18.62 ± 1.66 mean cell count per section).

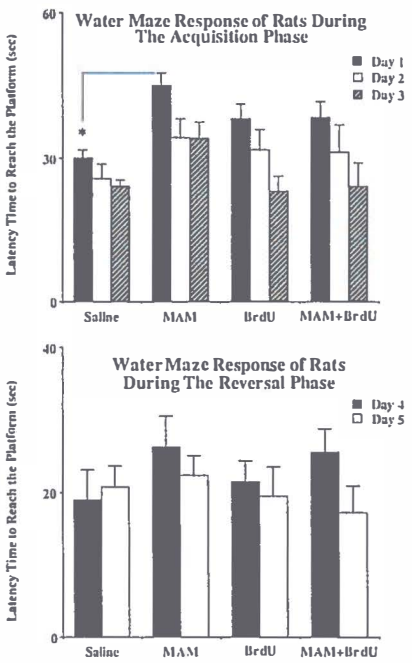

Fig. 1. Water maze responses in the acquisition and reversal phases of MAM or BrdU or both rats and controls. Data represent mean levels (±SEM). Asterisk indicates significant differences between groups (*$P < 0.05$).

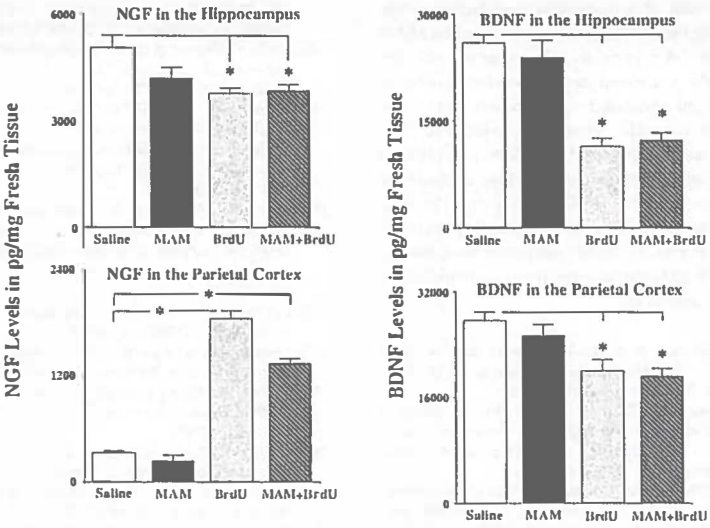

Fig. 2. Levels of endogenous NGF and BDNF (expressed as pg/g of fresh tissue) in the hippocampus and parietal cortex of prenatal MAM or BrdU or both rats and controls. Data represent mean levels (±SEM). Asterisks indicate significant differences between groups (*$P < 0.05$).

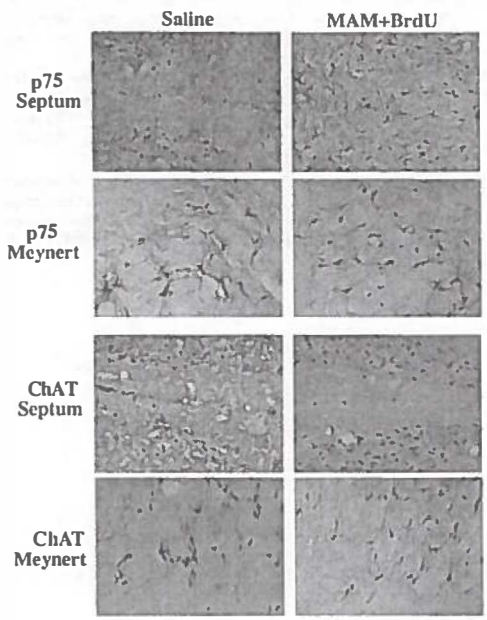

Fig. 3. ChAT and p75 immunopositivity in the septum and Meynert's magnocellular nuclei of controls (saline) and MAM + BrdU rats (the other groups were not included because similar to controls). ×150.

Our results demonstrate that embryonic exposure to MAM impairs learning performances [7], while concomitant treatment with BrdU elicits NGF potentiation in the parietal cortex and decreasing hippocampal NGF and BDNF. Prenatal exposure to MAM at GD11 led to mild effects on cognitive behavior as a result of CNS changes [16,17]. In fact, exposure to MAM early during brain growth induces abnormal development of the cortical/hippocampal axis with reduced cortical thickness, disorganized brain cytoarchitecture, and abnormal temporal asymmetry. This observation is consistent with findings showing that the hippocampus and related cortical regions are brain structures playing a key role in processing associational information and spatial memory [5]. Interestingly, animals treated with both MAM and BrdU behaved as controls suggesting that some factors may have contributed in increasing attentional levels of these rats. Indeed, septal cholinergic pathways playing a key role in spatial memory [8,9] or p75 immunoreactivity were potentiated in MAM + BrdU rats following the NGF/BDNF changes in the hippocampal/cortical axis. NGF is known to be produced in the hippocampus and cortex [12,13] and NGF changes may have stimulated in MAM + BrdU animals septal or Meynert's nuclei ChAT/p75 cell positivity (for review see Refs. [4,10]). The enhanced expression of ChAT immunopositivity observed in basal forebrain cholinergic neurons of MAM + BrdU rats could also suggest an impaired ChAT accumulation and/or release while the p75 increase might be linked to apoptotic mechanisms [4,10]. In addition, we show that BrdU inhibits neurotrophin expression in the hippocam-

pus possibly as a result of a functional long-lasting DNA-damage emphasizing the irregular changes caused by MAM and BrdU. However, NGF is produced in neurons and glial cells [12,13] and the increased parietal cortex level may represent not only an increased function but rather that parietal cortex is permanently stimulated to produce NGF in an attempt to restore impaired development [10]. In conclusion, the present findings indicate that exposure to neurotoxins such as MAM or BrdU during specific time points of prenatal development provides a useful approach for the study of early brain maldevelopment and for the analysis of structural and neurochemical mechanisms implicated in behavioral disorders.

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Long-lasting effects of prenatal MAM treatment on water maze performance in rats: Associations with altered brain development and neurotrophin levels

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Abstract

We previously reported that prenatal methylazoxymethanol (MAM) administered on days 11 and 12 of rat pregnancy induces structural changes in the cytoarchitecture of the hippocampal–entorhinal axis. We also showed that young and middle-aged prenatally treated MAM animals displayed changes in brain neurotrophin levels [Neurosci. Lett. 309 (2001) 113; Physiol. Behav. 71 (2000) 57.]. To continue this line of investigation, the working hypothesis adopted was that prenatal MAM administration, by interfering with limbic neurogenesis, could impair learning and memory ability of aged animals in the water maze. It was found that injection of MAM during early rat brain development induced deficits in both the acquisition and retention phases of the Morris maze. These behavioral changes were associated with significant changes in brain nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), reduced choline acetyltransferase (ChAT) immunoreactivity in forebrain cholinergic neurons and loss of neuropeptide Y (NPY) immunodistribution in cells of the entorhinal cortex. This finding, as well as confirming previous studies showing that injection of prenatal MAM administration induces significant changes in hippocampal–entorhinal axis neurogenesis and marked behavioral deficits in adult life, provides additional experimental evidence supporting the hypothesis that loss of NGF and/or BDNF-receptive or producing cells can co-occur at the onset of neurodevelopmental disorders. © 2002 Published by Elsevier Science Inc.

Keywords: NGF; BDNF; Behavior; ChAT; NPY

1. Introduction

It is known from developmental mammalian brain studies that neurogenesis is characterized by cell migration and differentiation before a given group of neurons reaches their final structural and topographical localization within the CNS. The time schedule for cell migration and differentiation is known to be regulated by neurotrophins [8,29]. Failure of or impairments in these processes can lead to severe neurological deficits in postnatal life [19]. One specific aspect of this development strategy is that disruption of cell migration from the cortical proliferative zone can produce long-lasting changes in the neuronal structural organization of the entorhinal cortex and hippocampus leading to severe neuropathologies in humans [11,49]. Selective lesions of the entorhinal–hippocampal axis have been carried out in animal models of neurodevelopmental disease by injecting drugs, which interfere with cell division and/or migration during specific time points of brain neurogenesis.

Prenatal methylazoxymethanol acetate (MAM) treatment at different gestational days (GD) has been used to produce animal models for neuropathologies characterized by disorders in neuronal cells and for human brain dysgenesis including epileptogenic cortical malformations [13,23,57]. In particular, to induce maldevelopment of the entorhinal–hippocampal axis, we administered MAM at GD11 or GD12, which kills neuroblasts undergoing mitosis of rat fetal life [26,52]. Indeed, prenatal MAM exposure at GD13 or later elicits marked microencephaly with gross changes in all brain areas and behavior [13]. We demonstrated that prenatal MAM treatment at GD11 and GD12 induced changes in the behavior [25,26,52,53]. Young rats prenatally

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exposed to MAM showed slight impairments in passive avoidance acquisition suggestive of modifications in the elaboration of data acquisition and also had impaired pain sensitivity on the hot plate [25] while no changes were observed in social behavior and open-field activity [25,26]. Adult rats prenatally exposed to MAM at GD11 also showed slight changes in a Morris maze test while no differences were found in locomotion during an open-field [24] test suggestive of a retardation in learning capabilities [24,26]. Adult rats did not also show any changes in social behavior [25]. The behavioral effects induced by MAM in young and adult rats were found to be associated with altered brain neurotrophin production such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and the results were discussed in reference to the neurodevelopmental hypothesis of psychiatric disorders. However, no data are available on the long-term effects of prenatal MAM administration in the rat. Indeed, brain neurotrophin levels change with age, inducing alterations in brain neurotrophin receptors and brain neuropeptides regulated by neurotrophins [i.e. choline acetyltransferase (ChAT)] [7,17,18,33,38].

The purpose of this study was to continue the line of investigation on the prenatal MAM model [25,26] in aged rats. Accordingly, we studied whether in an animal model of brain maldevelopment learning and memory capabilities of aged rats were associated with alterations in NGF/BDNF protein and their respective mRNA distribution. In addition, since aging lowers learning performance and disrupts cholinergic pathways and neuropeptide Y distribution (NPY) [14,18], the immunodistribution of ChAT and NPY was also investigated in target brain regions of the same animals as in the behavioral tests. NPY and ChAT are molecules also known to be regulated by neurotrophins [20,32,36]. We predicted that prenatal MAM administration in rats would induce changes in water maze responses, alter brain neurotrophin levels and disrupt ChAT immunopositivity.

2. Materials and methods

2.1. Subject housing and MAM treatment

Gestating Wistar Wl rats (*Rattus norvegicus*) were obtained from an animal farm (Charles River, Germany), 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 an air-conditioned room (temperature 21±1 °C, relative humidity 60±10%), with white lights on from 7.00 a.m. to 7.00 p.m. in Plexiglas boxes with a metal top and sawdust as bedding. Regular rat pellet food (Hope farms, Woerden, The Netherlands) and water were available ad libitum. According to procedures previously described [24–26,50,51–53], pregnant rats were randomly divided in three groups: GD11, GD12 and Saline. Five pregnant rats for each GD group underwent a single intraperitoneal injection of MAM (20 mg/kg) on GD11 or GD12. As controls, we used dams (five rats), which received 20 mg/kg of saline solution on one of the GD11 and GD12. At birth, animals were raised by the biological dams and litters were reduced to four males following behavioral procedures previously described with minor modifications [17,57]. Postweaning, prenatally MAM-exposed male rats were housed in pairs and used for the behavioral and biochemical studies at 15 months of age. No behavioral test was carried out on the animals before the water maze, which took place in an experimental room with an air-conditioned system. Environmental conditions such as humidity and temperature levels inside the room were very similar to those of the housing facility. All efforts were made to minimize and reduce animal suffering and for limiting the number of animals 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).

2.2. Morris water maze testing

For the behavioral studies, we used the Morris water maze test [41] that permits evaluation of learning abilities of developing and adult rodents. The apparatus consisted of a black Plexiglas circular pool 150 cm in diameter and 50 cm in height designed according to Morris, placed in the middle of an experimental room (dimension 5×4×3 m). The pool was filled with water kept at a temperature of 24–26 °C. A plastic black platform (20 cm in diameter) was placed 0.5 cm below the water surface and 15 cm from the edge of the pool.

Male rats were tested (GD11 $n=8$, GD12 $n=8$ and Saline $n=11$). All tests were conducted between 9:00 and 14:00 h under red light conditions. The day before the first day of the acquisition phase, rats were gently trained by placing them on the platform until they stayed, three times consecutively, 10 s without leaving the platform. The entire procedure took 5 days. The position of the hidden platform remained fixed for the first 3 days (acquisition phase). Each subject was allowed six individual trials for the first 2 days and five trials the third day. In addition, a sixth trial without platform during the third day was considered as a "probe" test. Rats were also tested for the reversal phase of the Morris maze during the fourth and fifth day, in which the platform was subsequently moved to a location symmetrically opposite with respect to the center of the pool for six individual trials for day.

To avoid visual orientation prior the release, rats were transferred from their cages into the pool in a nontransparent plastic cup, from which they glided into the water facing the pool wall. Release points were balanced across four symmetrical positions on the pool perimeter. Animals were left swimming either until they found the platform or until 60 s

had elapsed. Platform finding was defined as staying for at least 3 s on it. If rats crossed the platform without stopping (jumping immediately into the water), they were left swimming until they met the above criteria. After staying for about 10 s on the platform, the rats were given the opportunity to climb on a wire-mesh grid attached to a stick, which was also used to pick up the animals from the water after the cut off time. Between trials, the animals were gently placed under infrared lamps and allowed to warm up and dry for about 3 min. Intertrial times varied between 40 and 50 min.

The length of the swim path (total distance moved), the time to reach the platform (escape latency) and mean swimming speed of the rats were recorded by means of a computer-based video-tracking system Ethovision (Noldus, Wageningen 6700, The Netherlands). In the probe test, the time to reach the platform was not a measure but the time spent in each quadrant was measured.

2.3. Tissue dissection for NGF and BDNF analyses

Animals ($n=4$ for each group) were anaesthetized with an overdose of Nembutal and brain areas were quickly removed using a mouse brain matrix (ASI Instruments, USA; see also the methodology described by Cuello [21]. Tissues were then homogenized and centrifuged at 8500 rpm and the supernatant used for NGF and BDNF assays. All tissues were stored at −70 °C until analysis.

2.4. NGF determination

NGF evaluation was carried out in the hippocampus, parietal cortex, entorhinal cortex and striatum of prenatally MAM-treated rats. NGF was measured using a highly sensitive two-site immunoenzymatic assay, which recognized both mouse and human NGF as previously described [12,59]. Briefly, polystyrene 96-well microtubes immunoplates (Nunc) were coated with affinity-purified monoclonal mouse anti-NGF antibody (Boehringer Mannheim, Germany), which does not cross-react with BDNF diluted in 0.05-M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA) for evaluation of the nonspecific signal. After an overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05-M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris–HCl (pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatin, 0.1% Triton X-100). After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100-mM Tris–HCl, pH 7.2, 400-mM NaCl, 4-mM EDTA, 0.2-mM PMSF, 0.2-mM benzethonium chloride, 2-mM benzamidine, 40-U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4-mu/well anti-β-NGF-galactosidase (Boehringer Mannheim) for 2 h at 37 °C, and after further washing, 100 μl of substrate solution (4-mg/ml chlorophenol red, Boehringer Mannheim, substrate buffer: 100-mM HEPES, 150-mM NaCl, 2-mM $MgCl_2$, 0.1% sodium azide and 1% BSA) were added to each well. After an incubation of 2 h at 37 °C, the optical density was measured at 575 nm using an ELISA reader (Dynatech), and the values of standards and samples were corrected by taking into consideration the nonspecific binding. NGF concentrations were determined from the regression line for the NGF standard (ranging from 15 to 1000 pg/ml purified mouse NGF) incubated under similar conditions in each assay. The recovery of NGF during assay procedure was estimated by adding a known amount of highly purified NGF to the samples or to the homogenization buffer as internal control. The yield of the exogenous NGF was calculated by subtracting the amount of endogenous NGF from the value of endogenous plus exogenous values. 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 of NGF and cross-reactivity with other related neurotrophic factors (BDNF, NT-3 and NT-4) was less than 3%. Data are represented as pg/g wet tissue and all assays were performed in triplicate.

2.5. BDNF determination

The concentrations of BDNF in brain samples of MAM-treated rats and controls were measured with an ELISA kit "BDNF Emaxtm ImmunoAssay System number G6891" by Promega (Madison, WI, USA) following the instructions suggested by the manufacturer. Entorhinal cortex, parietal cortex, hippocampus and striatum were homogenated in the kit calibration buffer and centrifuged. The brain tissues were homogenized with ultrasonication in extraction buffer 0.2% triton. Briefly, 96-well immunoplates (Nunc) were coated with 100 μl/well of monoclonal anti-mouse BDNF antibody. After an overnight incubation at 4 °C, the plates were washed three times with wash buffer and the samples were incubated in the coated wells (100 μl each) for 2 h at room temperature with shaking. After additional five washes, the immobilized antigen was incubated with an anti-human 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 1-M phosphoric acid (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). BDNF concentrations were determined from the regression line for the BDNF standard (ranging from 7.8 to 500 pg/ml purified mouse BDNF) incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/g of BDNF and cross-reactivity with other related neurotrophic factors (NGF, NT-3 and NT-4) was less than 3%. Data are represented as pg/g wet tissue and all assays were performed in duplicate.

2.6. RT-PCR ELISA for NGF and BDNF

RT-PCR ELISA for NGF and BDNF was carried out following methods recently described [55]. Briefly, total RNA was extracted from hippocampus, entorhinal and parietal cortex and striatum of MAM rats and controls by using the method of Chomczynski and Sacchi [16] as modified in the TRIzol Kit (Gibco). RNA was analyzed by gel electrophoresis and its concentration was measured by spectrophotometer reading at 260 and 280 nm (1 A_{260} adsorbance unit equal to 40 mg/ml).

Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using 200 u of M-MLV reverse transcriptase (Promega Italia, Milano, Italy) in 20 μl of total volume reaction. To optimize reproducibility of cDNA synthesis, a master mix solution containing 250-ng Oligo $(dT)_{12-18}$ primer, 0.5-u RNasin ribonuclease inhibitor and 0.5-mM dNTP in 5× reaction buffer (250-mM Tris–HCl pH 8.3, 375-mM KCl, 15-mM $MgCl_2$, 50-mM DTT) was used. The mixture was incubated at 42 °C for 1 h and the reaction was terminated with a further incubation at 95 °C for 5 min. Dilutions (1:10) in H_2O DEPC of the synthesized cDNAs were aliquoted and stored at −20 °C until use.

PCR amplification is carried out using 5′ biotinylated primers to generate biotinylated PCR products detectable by digoxygenin-labeled probes in an immunoenzymatic assay (ELISA). Briefly, an aliquot of cDNA was mixed with 5-μl 10× buffer, 200-μM dNTPs, 1.5-mM $MgCl_2$, 2.5-u Taq DNA polymerase (Promega), 12.5-pmol NGF (5′TCCAC CCACCCAGTCTTCCA3′; 5′GCCTTCCTGCTGA GCACACA3′), 7.5-pmol BDNF (5′AGCTGAGCGTGTGT GACAGT3′; 5′TCCATAGTAAGGGCCCGAAC3′) and 3.12-pmol GAPDH (5′CACCACCATGGAGAAGGCC3′; 5′CACCACCATGGAGAAGGCC3′) primers in a final volume of 50 μl. A sample containing all reaction reagents except cDNA was used as PCR negative control in any amplification. Ten microlitres of 1:10 RT mixture without enzyme was used as further PCR negative control. The mixes were incubated for the indicated cycles (denaturation 1 min at 95 °C, annealing 1 min at 55 °C, extension 2 min at 72 °C) in a GeneAmp PCR System 9600 (Perkin-Elmer). The correct size of all PCR products was confirmed by comparing with a DNA standard on agarose gel and the identity was confirmed by Southern blotting (data not shown).

Biotinylated PCR products diluted in PBS containing 3% bovine serum albumin (PBSB) were distributed in triplicates (100 μl/well) onto avidin-coated microplates and incubated for 1 h at room temperature. After incubation, the microplates were washed three times with PBS containing 0.02% Tween 20 (washing buffer). DNA was denatured using 0.25-M NaOH at room temperature for 10 min. Following the washing, 100 μl/well of 4-pmol/ml digoxygenin-labeled probes (for NGF 5′TCCTGTTGAGA GTGGTGCCGGGGCATCGA3′, for BDNF 5′TAACCCA TGGGATTACACTTG-GTCACGTAG3′ and for GAPDH

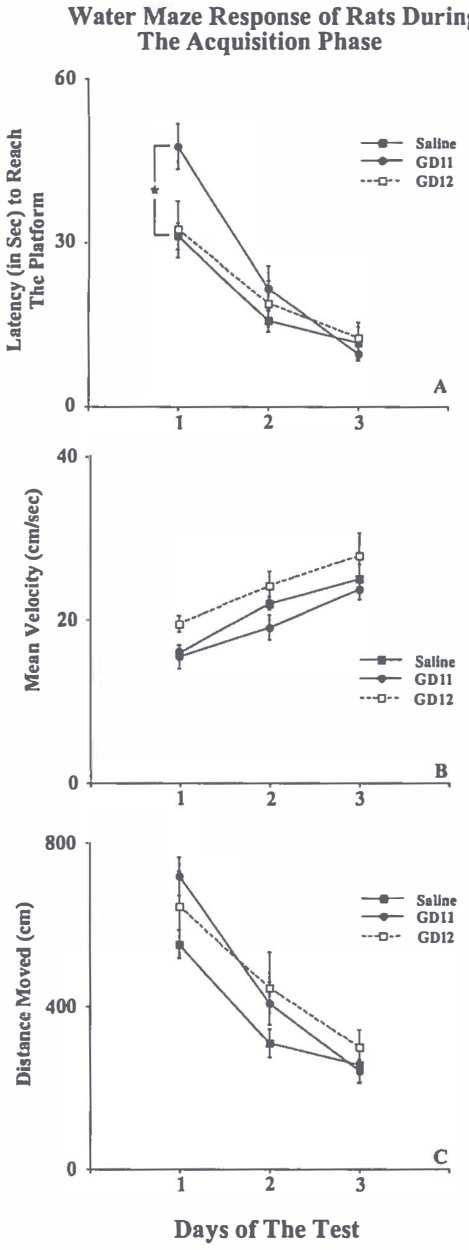

Fig. 1. Water maze responses in the acquisition phase of prenatal MAM-exposed rats and controls. (A) The time spent to reach the platform (escape latency), (B) the total distance moved in the pool and (C) evidence of the mean swimming velocity. Data represent mean±S.E.M. levels. Asterisks indicate significant differences between groups (* $P<.05$).

5′ACAATCTTGAGTGAGTTGTCATATTTCTCG3′) in DIG Easy Hybridisation buffer (Boehringer Mannheim) were added and incubated at 42 °C for 2 h. After washes, anti-digoxygenin POD-coupled antibody (Boehringer Mannheim) was added (1:1000 in PBSB) and incubated 1 h at 37 °C. The reaction was developed by TMB (3,3′,5,5′-tetramethylbenzidine; 0.6 mg in citrate buffer, pH 5.0) and blocked after 30 min with 2-M HCl. The amount of amplified products is measured as optical density at 450/690 nm ($OD_{450/690}$) using a Dynatech ELISA Reader 5000. GAPDH $OD_{450/690}$ level is used to normalize for the relative differences in sample size, integrity of the individual RNA and variations in reverse transcription efficiency.

2.7. Immunocytochemistry

Polyclonal antibody against NPY was purchased from Peninsula Laboratories, UK. Monoclonal ChAT antibody was obtained following methods previously described [4,5]. Immunoreactivity was carried out on serial brain sections of the hippocampus, parietal cortex and entorhinal cortex of prenatally treated MAM rats ($n=4$ for each group). ChAT immunopositivity was also measured in the septum and in the Meynert's nuclei. Animals were deeply anesthetized with pentobarbital and perfused transcardially with 100 ml of 0.1-M phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium nitrite, followed by 200 ml of 4% paraformaldehyde in PBS. Brains were then removed, post-fixed in PBS-buffered 4% paraformaldehyde at 4 °C for 12–18 h and cryoprotected in PBS 20% sucrose for 24 h. Each brain was then mounted on a stage of a freezing microtome and serial coronal sections were cut and stained with toluidine blue for general histology or for immunohistochemical analysis.

For immunolocalization, we used anti-NPY diluted 1:100 and anti-ChAT (μg/ml). Free-floating brain sections were first incubated in 0.1-M PBS (pH 7.4) containing 10% normal goat serum, 5% BSA and 0.1% Triton X-100 for 1 h at room temperature and then overnight at 4 °C with NPY or ChAT antibodies (2 μg/ml) in PBS containing 0.1% Triton X-100. Sections were washed three times in diluent (0.1-M PBS containing 0.1% BSA and 0.1% Triton X-100) followed by incubation with biotinylated secondary IgG anti-rabbit (1:300) and then with avidin biotin complex kit conjugated to horseradish peroxidase as suggested (Vectastain Elite ABC kit, Vector Laboratories) for 1 h at room temperature. Antibody staining was visualized using 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxide with 0.025% cobalt chloride and 0.02% nickel ammonium sulfate as chromogens. Stained sections were mounted onto gelatin-coated slides, dried and covered for immunostained cells identification. Control experiments were carried out to ensure the specificity of the immunostaining by omission of the primary antibodies or by preadsorbtion with an excess of antigen. No

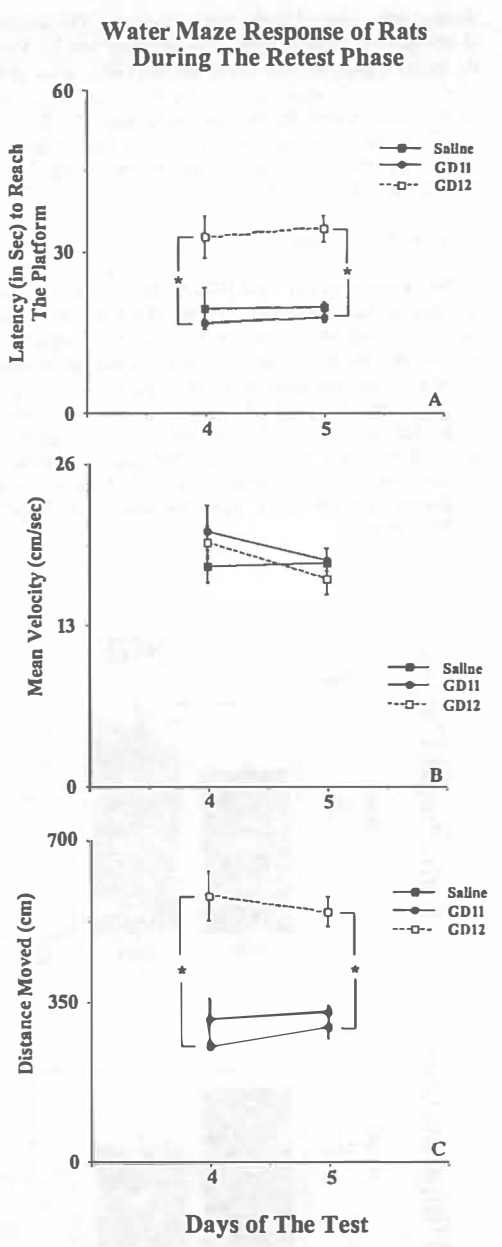

Fig. 2. Water maze responses in the reversal phase of prenatal MAM-exposed rats and controls. (A) The time spent to reach the platform (escape latency), (B) the total distance moved in the pool and (C) evidence of the mean swimming velocity. Data represent mean ± S.E.M. levels. Asterisks indicate significant differences between groups (*$P<.05$).

staining was observed under these conditions. The nomenclature of the brain areas applied to this study was based on the rat brain atlas of Paxinos and Watson [44]. Comparable 25-μm thick brain sections of each control and treated rats were immunostained for immunolocalization of NPY and ChAT, and cell count was carried out by a Zeiss Axiophot microscope and an image analysis program (IAS 2000, Delta Sistemi, Rome, Italy).

2.8. Statistical analysis

Data were analyzed by ANOVA considering the litter as blocking factor and the prenatal MAM treatments as variables. In the Morris water maze session, we took into account also the 5 days of the test as a repeated-measure factor. Post hoc comparisons within logical sets of means were performed using the Tukey's test, the use of which is permissible or even recommended also in the absence of significant main or interaction effects in the ANOVA, in order to minimize frequency errors of both types 1 and 2 following the indications given by Wilcox (Ref. [61], pp. 187–189).

3. Results

3.1. Morphological observations

Body weight of rats was affected by prenatal MAM administration. Indeed, GD12 treatment resulted in lighter body weight compared to Saline and GD11 animals ($P < .01$; Saline, 838.90±13.51 g; GD12, 705.66±7.47 g; GD11, 799.88±19.84 g). Prenatal MAM treatment, as used in this study, induced also significant changes in the morphology of the hippocampus and entorhinal cortex, which were smaller of those of controls (P's < .05 in the ANOVA; GD11: hippocampus 20.3%, entorhinal cortex 19%; GD12: hippocampus 24.2%, entorhinal cortex 28.9%), confirming data previously described [52,53].

3.2. Morris water maze testing

Prenatal MAM-treated animals displayed impairments in the learning processes (Fig. 1). Animals treated on GD 11 showed an increase in the latency to reach the platform during the acquisition phase [main effect of MAM treatment

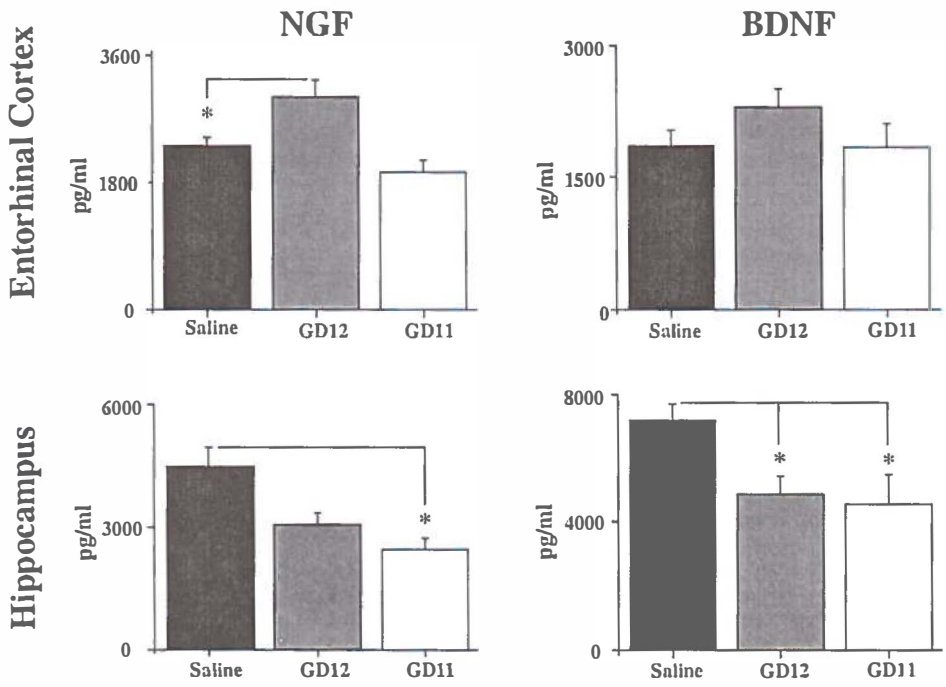

Fig. 3. Levels of endogenous NGF and BDNF (expressed as pg/g of fresh tissue) in the hippocampus and entorhinal cortex of prenatal MAM-exposed rats and controls. Data represent mean±S.E.M. levels. Asterisks indicate significant differences between groups (*$P < .05$).

$F(2,8)=4.04$, $P<.05$]. Interestingly, the impairments in GD11 animals were limited to the first day of the acquisition phase as revealed by the interaction [Repeated measures ×Main effect of MAM treatment $F(4,16)=4.51$, $P<.01$]. In fact, post hoc comparisons showed a significant delay in water maze acquisition ($P<.05$ at the first day testing) in GD11 rats when compared to the control animals (Fig. 1A). Overall, during the 3 days of the test, animals learned to reach the platform as revealed by the reduced escape latency evidence of a water maze acquisition [repeated measures $F(2,16)=75.44$, $P<.01$].

The total distance moved and mean swimming speed during the acquisition phase (Fig. 1B and C) in the pool were unaffected in MAM-treated rats and ANOVA of the repeated measures for the total distance moved revealed a general decrease during the 3 days of the test [$F(2,16)=48.79$, $P<.01$]. ANOVA also revealed that over the 3 days of the test, rats swam faster (Fig. 3C) to reach the platform [repeated measures $F(2,16)=36.00$, $P<.01$].

ANOVA of the probe test (data not shown) did not reveal behavioral changes in prenatal MAM-treated rats. No differences were observed in the total distance moved in the pool, the mean swimming speed or the time spent in each quadrant of the pool.

During the 2 days of the reversal session (Fig. 2), GD12 rats showed differences in the behavior if compared to

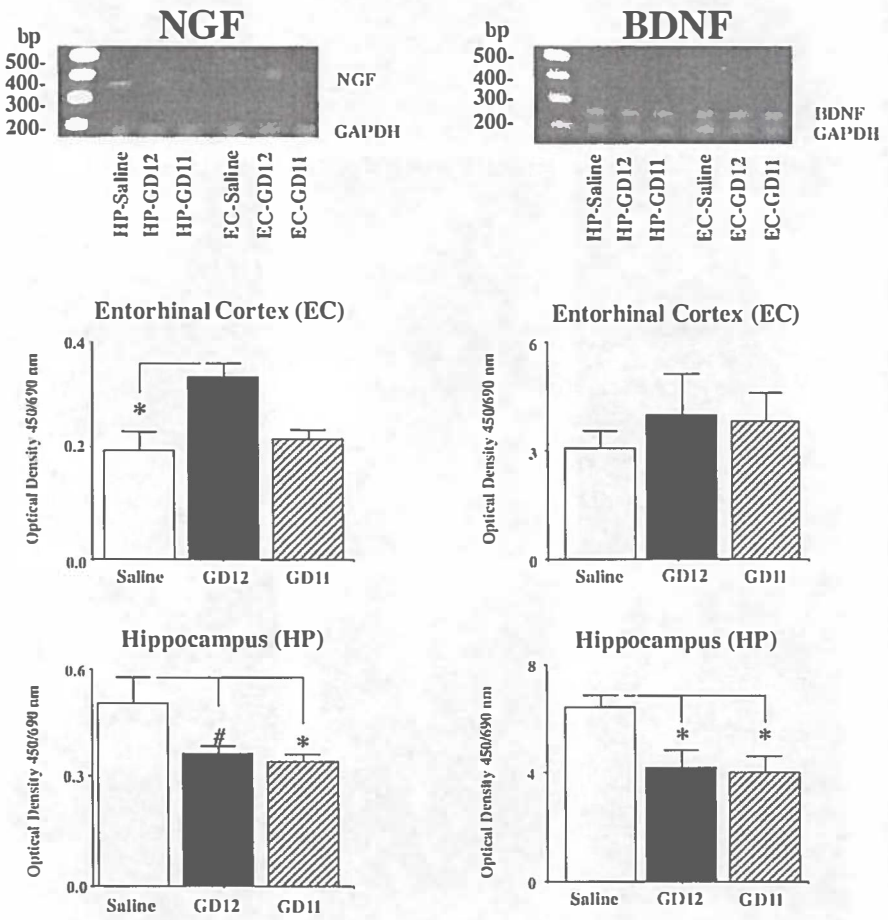

Fig. 4. NGF/BDNF mRNA expression in the hippocampus (HP) and entorhinal cortex (EC) of MAM rats. Pictures show the ethidium bromide-stained agarose gels of the RT-PCR and respective optical density measured by ELISA (see Materials and methods). Data represent mean ± S.E.M. levels. Asterisks indicate significant differences between groups ($^{*}P<.05$, $^{\#}P=.07$).

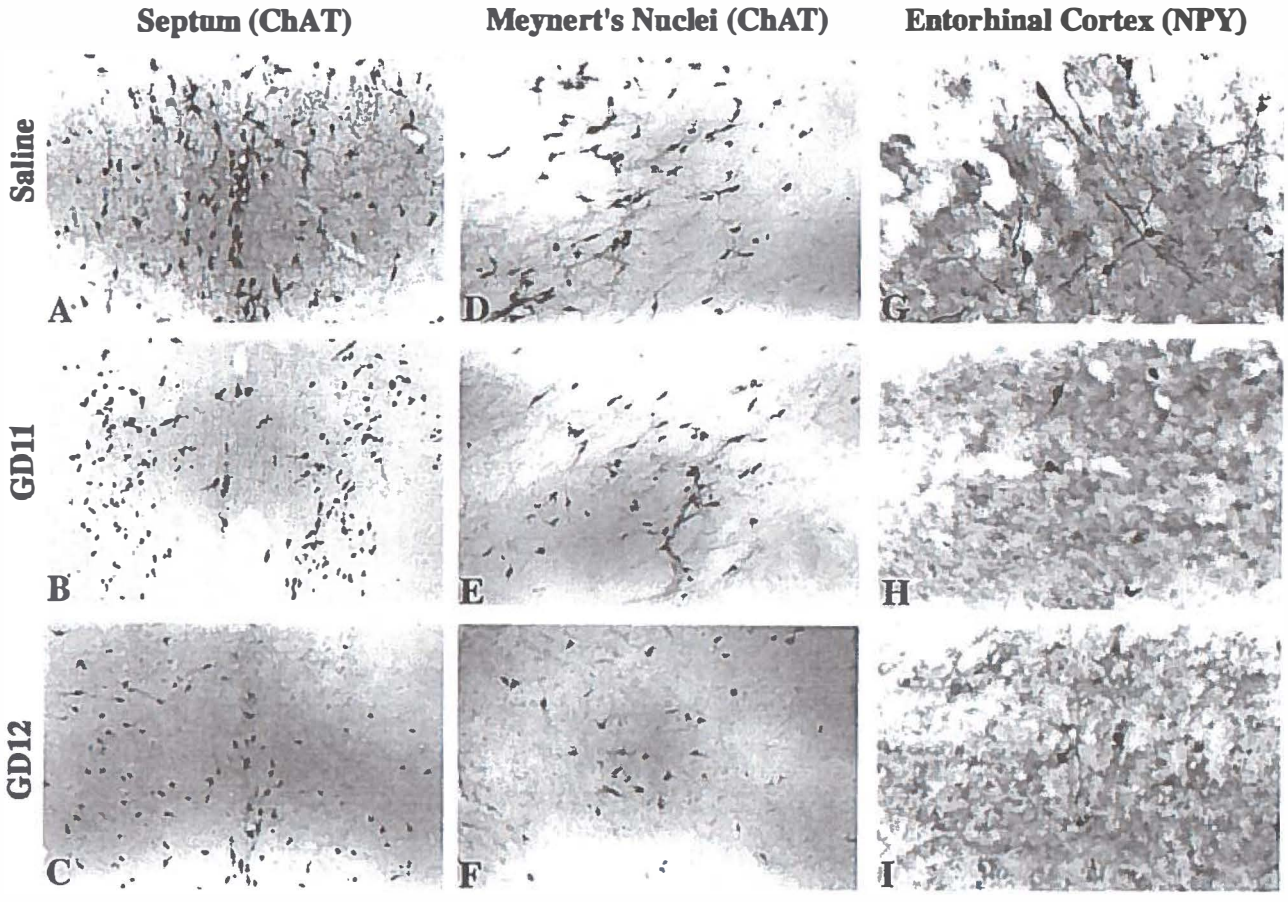

Fig. 5. ChAT immunopositivity in the septum of control rats (A), GD11 (B) and GD12 (C) animals in the Meynert's magnocellular nuclei of control rats (D), GD11 (E) and GD12 (F) rats. (G–I) The NPY immunopositivity in the entorhinal cortex of control rats (G), GD11 (H) and GD12 (I) (X-150 for A–F and X-300 for G–I).

Saline animals because they displayed an increase in the time spent to reach the platform [$F(2,8)=46.32$, $P<.01$ for the main effect MAM treatment]. GD12 rats also had increased distance moved during the 2 days of the reversal phase if compared with Saline or GD11 animals [$F(2,8)=40.31$, $P<.01$ for the main effect MAM treatment]. However, mean swimming speed did not change during the reversal phase in prenatal MAM-exposed rats.

3.3. NGF determination

Prenatal MAM administration affected the brain NGF levels expressed in pg/g wet weight in rats (Fig. 3, left). ANOVA revealed that in the entorhinal cortex of GD12 rats, NGF increased ($P<.05$ for the main effect of MAM treatment) when compared to Saline animals ($P<.05$ in post hocs). In the hippocampus, NGF content decreased ($P<.01$ for the main effect of MAM treatment) in GD11 (P's$<.05$ in post hocs). In the parietal cortex, NGF was unaffected, whereas in the striatum, NGF strongly decreased in GD12 and GD11 animals ($P<.01$ for the main effect of MAM treatment) when compared to controls (P's$<.01$ in post hocs; Saline, 1540.88±98.20; GD11, 494.85±66.41; GD12, 470.33±134.34).

3.4. BDNF determination

Prenatal administration of MAM affected brain BDNF distribution in rats (Fig. 3, right). Statistical analysis showed a decrease in BDNF levels in the hippocampus ($P<.05$ for the main effect of prenatal MAM treatment) of both groups of MAM rats when compared to Saline animals (P's$<.05$ in post hocs). However, in the parietal cortex, entorhinal cortex and striatum, BDNF content was not affected by MAM (not shown).

3.5. RT-PCR ELISA for NGF and BDNF

RT-PCR ELISA (Fig. 4, left) for the NGF-mRNA showed an effect of prenatal MAM administration. ANOVA revealed a slight increase in NGF-mRNA presence in the entorhinal cortex of GD12 rats and a decrease in the hippocampus of GD11 animals (P's$<.05$). No differences were observed in the parietal cortex and striatum (not shown). As for BDNF (Fig. 4, right), statistical analysis revealed that BDNF-mRNA decreased in the hippocampus of MAM-treated rats (P's$<.05$ in the ANOVA) when compared to saline, whereas no changes were observed in the entorhinal cortex. In the parietal cortex and striatum, BDNF-mRNAs were unaffected (not shown).

3.6. ChAT immunopositivity

Prenatal MAM administration affected MAM distribution in the rat brain. In the septum, GD12 rats showed (Fig. 5A–C) a reduction in the immunopositive cells ($P<.05$ in the ANOVA; Saline, 59.00±6.56; GD11, 53.05±4.94; GD12, 34.38±3.62). ChAT immunopositivity in the entorhinal and parietal cortices of GD11 rats was not affected by prenatal MAM administration (data not shown). In the Meynert's magnocellular nuclei cell counting (Fig. 5D–F), ANOVA revealed that prenatal MAM administration induced a decrease ($P<.01$ for the main effect of MAM administration) in cell number in both GD11 and GD12, although more evident in the latter group (P's$<.05$ in post hocs; Saline, 18.63±2.00; GD11, 12.87±1.23; GD12, 10.05±0.92).

3.7. NPY immunopositivity

Prenatal MAM administration affected the NPY immunodistribution (Fig. 5G–I) in the entorhinal cortex of rats ($P<.05$ for the main effect of MAM treatment). As revealed by post hocs, GD11 animals had lower number of immunopositive entorhinal cortex cells when compared with controls (P's$<.05$), whereas in GD12 rats, the decrease was not significant ($P=.08$ in post hoc comparisons; Saline, 23.83±0.76; GD11, 15.00±1.19; GD12, 18.20±2.28). No differences in NPY staining were observed between groups in the hippocampus and in the parietal cortex (data not shown).

4. Discussion

In the present work, we show that prenatal MAM administration during a specific developmental phase induces subtle deficits in a Morris maze test and a marked alteration in the levels of NGF and BDNF measured as protein or as relative mRNA (see Table 1 for a summary of selected biochemical and immunohistochemical data obtained with young, middle-aged and aged MAM rats). We found that rats treated on GD11 had reduced capability to reach the platform during the acquisition phase, whereas GD12 animals showed impairments in escape latency during the reversal phase. The morphological effects of prenatal MAM administration on GD12 were distributed throughout the brain [52,53], while on GD11 the effect was limited to "associative" regions such as the hippocampus and the entorhinal cortex [11,35]. This specific issue could be responsible for the slowdown in learning processes during the acquisition phase in GD11 rats and for the higher latency response of GD12 animals during the reversal phase. This hypothesis is consistent with studies showing that the hippocampus and related cortical regions are brain structures playing a key role in processing associational information (e.g., Ref. [15]). In addition, memory and learning processing, as well as "memory consolidation" phenomena, involve interactions between the hippocampus and parahippocampal regions, including also the entorhinal cortex [22].

Our findings also indicated that during the acquisition phase, GD11 rats displayed a longer latency in finding the

Table 1
Selected biochemical effects of prenatal MAM given in rats at GD11 or GD12

Age reference	Young [26]	Middle aged [25]	Aged (current paper)
NGF entorhinal cortex			
GD12	increased	increased	increased
GD11	normal	normal	normal
BDNF entorhinal cortex			
GD12	not measured	increased	normal
GD11	not measured	normal	normal
NGF hippocampus			
GD12	slightly increased ($P = .07$)	increased	decreased
GD11	normal	normal	normal
BDNF hippocampus			
GD12	not measured	increased	decreased
GD11	not measured	normal	decreased
NGF striatum			
GD12	normal[a]	normal	decreased
GD11	normal[a]	normal	decreased
BDNF striatum			
GD12	not measured	normal	normal
GD11	not measured	normal	normal
NGF parietal cortex			
GD12	normal	normal	normal
GD11	normal	normal	normal
BDNF parietal cortex			
GD12	not measured	normal	normal
GD11	not measured	normal	normal
ChAT Meynert's nuclei			
GD12	not measured	normal	decreased
GD11	not measured	normal	decreased
ChAT septum			
GD12	not measured	normal	decreased
GD11	not measured	normal	normal

[a] Unpublished data.

platform in the water maze. This effect was not, however, associated with changes in the swimming speed or the total distance moved by the animals in the pool. Because all rats had a day of being placed on the platform prior to the first day of testing, this suggests that GD11 rats, when compared to controls, were unable to cope with the new positional information either because of forgetting or of motivational impairments due to the new stressing environmental situation. Alternatively, GD11 rats could be more "confused" in learning tests, which required more complex information processing such as retrieving the platform position. Similar results in the Morris maze were found in adult MAM animals exposed at GD11 [24], whereas young GD11 MAM rats displayed disrupted responses in the passive avoidance test [26]. These findings suggest that prenatal MAM administration at GD11 induced mild impairments in rat cognition, which we discussed as a result of CNS changes. GD12 animals showed slight impairments in the reversal phase of the water maze, suggestive of a reduced flexibility in the data elaboration, which may be due to the hippocampal and/or cortical damage in the absence of both brain inflammation and changes in locomotion [48].

We also found that hippocampal NGF and BDNF levels measured as pg/g of analyzed tissue are significantly reduced by MAM treatment, and NGF and BDNF are known to regulate neuronal plasticity and ChAT activity in brain cholinergic neurons localized in the septum, nucleus of Meynert and striatum [8,46]. Since structural and biochemical impairments of cholinergic neurons are associated with deficits described in neuropsychiatric diseases [7,33], our results point to a possible functional link between NGF distribution and changes in brain development. Abnormalities of ChAT in the brain have been demonstrated in schizophrenia and sudden infant death syndrome [43]. Decreasing ChAT has been also observed in the aged brain [18,43]. With regard to aging, one point made in the present work is that rats prenatally treated with MAM present decreased ChAT immunoreactivity with aging. Indeed, prenatal MAM exposure did not induce lower ChAT immunopresence in 6-month-old rats [25], which demonstrates that brain maldevelopment may speed up aging processes as observed in human neuropsychiatric diseases. It should be noted that prenatal maldevelopment of cholinergic neurons could be a risk factor for Alzheimer dementia [33,43] due to a possible age-dependent decrease of the cholinergic system, which might turn pathological earlier if there are fewer cholinergic neurons at birth. Furthermore, cognitive defects and changes in neurotrophin expression play a key role in depression as well [3,56]. In the entorhinal cortex, NGF increased in GD12 animals as had been previously shown

[25,26], and this finding appears to be a specific MAM effect in the rat brain. We suggested [24] that since NGF is produced in neurons and glial cells [37,38], the NGF potentiation in the entorhinal cortex might indicate not only an increased function but also that the entorhinal cortex is permanently stimulated to produce NGF in an attempt to restore impaired development [33].

Interestingly, the changes in NGF/BDNF protein in MAM rats in both entorhinal cortex and hippocampus were found to be associated with similar levels of their respective mRNAs, suggesting that under these experimental conditions the hippocampal–entorhinal axis was specifically affected by prenatal MAM administration. However, in the striatum, NGF protein level decreased, whereas its mRNA remained unchanged. The functional significance of this difference is not known at the present time.

In the present study, it was also found that NPY decreased in the entorhinal cortex of GD11 animals and these rats also displayed a mild learning impairments in the water maze. NPY is a neuropeptide, which is widely distributed in the CNS of mammals, including humans, and there are consistent data indicating that NPY is also involved in regulating behavior and in a variety of neuropsychiatric diseases [45,62]. NPY modulates memory processing and attenuates learning impairments induced by neurotoxins [10]. This finding suggests that NPY can in some way interact with cognitive processes associated with prenatal MAM treatment. A previous study on prenatal MAM administration demonstrated that at GD15 MAM treatment caused a decrease in hippocampal NPY-positive cells [63] and this is in line with our results. In humans affected by neuropsychiatric disorders, the concentration of NPY has been said to be altered in the brain and cerebrospinal fluid [40,60], and analysis of postmortem tissues indicates a decrease in NPY in the temporal cortex but not in the hypothalamus [27]. It should also be noted that since NPY is known to be regulated by neurotrophic factors [20], disrupted levels of both NGF and BDNF could have contributed to down-regulating NPY.

4.1. General considerations

The findings for the prenatal MAM model as presented here and previously [25,26] indicate that the baseline behavior of rats in the open-field and social interaction tests was not disrupted by prenatal MAM, whereas animals showed a retardation in the acquisition of information of specific learning tests as the passive avoidance paradigm and the water maze. These learning changes were more substantial in aged rats when MAM was administered at GD12 of brain development. Indeed, GD12 aged rats displayed more widespread changes in brain neurotrophin levels and in their related neuropeptides, suggesting that MAM rats might behaviorally age faster. Recent findings have provided intriguing evidence that some neuropsychiatric diseases are characterized by neuropsychological abnormalities associated with significant structural and neuronal changes occurring in the developing brain. One novel, although still debated, hypothesis suggests that schizophrenia is related to cytoarchitectural alteration of the hippocampal–entorhinal axis [1,6,31,58] and basic studies have raised the question as to whether neurotrophins, particularly NGF and BDNF, play a role in regulating behavior and in neurodevelopmental disorders [2,9,42,47,50]. Human brain neurotrophin levels undergo significant changes following neuropsychiatric discases. Specifically, adult schizophrenic patients showed increased BDNF levels in the hippocampus and anterior cingulate cortex [50]. No data are available in aged schizophrenics about the distribution of BDNF. However, neonatal hippocampal lesions used as an animal model for schizophrenia suppress BDNF mRNA expression in the dentate gyrus and tend to reduce its expression in the prefrontal cortex when measured in young adult rats [39]. Brain NGF levels decreased at the early phases of Alzheimer's disease, whereas, as the disease progresses, NGF appears to be potentiated [33]. All together, these findings suggest that the deficits in cognitive abilities observed in schizophrenic patients [30] and the altered immunopresence of ChAT and NPY found in MAM-treated rats may be linked to the disrupted availability of NGF and/or BDNF in the hippocampal/entorhinal axis. Our working hypothesis is that the lack of one or both of these neurotrophins may contribute to the maldevelopment of the hippocampal/entorhinal axis and ultimately to the development of some neuropsychiatric manifestations.

A wide variety of pharmacological strategies has been tested to see whether they augment cognitive performance in the treatment of disorders such as anxiety, memory dysfunction and neuropsychiatric diseases [34]. The finding that the utilization of drugs influencing the synthesis and release of cholinergic neurotransmitters might be useful in enhancing cognition in healthy subjects suggests that cholinomimetics may be used for treating cognitive deficits [28,54]. Consequently, NGF and BDNF, which are regulators of ChAT and NPY activity, could deploy a potential therapeutic action in the treatment of neuropsychiatric diseases. Thus, the prenatal MAM model could be considered a useful tool for investigating the pathogenic mechanisms involved in human brain maldevelopment.

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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 1. 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±1°C, relative humidity 60±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°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°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 × 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 × 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 × 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

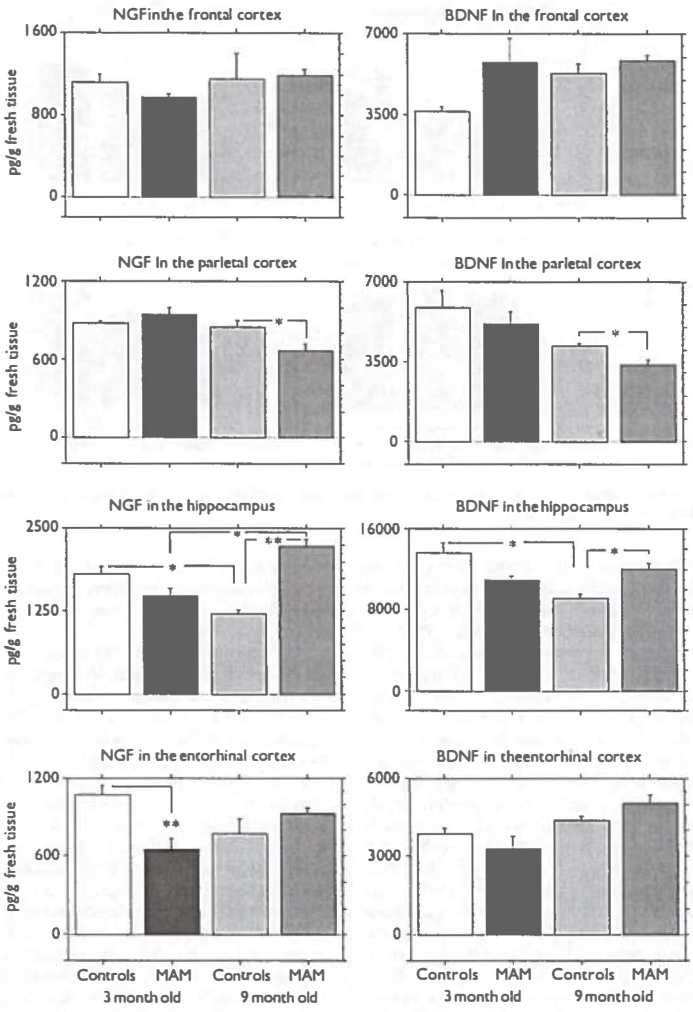

Fig. 1. NGF and BDNF in the hippocampus and in the frontal, parietal and entorhinal cortex of rats exposed *in utero* to MAM (GD17). Data represent mean levels (±s.e.m.). Significant differences between groups: $^{*}p < 0.05$; $^{**}p < 0.01$.

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].

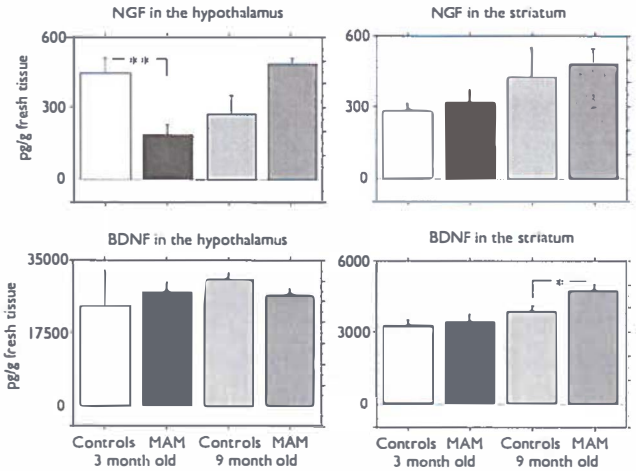

Fig. 2. NGF and BDNF in the striatum and hypothalamus of rats exposed *in utero* to MAM (GD17). Data represent mean levels (± s.e.m.). Significant differences between groups: $^{*}p < 0.05$; $^{**}p < 0.01$.

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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Nerve Growth Factor and Brain-Derived Neurotrophic Factor in Schizophrenia and Depression: Findings in Humans and in Animal Models

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Abstract: Depression and schizophrenia are major psychiatric disorders. Recently it has been documented that these diseases are characterized by deficits and/or loss of neurons in specific brain regions. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are endogenous biological mediators involved in neuronal survival and plasticity of dopaminergic, cholinergic, and serotonergic neurons in the central nervous system (CNS). Structural, biochemical, and molecular findings led to the hypothesis that these molecules play a role in the pathophysiology of psychiatric disorders and suggested that alterations in expression of neurotrophic factors could be responsible for neural maldevelopment and disturbed neural plasticity both in young, adult and aged subjects. Studies aimed at understanding the mechanisms regulating these events might be an important line of research for analyzing the etiopathogenesis of psychiatric disorders and eventually identifying new methods for diagnosis and new therapeutic strategies.

NEUROTROPHINS

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are supposed to derive from a common ancestral gene, and are therefore collectively called neurotrophins [48,89]. Neurotrophins (NTs) regulate development, maintenance, and function of the peripheral (PNS) and central (CNS) nervous systems. Neurotrophins also regulate many aspects of neural processes. In the mature nervous system, they control synaptic function and synaptic plasticity and modulate neuronal survival. All neurotrophins display a marked structural homology and a specific binding homology [reviewed in 107].

Nerve Growth Factor

Nerve growth factor (NGF) is the first neurotrophin to be discovered [reviewed in 132], and is a dimer of two identical polypeptide chains, each of 118 amino acid residues [144]. NGF was purified as a factor able to support survival of sympathetic and sensory spinal neurons in culture [132]. Injections with anti-NGF showed an important NGF role in maintaining the survival of sympathetic neurons *in vivo* and *in vitro*. In the PNS, NGF is synthesized and secreted by sympathetic and sensory target organs [reviewed in 126]. From these sources, it is captured in nerve terminals by receptor-mediated endocytosis and is transported through axons to neuronal cell bodies to promote neuronal survival and differentiation. The NGF expression in the CNS is much more restricted; NGF mRNA and protein are expressed in a number of brain regions, with the hippocampus providing the single largest source of NGF in the entire CNS [125]. In the hippocampus, NGF mRNA and protein are expressed by the principal excitatory (glutamate) neurons, as well as by a subset of γ-aminobutyric acid (GABA)-containing inhibitory neurons [171]. These hippocampal cells receive rich innervations from ascending neurons with their cell bodies in the basal forebrain.

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Brain-Derived Neurotrophic Factor

BDNF was purified from the pig brain, for its effect on survival-promoting action on a subpopulation of dorsal root ganglion neurons [20]. The amino acid sequence of mature BDNF has a strong homology with that of NGF [131,172]. BDNF is necessary for survival of peripheral sensory neurons, notably those in the vestibular ganglia and nodose-petrosum ganglia. Some trophic effects of BDNF in the PNS seem to depend on autocrine loops and paracrine interactions between adjacent neurons, since sensory neurons can express both BDNF and its high affinity receptor TrkB.

BDNF is more highly expressed and widely distributed than NGF in the CNS, and has survival promoting actions on a variety of CNS neurons including hippocampal and cortical neurons [82,133], cholinergic neurons [4], and nigral dopaminergic neurons [105]. Recent findings showed that BDNF is anterogradely transported in the CNS, a fact that could considerably expanded the concept of neuronal-derived trophic support, and sustained the hypothesis that BDNF act at the synaptic level [9] and might be a more multifunctional compound than thought before.

NEUROTROPHIN RECEPTORS

Two classes of membrane receptors mediate the biological actions of NTs [19,32,43,53]: the Trk family of receptor tyrosine kinases, and a protein called p75 a member of the TNF receptor superfamily. NTs bind with distinct selectivity to three highly related receptor protein-tyrosine kinases, known as high-affinity (K_d~10^{-11} M) NT receptors

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TrkA, TrkB and TrkC. NGF specifically binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 binds preferentially to TrkC but also to TrkB and TrkA with lower efficacy [112,117]. Through these receptors, neurotrophins activate many signaling pathways, including those mediated by RAS, by members of the cdc-42/ras/rho G protein families, by the MAP kinase, PI-3 kinase, and by Jun kinase cascades. Within neural precursors and neurons, the pathways regulated by tyrosine kinases include survival and differentiation, axonal and dendritic growth and remodeling, assembly of the cytoskeleton, membrane trafficking and fusion, and synapse formation and function. All neurotrophins bind with similar affinities (K_d~10^{-9} M) to p75NTR. The mechanisms of transduction mediating the biological effects of p75NTR in neurons are poorly understood. p75NTR is known to modulate cellular responses to neurotrophins by interacting with their high-affinity Trk. Modulation of Trk interaction with neurotrophins has been considered as the main p75NTR mechanisms of action since the discovery of Trk receptors [19,33,44]. However, it seems that p75NTR can induce cellular responses in the absence of Trk receptors inducing cell death when is activated by NGF [38,72].

NEUROTROPHINS AND CNS NEURONS

NGF-Producing Cells

NGF is produced in the CNS during development and throughout adult life. Most of NGF-producing cells are cholinergic neurons of the basal forebrain including cortex and hippocampus. In the hippocampal formation, pyramidal and dentate granule neurons express NGF, as do subpopulations of GABAergic interneurons [74,77,162]. Glutamatergic and cholinergic neurotransmission increases NGF expression in the hippocampus while GABAergic neurotransmission decreases NGF expression [29,74,119]. In the striatum, NGF is produced by a subpopulation of small interneurons [30].

NGF is produced by astrocytes and microglia, and NGF expression is markedly up-regulated by local tissue injury, inflammation, cytokines, and bacterial lipopolysaccharide (both *in vivo* and *in vitro*) [16,61,93, 146,214]. Increased NGF levels in the injured CNS suggest that astrocytes and microglial cells could serve as local sources of NGF for the recovery of injured neurons and other NGF responsive cell types contributing to the response to NGF in the whole injured tissue. Up-regulation of NGF has been found following acute brain lesions due to chemical or mechanical insults in an intrinsic effort to regenerate NGF receptive neurons. However, following chronic brain lesions due to noxious processes the NGF increase cannot be maintained and NGF may decrease. The amount of this NGF up-regulation following lesions depends on the age and the type of the NGF receptive brain cells. It has been hypothesized that very high levels of NGF, or its chronic potentation result to be harmful to neuronal functioning at least under certain physiopathological conditions. Indeed in the Alzheimer's disease, NGF levels are lower during the onset of the disease. However when the brain degeneration is well established, brain NGF appears to elevated [reviewed in 96].

NGF Receptor Expressing Cells

p75NTR gene expression in the CNS is widespread, mainly during development [43]. p75NTR expression is more limited in the adult. Several populations, including cholinergic neurons of the caudate-putamen and cranial nerve nuclei of the brainstem, show markedly reduced or no expression at the adult stage [121]. Cerebellar Purkinje neurons, hippocampal pyramidal neurons, and retinal ganglion neurons also have low NGF expression in adults but may express again p75NTR after injury [35,59,143,212].

Cholinergic neurons of the septal-basal forebrain complex express both TrkA and p75NTR during development and throughout adult life [102]. Expression of both TrkA and p75NTR in forebrain neurons is upregulated by NGF [76,102]. Adult cholinergic neurons of the extended striatal complex (caudate, putamen, accumbens, etc) mainly express TrkA: however, p75NTR and TrkA expressions are increased by local tissue injury or NGF infusions [76,101]. Adult neurons expressing TrkA, but not p75NTR, are found in the thalamic paraventricular nuclei, rostral and intermediate subnuclei of the interpeduncular nucleus, and various other brain regions [101,203], and also in the spinal cord in regions associated with the regulation of the autonomic outflow [147]. Some hippocampal pyramidal neurons may also express very low levels of TrkA [42], and a recent study indicates the presence of TrkA and p75NTR proteins in pyramidal cells of the somatosensory cortex of the mature rat [164].

Low levels of p75NTR are expressed in many mature astrocytes and TrkA is also expressed by astrocytes, particularly after exposure to NGF or inflammatory cytokines [104,128]. Oligodendrocytes express p75NTR [39,128]. Microglia have the capacity to express p75NTR and TrkA and may be modulated by inflammatory stimuli, such as cytokines, and bacterial lipopolysaccharides [62].

BDNF-Producing Cells

BDNF mRNA is highly expressed by brain neurons. Levels of BDNF mRNA expression are present in the rodent [65,66,100,209] and human brain [163]. BDNF and TrkB are co-expressed by neurons in the rat cerebral cortex and in the hippocampal formation displaying paracrine mechanisms [123]. BDNF protein has been found in striatal fibers but not in striatal neuronal cells bodies [47,55,115,213] where its mRNA is expressed in very low amounts [47,209]. Rat studies show that a minority of cells in the basal forebrain expresses BDNF mRNA, but many cells contain BDNF mRNA in the medial septum [47,213]. BDNF is also synthesized by a small number of cholinergic neurons projecting to the cerebral cortex and hippocampal formation. Indeed, BDNF-immunoreactivity [47,55,115,213], and BDNF mRNA expression [47,209] have been observed in rat and monkey nigral neurons. There has been revealed [182] the dopaminergic nature of BDNF-containing nigral neurons in double labeling experiments, showing the co-localization of BDNF mRNA and tyrosine hydroxylase in the rat mesencephalon. In the rat brain, BDNF mRNA is displayed by granule cells, but not by Purkinje cells [170,209]. However, neurons of the locus coeruleus display BDNF

mRNA [40,47,213] and BDNF-immunoreactive cell bodies [115] in rodents. A BDNF mRNA expression in rodent cultured glial cells has also been demonstrated. BDNF expression by Schwann cells in culture has been reported [1], whereas there has been reported low levels of BDNF mRNA in primary rat astroglial cultures [173], and that the rodent microglia expresses BDNF mRNA *in vitro* [61]. BDNF expression and release by glial cells can be regulated by different signals and injuries [21].

BDNF Receptor Expressing Cells

Numerous brain neurons co-express both BDNF and TrkB [123], indicating that BDNF may be involved in different mechanisms: (1) a kind of neurotransmitter-like axo-dendritic communication, (2) in autocrine loops and paracrine interactions between neighboring cells; (3) in trophic mechanisms, as retrograde communication from dendrites to axon terminals. Cortical and hippocampal interneurons express full-length TrkB but not BDNF [85] and TrkB mRNA is expressed in neurons of the human cerebral cortex, and pyramidal and granule cell layers of the hippocampus [26]. In the rat, most nigral dopaminergic neurons express both TrkB mRNA and protein [10,145,156] and in humans TrkB is expressed by nigral dopaminergic neurons [5,26]. Cholinergic neurons of the nucleus basalis of Meynert also express TrkB mRNA and protein in the human brain [26,31]. In the rodent cerebellum, TrkB-immunoreactivity [180] and TrkB mRNA [80], were found both in granule and Purkinje cells. Cultured microglia not only express p75NTR and all Trks receptors for neurotrophins but also respond to BDNF [61,149].

CHOLINERGIC NEURONS

The distribution of NGF and its receptors suggests a close interrelationship between cholinergic neurons and their NGF-producing targets. Indeed cholinergic neurons are receptive to the action of NGF; however, there is no strong evidence that brain cholinergic cells produce NGF. Cholinergic neurons account for almost all of the NGF-responsive neurons in the CNS, although there are groups of noncholinergic neurons that express TrkA, including some hippocampal cells [42]. In the adult forebrain cholinergic neurons, NGF influences the structural plasticity and the response to injury. Under normal condition administration of NGF to the unaffected CNS causes several effects on cholinergic neurons, including increasing hypertrophy, sprouting, upregulation of NGF receptors, increased levels of choline acetyltransferase (ChAT), and increased choline uptake [95,99,130]. These observations lead to the hypothesis that NGF plays a role to maintain the cholinergic phenotype through retrograde transport of the NGF/TrkA signaling complex from cholinergic nerve terminals in the hippocampus to the cell bodies of the basal forebrain [181].

This hypothesis is consistent with the findings showing that administration of NGF prevents cholinergic neuron atrophy caused by injuries of the septohippocampal and basal forebrain-cortical systems [94]. Basal forebrain cholinergic neurons are responsive not only to NGF but also to other trophic factors. It has been shown [4] that BDNF promotes survival of rat embryonic septal cholinergic neurons *in vitro*. BDNF increased the activity of enzymes involved in ChAT synthesis and degradation. BDNF administration provides limited recovery of the basal forebrain cholinergic neurons against the damage induced by axotomy or target cell destruction. It should be mentioned, however, that in forebrain cholinergic neurons, BDNF is not as effective as NGF in both protection and recovery [124,203].

DOPAMINERGIC NEURONS

There is evidence showing that injured brain dopaminergic neurons are receptive to the action of NGF but NGF influences dopaminergic neurons also following neurotoxin injury [81] and in Parkinson's disease [136]. These effects appear to be mediated by dopaminergic neurons expressing NGF-receptors [155]. Moreover different groups showed that BDNF prevents the spontaneous death of dopaminergic neurons in rat primary mesencephalic cultures [106,120]. Data have also been provided [105] revealing that BDNF administration protects TH-immunoreactive neurons from the selective toxin MPP^+ (1-methyl-4-phenylpyridinium). These results raised interest in BDNF as a putative novel therapeutic agent for Parkinson's disease. Cultured dopaminergic neurons treated with BDNF show increased TH activity, dopamine uptake and dopamine content, release of dopamine upon depolarization, and display an increased cell body size and higher neuritic complexity [24,106]. The protective effect of BDNF *in vitro* against specific toxins like MPP^+ and 6-OHDA, has likewise been confirmed [25]

Repeated intrastriatal injections of BDNF protected by the damage induced by intrastriatal administration of 6-OHDA [186] with moderate preserving of striatal dopaminergic nerve endings around the injection site. Likewise there has been reported a protective effect of direct nigral infusion of BDNF against reductions in striatal dopamine content induced by neurotoxins in the mouse [103]. In summary, there is a body of work suggesting that exogenous BDNF administration can increase survival and/or promote recovery of injured mesencephalic dopaminergic neurons.

SEROTONERGIC NEURONS

Besides the well-established role of NGF and its high-affinity NGF receptor TrkA for the cholinergic basal forebrain survival and function, it has been reported that 45% of the serotonergic raphe neurons also express both TrkA [83,191] and p75NTR [122]. However, a functional relationship between NGF and serotonergic neurons remains to be established. Serotonergic lesion of the median raphe nucleus results in biphasic changes of NGF protein content and in a delayed increase in the vulnerability of septohippocampal cholinergic neurons [97].

Neuroprotection by the $5\text{-}HT_{1A}$ receptor agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) has been demonstrated against excitotoxic neuronal damage in cholinergic neurons of the rat magnocellular nucleus basalis [157]. Stimulation of $5\text{-}HT_{1A}$ receptors inhibits apoptosis

initiated by serum deprivation *in vitro*. However these effects seem to be associated with the induction of NGF synthesis [2] and mediated by the neurotrophic effect of 5-HT [168,212].

Also BDNF has been demonstrated to exert potent neurotrophic effects on 5-HT neurons when infused into midbrain [187] while infusion of BDNF into forebrain results in a dramatic elevation of 5-HT neuronal fiber density, and protection of neurons from neurotoxic damage [141]. In addition, BDNF expression can also be regulated by serotonin [154,202,216].

NEUROTROPHINS AND SCHIZOPHRENIA

Although a number of hypotheses have been proposed in an effort to explain the pathophysiology of schizophrenia, no single theory seems to account for all the physiopathological manifestations of the disease. Each hypothesis explains some of the phenomena associated with schizophrenia and it is likely that many of the variables described interact to produce the disorder. Studies on animal models and in humans suggest that the developmental manifestation of schizophrenia might be a part of an ongoing specific brain neurodegenerative processes [18]. There is also strong evidence suggesting that non-heritable factors in the pathogenesis of schizophrenia are associated with abnormalities during prenatal development. Indeed, epidemiological researches show increased incidence of schizophrenia in humans prenatally exposed to virus [183], gestational complications or malnutrition [51,54]. In addition, post mortem studies have indicated that certain limbic or associative brain areas of schizophrenic subjects are characterized by decreased cell numbers and volume [37,67], disorganized cytoarchitecture and reduced content of certain microtubule associated proteins [17]. These findings have suggested a failure of both setting and migration of young neurons into their appropriate cortical target sites, particularly in the hippocampus and entorhinal cortex [34,206].

The entorhinal cortex is a brain structure contained in the limbic allocortex of the medial temporal lobe having a key role in processing associational information [46] and is connected to the hippocampus and other subcortical structures [211]. It has also been shown that memory and learning process as well as memory consolidation are regulated in the hippocampus and parahippocampal regions, including the entorhinal cortex [60]. An involvement of the latter structure in human learning capabilities is suggested by a PET-study [118] and by the fact that hippocampal damage is associated with cognitive impairments [84,207].

Neurotrophic factors play key roles in the development and maintenance of hippocampal and cortical structures. More specifically NGF participates in the neuroregulation of the mechanisms leading to the consolidation of hippocampal spatial memory through cholinergic pathways while BDNF promotes actions on neurons of hippocampal, nigral and cortical structures [4,82,105,133]. Thus, the neurotrophic factor hypothesis of schizophrenia has been proposed [198], explaining the changes in the brain of schizophrenic patients as the result of disturbances of developing processes involving trophic factors. Neurotransmitter deficits are thereby considered as epiphenomena of underlying neurotrophic factor disorganization. This hypothesis is supported by studies showing that NGF and BDNF are abnormally regulated in the CNS of animal models of schizophrenia [8,28,68-71], by the evidence that schizophrenic patients have low circulating levels of NGF [27] and by the fact that schizophrenics are characterized by reduced BDNF in the serum [199]. A link between neurotrophins and schizophrenia is potentiated by the evidence that neuronal development of embryonic brain tissue derived from schizophrenic women has shown neurite-growth deficits [73] and the polymorphism gene of neurotrophin-3 is resulted to be associated with schizophrenia [92,150]. However it should be noted that the hypothesis of a genetic origin of schizophrenia is still debated [153,189,204]. Findings obtained with studies on human post-mortem tissues have also revealed a significant increase in BDNF concentrations in cortical areas and a decrease in this neurotrophin in the hippocampus of patients when compared with controls [57], whereas other authors have found that BDNF protein is elevated in the anterior cingulated cortex and hippocampus of schizophrenic patients [193]. In addition, analysis of post mortem brain tissues of schizophrenic patients revealed decreased mesopontine ChAT levels in schizophrenia, which were correlated with the cognitive impairments evidenced by schizophrenics [114]. All together these findings suggest that neurotrophic abnormalities are associated with the corticolimbic structures of schizophrenic patients providing the molecular substrate for pathological manifestations of the disease. Moreover these data were discussed as further evidence to the neurotrophin hypothesis of schizophrenic psychoses proponing that alterations in the expression of neurotrophins might participate in the neural maldevelopment and in the disturbed neural plasticity as important event in the etiopathogenesis of schizophrenia.

It is known that antipsychotic drugs as haloperidol and risperidone are widely used to reduce or overcome the clinical manifestation of schizophrenia. Haloperidol mainly aets on the dopamine D_2 and D_3 receptors [88]. The latter receptor is also susceptible to the action of BDNF. Risperidone binds to both DA and serotonin (5HT) receptors, particularly in the neurons of striatal and limbic structures. Findings revealing a link between antipsychotic administration and synthesis, and release of neurotrophins and some neurotransmitters regulated by NGF and BDNF have been recently reported [12,13]. We showed that haloperidol and risperidone increased the NGF concentrations in hypothalamus but decreased NGF levels in the striatum and hippocampus, which resulted to be associated with a significant decrease in ChAT-immunoreactivity in large-size neurons following both haloperidol and risperidone treatments in the septum, as well as the Meynert's nucleus. These studies also revealed that haloperidol and risperidone decreased the basal levels of BDNF in frontal and occipital cortex and hippocampus, whereas haloperidol alone significantly reduced the number of TrkB immunoreactive neurons of the hippocampus, substantia nigra and ventral tegmental area. Administration of haloperidol potentiated NPY expression in the occipital cortex, while risperidone increased NPY in the occipital cortex, hippocampus, and hypothalamus. All these studies seem to provide evidence that a mechanism through which

antipsychotic drugs exert their effect might be to modify the synthesis and distribution of neurotrophins in selected brain regions.

Animal Models of Schizophrenia

Animal models of brain disorder, particularly those resembling analogous defects in cortical development, could be useful to study how such defects are translated into a disease with cognitive, and behavioral characteristics of schizophrenia. Such models might also offer a chance to verify the hypotheses whether specific etiologic factors, such as changes in growth factor levels, or drugs are able to affect the cortical development producing schizophrenia-like abnormalities. The administration of drugs producing or exacerbating humans' schizophrenia symptoms or brain lesion models has been carried out widely [23,111,165,175]. For example, neonatal damage of the ventral hippocampus or amygdala [50,134,135], or administration of amphetamine [63] inducing disruption of latent inhibition (and probably blocking), or phencyclidine [174] inducing changes in the startle response provided interesting animal models with construct, face, and predictive validity for schizophrenia.

It has been shown [194-195] that interference with neurogenesis in the mediotemporal allocortex of rat embryos, during the earliest stages of cortical proliferation, results not only in a thickness reduction of the adult entorhinal cortex and hippocampus [68,195] but also in other morphological characteristics resembling those observed in patients with schizophrenia [34]. An animal model displaying these structural brain deficits (Table 1) was obtained administering a single injection of methylazoxy-methanol acetate (MAM) in pregnant rats on gestational day 11 or 12 [see 41] when the entorhinal-hippocampal axis was supposed to be in major cell proliferation [22].

Analysis of the forebrain in adult animals showed reduction in the entorhinal cortex size and this effect shifts from lateral to medial divisions of the entorhinal cortex with later administration of MAM, following a known developmental gradient. Morphological consequences of MAM administration appear to be largely confined to the hippocampal-entorhinal axis although slight reductions of the frontal and occipital neocortex were also observed. MAM treatment on gestational day 12 revealed relatively more widespread damage, as reflected among others in a small decrease in brain weight.

Animals prenatally exposed to MAM were tested for both behavioral performances [68-71,194] and neurotrophins' levels [68-71]. Young animal prenatally treated with MAM didn't show gross behavioral changes in social interaction, open-field and novel object investigation tests. On the contrary, young MAM treated rats had retardation in passive avoidance acquisition suggestive of slight changes in memory and learning processes. Moreover, young rats exposed to MAM on gestational day 12 revealed reduced pain sensitivity and increased NGF in the entorhinal cortex and remaining cortex. However, young MAM treated animals showed no changes in paw NGF or substance P levels, suggesting that the altered nociceptive response is not linked to the local down-regulation of these two peptides [71]. Indeed, it was shown that schizophrenic patients had altered peripheral sensory responses leading to a strong analgesia [58,87].

At the adult age the behavioral studies showed that rats treated with MAM had moderate to severe social impairment depending on the exact timing of prenatal exposure. Working memory was not importantly affected in any group examined [194] but spatial learning was delayed in the Morris maze [69]. Adult rats exposed to MAM showed changes in locomotion and displacement activities [68]. Immuno-enzymatic assays revealed that rats treated on gestational day 12 had increased concentration of NGF and BDNF in the entorhinal cortex and hippocampus compared to control rats. However, prenatal MAM administration did not affect significantly p75 and ChAT distribution in the EC and septum [68-69].

Based on the above evidence our working hypothesis was that prenatal MAM administration, by interfering with limbic neurogenesis, could affect learning and memorizing ability of aged animals measured in the water maze [70]. This hypothesis is in line with the observation that injection of MAM during early rat brain development induced deficits in both the acquisition and retention phases of the Morris maze. These changes in the behavior were associated with significant increase in brain NGF in the entorhinal cortex, while in the hippocampus a decreases in NGF and BDNF [70]. We also evidenced in the aged MAM rat reduced ChAT immunoreactivity in forebrain cholinergic neurons and loss

Table 1. Selected Features of the MAM Rat Model of Schizophrenia (— = Unknown)

Functional or Biochemical Changes	MAM Model	Schizophrenic Patients
Cognitive Deficits	Yes	Yes
Reduced Pain Sensitivity	Yes	Yes
Decreased Enthorinal Cortex and Hippocampus	Yes	Yes
Altered Social Behavior	Yes (mild)	Yes
Abnormal Regulation of BDNF and NGF	Yes	Yes
Abnormal Expression of ChAT in the Mesopontine Tegmentum	---	Yes
Abnormal Expression of ChAT in the Forebrain	Yes	---

of NPY immunodistribution in cells of the entorhinal cortex suggestive of a link between neurotrophins' and neurotransmitters' changes.

NEUROTROPHINS AND DEPRESSION

There are findings suggesting that neurotrophin action could be impaired in depression and stress-related affective disorders and that these endogenously produced molecules, particularly BDNF, are involved in the etiology of these illnesses. Indeed, chronic stress has been shown to down-regulate neurotrophin synthesis causing atrophy and, in severe cases, death of vulnerable CA3 neurons in the hippocampus [138,176]. Stress is also reported to decrease the expression of BDNF in CA3 pyramidal and dentate gyrus granule cell layers of the hippocampus [190]. These observations lead to the hypothesis that down-regulation of BDNF could contribute to accelerate the atrophy of CA3 neurons rendering these neurons more susceptible to other factors, such as adrenal glucocorticoids that are induced in response to repeated stress. Brain imaging studies have reported that there is a small, but significant reduction in the volume of hippocampus in patients with depression or posttraumatic stress disorder [36,177,184]. Atrophy and decreased functions of the hippocampus could explain the reduction, observed in depressed patients, in negative feedback control that this brain region exerts on the hypothalamic-pituitary-adrenal axis [215]. Moreover, chronic infusion of BDNF is reported to have antidepressant effects in two behavioral models of depression, such as the forced swim and learned helplessness paradigms [188]. Therefore up-regulation of BDNF in response to antidepressant treatment could have similar behavioral effects, and could enhance 5-HT neurotransmitter function. These findings also indicate that there is a positive, reciprocal link between 5-HT and BDNF and up-regulated BDNF would be expected to increase 5-HT neuronal function. Other studies have shown decreased plasma levels of BDNF in patients with major depression [113] and an increased BDNF immunoreactivity in the hippocampus of patients treated with antidepressant drugs [45]. There has been also postulated a role of the prefrontal cortex in addition to the hippocampus as a site of neuropathology in depression evidencing relevance of cellular changes in mood disorders to stress and prolonged prefrontal cortex development with a role of neurotrophic factors [166].

There are few genetic studies on depression and neurotrophins [153,189] and they are essentially negative thus a direct involvement of neurotrophins is debated. In addition to monoamines, several neuropeptides, including opioid peptides [64,86] and neuropeptide Y [91,205,210], are believed to play a role in clinical depression or learned helplessness behavior in rats. Information regarding this possible role comes from studies on the effects of central administration of BDNF on neuropeptide distribution [49,151]. Therefore, the modulation of neuropeptide systems by BDNF, throughout a direct or indirect effect, may contribute to the antidepressant-like effects of this protein. Although these pilot studies have demonstrated an antidepressant-like effect of BDNF [185], the anatomical sites capable of mediating this effect remain to be determined. Furthermore, the possibility of BDNF-induced changes in monoamine and/or peptide receptors has not yet addressed.

Animal Models of Depression

To date, a wide range of animal models of depression has been developed. They include models in which "depressive behavior" is the result of genetic selection or manipulation, environmental stressors during development or in adulthood, or pharmacological treatments. Genetic models of depression include the Fawn-Hooded (FH/Wjd) rat, a rat inbred strain reported to show high immobility in the forced swim test [167], and the Wistar Kyoto (WKY) rat, characterized by hyper-reactivity to stress and depressive-like behavior in several standard behavioral tests [192]. A few studies were also made by using knockout, mice as, for example, those lacking the 5-HT transporter [129] or those lacking the alpha(2)-adrenergic receptors, which modulate norepinephrine release, as well as the release of serotonin and other neurotransmitters, and are therefore potential targets for antidepressant and anxiolytic drug development [179]. Early maternal separation inducing a response characterized by reduced activity and other depressive-related behaviors has been also used [99]. In adulthood, anhedonia (the loss of interest or pleasure in daily activities) induced in rats by a regimen of repeated, mild, unpredictable stressors, is also supposed to be a model of depression with predictive and etiological reliability [148]. In addition, learned helplessness, in which exposure to inescapable stress produces deficits in escape testing, has been proposed as an animal model of stress-induced behavioral depression [110]. To better characterize these models, many investigators have tested a wide variety of pharmacological treatments. It is found that administration of clomipramine, a preferential serotonin re-uptake inhibitor, to neonatal rats produces adult depression-like behaviors, such as decreased pleasure seeking, reduced aggressiveness, increased locomotor activity, diminished sexual activity and increased REM sleep [142]. The reliability of these animal models is based on both behavioral tests measuring traits that are homologous to symptoms of the human disorder and behavioral tests responsive to appropriate pharmacologic treatments.

The Flinders Sensitive Line (FSL) rats and their corresponding controls, the Flinders Resistant Line (FRL) rats (Table 2), were established by selective breeding for high and low sensitivity, respectively, to the anticholinesterase agent, diispopropyl fluorophosphate [159]. This rat line was originally developed based on the fact that an overactive cholinergic system seems to be associated with depression-like behavior [108] while patients with affective disorder displayed increased sensitivity to cholinergic agonists [109,169]. These rats show a severe immobility in the forced swim test compared to their control FRL rats [158]. Immobility in the swim test is considered a reliable marker of anergia or depressive characteristics of the rats, which may be used for testing the efficacy of antidepressant drugs [137]. Indeed, the exaggerated immobility in the forced swim test in the FSL rats can be counteracted by the administration of antidepressant drugs [160,178]. Thus, the FSL rats seem to meet the criteria of face, construct, and predictive validity for an animal model of depression [161] for studying the neurochemical mechanisms involved in

Table 2. Selected Features of the FSL Rat Model of Depression (— = Unknown)

Functional or Biochemical Changes	FSL Model	Depressed Patients
Immobility in Forced Swim Test	Yes	Yes
Reduction in Symptoms after Antidepressants	Yes	Yes
Vulnerability to Stress	Yes	Yes
Supersensitivity to Muscarinic Agonist	Yes	Yes
Cognitive Deficits	Yes	Yes
Amount of REM	Yes	Yes
Increased NGF and BDNF in the Occipital and Frontal Cortex	Yes	—
Increased BDNF in the Hypothalamus	Yes	NK

depressive-like behavior and in the mode of action of antidepressants.

We have recently shown that brain regions of FSL rats expressed higher concentrations of BDNF and NGF as compared to brain regions of FRL controls. The amount of neurotrophic factors was higher in both male and female "depressed" FSL rats. Specifically, in the frontal cortex, the female FSL showed significantly higher concentrations of both BDNF and NGF. In contrast, in the occipital cortex, the male FSL had significantly higher concentrations of BDNF and NGF. In the hypothalamus, higher levels of BDNF were found in FSL, both male and female, rats. Moreover, in the frontal cortex, higher NGF amounts were found in the male compared to female rats in both strains [14]. Cumulatively these observations suggest that BDNF and NGF may play a role in depression and, hypothetically, different brain regional concentrations of BDNF and NGF in male and female animals may be relevant to gender differences in vulnerability to depression. We also demonstrated that the effects of repeated electroconvulsive stimuli (ECS) used as model of electroconvulsive treatment (ECT) induced changes in NGF and BDNF in FSL rats. In the hippocampus, ECS increased NGF concentration in FSL, but not FRL rats. ECS decreased NGF concentration in the frontal cortex of FSL rats. In both FSL and FRL rats, ECS increased NGF levels in the striatum. In contrast, ECS did not change BDNF concentrations in hippocampus, frontal cortex and striatum of FSL and FRL rats (unpublished data).

In Sprague Dawley rats used as further controls of FSL rats, ECS increased the concentrations of NGF in the frontal cortex and the concentrations of BDNF in hippocampus, striatum and occipital cortex. In contrast, ECS decreased GDNF concentrations in hippocampus and striatum [15]. Our data indicate that neurotrophic factors play a role in the mechanism of action of ECS and, by extrapolation, therapeutic mechanism of action of electroconvulsive treatment.

CONCLUSION

Although still debated, the evidence so far available both in humans and animal models suggest that schizophrenia may be the result of developmental cytoarchitectural alterations of the hippocampal–entorhinal axis and that the depression may be associated with the inability of neuronal systems to exhibit appropriate adaptive plasticity [3,17,90,201,207]. However, basic studies on animal models have raised the question as to whether neurotrophins, particularly NGF and BDNF, play a role in regulating behaviour and in neurodevelopmental disorders [6,27-28,152,193]. Supportive evidence related to these hypotheses comes from studies on human brain neurotrophin levels showing that neurotrophins' levels undergo significant changes following neuropsychiatric diseases. Schizophrenic patients showed changes in neurotrophins' levels in both the plasma and CNS [152,193] suggesting that changes in the expression of neurotrophins might contribute to the neural maldevelopment and to the disturbed neural plasticity as important event in the etiopathogenesis of schizophrenia. Indeed, schizophrenic patients showed low circulating levels of NGF and BDNF [27,199], elevated BDNF protein in the cortex, and reduced BDNF in the hippocampus [57,193]. We speculated that some deficits in cognitive processes observed in schizophrenic patients [84] were associated with decreased brain ChAT immunopresence [114] and the altered immunopositivity of ChAT and NPY found in MAM exposed rats might be related to the disrupted availability of NGF and/or BDNF in the hippocampal/entorhinal axis. Our working hypothesis is that the deficiency of one or both of these neurotrophins may contribute to the maldevelopment of the hippocampal/entorhinal axis and participate in the development of some specific neuropsychiatric manifestations. A wide variety of pharmacological trials have been tested to check whether or not they might increase cognitive performance in the treatment of disorders, such as anxiety, memory dysfunction and neuropsychiatric diseases [75]. Based on the above observation, it seems reasonable to imply that the use of drugs influencing the synthesis and release of cholinergic neurotransmitters might be helpful in potentiating cognition in healthy subjects and suggests that cholinomimetics may be important factors for treating cognitive deficits [75,196]. Consequently, NGF and BDNF, which play key roles in regulating ChAT and NPY activity, could assemble a potential therapeutic action in the treatment of neuropsychiatric diseases. In the framework of this hypothesis the prenatal MAM model could be considered a useful tool to study some specific pathogenic mechanisms

involved in human brain maldevelopment. Indeed, the experimental disruption of limbic cortex development may present a good approach to investigate the relationship between abnormal ontogenesis and schizophrenia. Similar strategies might be considered to study other neuropsychiatric disorders with a presumed neurodevelopmental pathogenesis. Nonetheless, it should be noted that antipsychotic agents such as Haloperidol and Risperidone, commonly used by schizophrenics are known to reduce hippocampal neurotrophic factor expression [13]. These findings apparently do not fit with our working hypothesis of a reduced neurotrophic support during development. However, it should be considered that treatment with antipsychotics reduces dendritic spines in brain regions [116], decreases hippocampal and striatal ChAT activity [139] leading to strong impairments in cognitive abilities [78]. Thus an unexpected secondary effect of chronic antipsychotic administration is to disrupt cognition throughout the inhibition of neurotrophins' production. All together these data highly potentiate the debate on the real levels of the side effects of antipsychotics in schizophrenia [79,127,197]. In addition, in the case of schizophrenia the discrepancy between antipsychotics and animal models could also be due to the fact that antipsychotics do not really affect the course of schizophrenia. Essentially, the drugs only reduce the positive and not so much the negative symptoms and also reduce the anxiety that often is associated with the disorder. In schizophrenia one might argue that because of some cerebral cortical abnormalities the mesolimbic dopamine system becomes dysregulated, thus causing some symptoms. So either by repairing the cortical deficit (which is at the present time an illusion) or by reducing dopamine activity antipsychotic effects may be obtained.

Chronic antidepressant treatments enhance BDNF expression within hippocampal and cortical neurons and clinical studies revealed the existence of significant hippocampal damage in cases of major and/or recurrent depression. Thus, antidepressant treatments through enhanced expression of growth and survival promoting factors like BDNF could prevent or reverse the atrophy and damage of hippocampal neurons. Indeed, findings have indicated that chronic antidepressant treatments enhance hippocampal neurogenesis, promote neuronal sprouting and prevent atrophy. The observations that BDNF may produce an antidepressant-like effect when injected into the brain [188] and that the stressing events alter the constitutive levels of neurotrophins [7,200] have raised the question as to whether changes in neurotrophin production and/or function are instrumental in the pathophysiology of depression [11]. The available data on the BDNF and NGF levels of the FSL animal model of depression showed increased neurotrophin presence in the brain but not in the hippocampus (the major area of synthesis of these two neurotrophins). Our findings are not consistent with the data obtained by others showing decreased levels of neurotrophins in depression [11]. An accumulation of neurotrophins could show a decreased release/breakdown or a compensatory increase in neurotrophin synthesis in response to primary neurotransmitter changes in the limbic system during depression [56]. Though this does not explain the obtained differences, it is possible that this discrepancy could be due to the different animal models and different experimental protocols used. Since gender differences in many neurotransmitters, e.g., the serotonergic and catecholaminergic systems, and neuropeptides both in the CNS and periphery have been showed [52], it was of interest to explore whether the same is true for neurotrophins. In addition, since women exhibited higher frequency and greater susceptibility to depression [140,208], we studied whether neurotrophins might be selectively affected in the brain of FSL female rats. Our study has indicated that the male and female brains, irrespective of the strain, contain different levels of neurotrophins, at least in some brain regions. Though the functional significance of these findings remains to be established, the data reveals clear-cut sex differences and, by inference, indicate that BDNF and NGF may also play a role in susceptibility to depression.

On the whole the data on NGF and BDNF in humans affected by neuropsychiatric diseases, and in animal models suggest that modulation of neurotrophic function may affect therapeutic interventions in depression and schizophrenia and indicate that this approach may be a new way to promote limbic system neuroplasticity.

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PART IV: GENERAL FINDINGS

It has been shown (Talamini et al. 1998; 1999) that interference with neurogenesis in the mediotemporal allocortex of rat embryos, during the earliest stages of cortical proliferation, results not only in a thickness reduction of the adult entorhinal cortex and hippocampus (Talamini et al. 1998) but also in other morphological characteristics resembling those observed in patients with schizophrenia (Braak and Braak 1992). The animal model displaying these structural brain deficits was obtained administering a single injection of methylazoxymethanol acetate (MAM) in pregnant rats on gestational day 11 or 12 (see Cattabeni and Di Luca 1997) when the entorhinal-hippocampal axis is supposed to be in major cell proliferation (Bayer and Altman 1987) (we administered also ad GD9 and GD10 to get additional informations).

Analysis of the forebrain in adult animals showed reduction of the entorhinal cortex size and this effect shifts from lateral to medial divisions of the entorhinal cortex with later administration of MAM, following a known developmental gradient. Morphological consequences of MAM administration appear to be largely confined to the hippocampal-entorhinal axis although slight reductions of the frontal and occipital neocortex were also observed. MAM treatment on gestational day 12 revealed relatively more widespread damage, as reflected among other in a small decrease in brain weight.

Results of Paper 1

Our results show that prenatal MAM exposure elicits changes, in both behavior and brain NGF levels in young MAM rats. Data on brain morphology show that interference in cell division during prenatal development, from gestational day 9 to day 12 in rats, progressively reduces the formation of the entorhinal cortex. Entorhinal cortex reduction is more effective caudally than rostrally in all groups. Furthermore the lateral entorhinal cortex is predominantly affected by treatment on GD9, GD10, or GD11, whereas GD12 treated rats also show abnormalities of the medial entorhinal cortex. Moreover, in GD12 animals, MAM treatment produced also cortical and hippocampal thinning, disorganised cortical layering and abnormal temporal asymmetries. Brain abnormalities failed to importantly disrupt social behavior suggesting that, during development, slight changes in cortical proliferation are not sufficient to affect social behavior. Because social and play behaviors are prominent in mammals, and mammals have a more elaborated cortical structure than non-mammalian species, it has been suggested that the cortex could play a crucial role in the regulation of these behaviors. This is in line with the supposed role of the hippocampal/enthorinal axis in processing complex

contextual information (Klinberg et al. 1994), which probably is more important in adult, than in pre-adolescent social interaction.

Abnormalities in locomotion and rearing in GD12 MAM rats were observed in the open-field. and under novel environmental situations may reflect impaired capability to cope with stressful conditions (Belzung and Le Pape 1994). Prenatal MAM administration on gestational day 12 induces slight but widespread cortical changes associated with a small brain reduction in rats (Talamini et al. 1998) which could be associated with the behavioral changes observed in the open-field test in locomotory activity and exploration of GD12 rats, since the neural substrates of these behavioral patterns are widely distributed all over the brain.

Another effect of prenatal MAM administration was that grooming increased in rats exposed at GD9 and GD10. In rodents, grooming can represent a common attitude of body care, a displacement behavior due to stressing environmental situation and/or a behavioral pattern which expression is affected by specific damage to its neural substrates (see for review Spruijt et al. 1992). Indeed, it has been reported that grooming is influenced by manipulation of the striatonigrocollicular pathway and by alterations in cholinergic neurons and muscarinic receptors (see references in Spruijt et al. 1992).

Notably GD11 and GD12 rats had a retardation in passive avoidance acquisition. The alterations in learning and memory processes were limited to the acquisition phase, since no effects were found during the retention phase, which took place 1 day later. This leads to a disruption in the associative mechanisms serving the learning and memory processes, active during acquisition and imply that prenatal MAM treatment on gestational days 11 and 12 may influences the neural systems underlying specific behavioral responses. The observation that the hippocampal/entorhinal formations are brain areas involved in learning and memory capabilities (Vnek et al. 1995) is suggestive of a link between the brain morphological MAM effects observed in GD11 and GD12 animals and the reported behavioral impairments.

Hot-plate responses showed that prenatal MAM treatment on GD12 increased analgesia. Pain sensitivity in rodents seems to be regulated by different peripheral and central pathways (Kavaliers 1988). For example, exposure to exogenous or endogenous TNF-α is able to induce changes in nociception (Fiore et al. 1996) and so does the administration of NGF, via the activation of several neuropeptides such as SP and calcitonin gene related peptide (Lewin et al. 1994). Our immunoenzymatic data indicate that both NGF and SP did not change in the paws of MAM treated rats, suggesting that this treatment might alter other central pathways regulating pain sensitivity.

Nerve growth factor increased in GD12 animals in entorhinal and in remaining cortex. Abnormal neuronal cytoarchitecture induced by MAM treatment disrupting

the NGF pathways and also altering the distribution of NGF-receptors on NGF-responsive cells may lead to an accumulation and non-utilization of NGF. Accumulation of NGF in the cortex due to deficits in NGF-receptors' distribution and/or failure of brain neurons responding to the action of NGF has been previously reported in human subjects affected by other neurological diseases such as Alzheimer's disease (Scott and Crutcher 1994). In addition, increased NGF-like activity in young rat brain has been also revealed after lesions (Scott et al. 1994). It may also be speculated that neurons resistant to the antimitotical effects of MAM, for some unknown reason, produced elevated amounts of NGF or alternatively, glial cells, which under normal condition do not extensively contribute to NGF release, could produce this neurotrophin after neuronal damage (Bakhit et al. 1991).

Results of Paper 2

The aim of our investigation was to gain potential useful information about the role of the entorhinal/hippocampal axis development in neurotrophin synthesis in adult rats treated prenatally with MAM. The results of this study show that prenatal MAM administration causes long-lasting structural alteration in adult rats and that the entorhinal cortex and hippocampus of GD12 rats are characterized by a gross increase in NGF and BDNF. As for behavior, in the social interaction test we found that MAM adult animals displayed increased locomotion, while in the open-field locomotion and exploratory activity were unaffected while displacement behaviors were elevated. Notably, animals showing increased NGF/BDNF levels had stronger changes in the behavior.

Although elements of social behavior appeared unchanged, nonsocial behavior was affected in GD12 treated rats with increased locomotion during the social interactions. Alterations in locomotion and exploration in some behavioral tests reflect impaired capability to cope with stressful conditions (Belzung and Le Pape 1994). In addition, it has been demonstrated that MAM microencephalic rats display hyperactivity compared to control rats (Watanabe et al. 1995). Since the hippocampus is involved in motor control hippocampal lesions and microencephaly with hippocampal reduction may result in notable hyperactivity (Cassel et al. 1998). The entorhinal cortex which is considered part of the hippocampal formation (Eichenbaum et al. 1994), might play a subtler role in motor control and might to some extent play a role in motor regulation.

MAM rats in a stressful situation as the novel object investigation potentiated some displacement and escaping behaviors indicating that prenatal MAM treatment on gestational day 12 can induce impairments in coping with mildly stressful condition and showing also that the behavioral alterations observed in GD12 rats could suggest that these animals might be abnormally reactive to external stimuli

(Ferguson et al. 1993; Ferguson and Holson 1997). This hypothesis is also supported by the role of the hippocampal/entorhinal formations in sensory integration (Klinberg et al. 1994; Braak and Braak 1992).

NGF and BDNF (Rylett and Williams 1994) regulate the naturally occurring neuronal death during neurogenesis and promote repair in several neurological central and peripheral insults (Hefti 1999; Kromer 1987; Scott et al. 1994) and play a crucial role in brain neuropeptide and neurotransmitter synthesis and release (Croll et al. 1994). Since the entorhinal formation is structurally and functionally connected to the hippocampus (Eichenbaum et al. 1994; Van Praag et al. 1996; Vnek et al. 1995), neurotrophins elevation is a main consequence of the their structural disorganization. The functional significance of this potentiation is at present not known. However, it is interesting to underline that the effect of prenatal MAM administration in the brain of our rats occurs in the absence of gliosis, a finding which is consistent with that observed in some neurodevelopmental diseases (Falkai et al. 1988; Weinberger 1987). Since neurotrophins prevent neuronal damage (Barde 1989; Hefti 1986), protect the cells from apoptosis (Shigeno et al. 1991), and counteract the effects of chemical and pharmacological insults (Fiore et al. 1997; Kromer 1987), an elevated synthesis of NGF and BDNF in the brain of MAM treated mice might be important in the regulation of neuronal survival. Indeed changes in NGF/BDNF could be related with the behavior of GD12 rats. In fact, stress-related behaviors lead to significant changes in the basal NGF levels both in humans and animals. In humans, the level of NGF increases in the blood of young soldiers who experienced their first parachute jump (Aloe et al. 1994). Because the release of NGF precedes the release of cortisol and ACTH, it was hypothesized a correlative event between released NGF and anxiogenic status. In rodents, highly stressing situations as aggressive or submissive behaviors altered the basal levels of NGF (Alleva et al. 1993) both in the brain and in the periphery. All these results lead to a NGF role in coping and neuroendocrine mechanisms.

This study also reveals that the expression of ChAT or p75 is unaffected in MAM rats (only in the septum we found a non significant increase in positive cell number in line with a previous work which demonstrated that ChAT specific activity increased following prenatal MAM administration at gestational dat 13 in the neocortex of rats). This finding suggests that these biological mediators are not directly implicated in the deficits due to MAM treatment or alternatively that other compensatory mechanisms for both the limbic or septal formations have been developed.

Results of Paper 3

Our results demonstrate that embryonic exposure to MAM impairs learning performances, while concomitant bromodeoxyuridine administration (BrdU) elicits

NGF potentiation in the parietal cortex and decreasing hippocampal NGF and BDNF. Exposure to MAM at GD11 led to mild effects on cognitive behavior as a result of CNS changes (Talamini et al. 1998; 1999). Indeed, exposure to MAM early during brain growth elicits abnormal development of the cortical/hippocampal axis with reduced cortical thickness, disorganized brain cytoarchitecture, and abnormal temporal asymmetry and the hippocampus and related cortical regions are brain structures playing a key role in processing associational information and spatial memory (Eichenbaum et al. 1994). We revealed also that animals treated with both MAM and BrdU behaved as controls suggesting that some factors may have participated in increasing the attentional levels of these rats as the septal cholinergic pathways playing a key role in spatial memory (Fisher et al. 1987; Hefti 1986) or p75 immunoreactivity that were potentiated in MAM+BrdU rats following the NGF/BDNF changes in the hippocampal/cortical axis. The elevated expression of ChAT seen in basal forebrain cholinergic neurons of MAM+BrdU rats could also indicate a ChAT accumulation and/or release while the p75 increase might be associated to apoptotic mechanisms (Connor and Dragunov 1998; Hellweg et al. 1998). Moreover, we show that BrdU inhibits neurotrophins in the hippocampus possibly as a result of a functional long-lasting DNA-damage emphasizing the irregular changes caused by MAM and BrdU.

Results of Paper 4

In the present work we show that prenatal MAM administration induces deficits in a Morris maze test, and a marked alteration in the levels of NGF and BDNF measured as protein or as relative mRNA. Rats exposed on GD11 had reduced capability to reach the platform during the acquisition phase whereas, GD12 animals showed impairments in escape latency during the reversal phase. The morphological effects of prenatal MAM administration on GD12 was distributed throughout the brain (Talamini et al. 1998; 1999) while on GD11 the effect was limited to "associative" regions as the hippocampus and the entorhinal cortex (Braak and Braak 1992; Klingberg et al. 1994). This aspect leads to a slowing down in learning processes during the acquisition phase in GD11 rats and for the higher latency response of GD12 animals during the reversal phase. Indeed the hippocampus and related cortical regions are brain structures playing a key role in processing associational information and spatial memory (e.g. Cho and Kesner 1996). In addition, memory and learning processing as well as "memory consolidation" phenomena involves interactions between the hippocampus and parahippocampal regions, including also the entorhinal cortex (Eichenbaum et al. 1994).

Our finding also shows that GD11 rats had longer latency to find the platform

without changes in the swimming speed or the total distance moved by the animals in the pool suggesting that GD11 rats when compared to controls were unable to cope with the new spatial information for forgetting or for motivational impairments due to the new environmental situation. GD11 rats could be also more "confused" in learning tests which require more complex spatial processing as retrieving the platform position. Similar results in the Morris maze were found in adult MAM animals exposed at GD11 (Paper 3) whereas young GD11 MAM rats had disrupted responses in the passive avoidance test (Paper 1) as a result of CNS changes. GD12 animals showed slight impairments in the reversal phase of the water maze suggestive of a reduced flexibility in the data elaboration associated with the hippocampal and/or cortical damage in the absence of both brain inflammation and locomotory changes.

We also show that hippocampal NGF and BDNF are significantly reduced by MAM treatment and NGF and BDNF regulate neuronal plasticity and ChAT activity in brain cholinergic neurons localized in the septum, nucleus of Meynert and in the striatum (Barde 1989; Rylett and Williams 1994). Since structural and biochemical changes of cholinergic neurons are associated with deficits described in neuropsychiatric diseases (Babic 1999; Hellweg et al. 1998), we show a functional link between NGF distribution and changes in brain development. Indeed abnormalities of ChAT in the brain have been demonstrated in schizophrenia and sudden infant death syndrome (Friedman et al. 1999) and decreasing ChAT has been also observed in the aged brain (Colombo and Gallagher 1998; Oda 1999). As for aging, a main issue of the present study is that aged MAM rats had decreased ChAT immunoreativity but prenatal MAM did not induce lower ChAT immunopresence adult MAM rats (paper 2) to demonstrate that brain maldevelopment may speed up aging processes as observed in human neuropsychiatric diseases. In the entorhinal cortex NGF increased in GD12 animals as previously shown (Papers 1, 2) and this finding appears to be a specific MAM effect in the rat brain. We proposed (Paper 1) that since NGF is produced in neurons and glial cells (Levi-Montalcini 1987; Lewin and Barde 1996) the NGF elevation in the entorhinal cortex might represent not only an increased function but rather that the entorhinal cortex is permanently stimulated to release NGF in an attempt to restore impaired development (Hellweg et al. 1998).

Interestingly, the changes in NGF/BDNF protein in MAM rats were associated with similar levels of their respective mRNAs suggesting that in the MAM models the hippocampal-entorhinal axis is specifically affected by prenatal MAM administration.

We found that NPY decreased in the entorhinal cortex of GD11 animals that had also the major impairments in the water maze. NPY is a neuropeptide which is widely distributed in the CNS of mammals, humans included and plays a role in

regulating behavior and in a variety of neuropsychiatric diseases (Redrobe et al. 1999; Wettstein et al. 2000). NPY modulates memory processing and attenuates learning impairments induced by neurotoxins (Bouchard et al. 1997) and can in some way interact with cognitive processes associated with prenatal MAM treatment. A previous study on prenatal MAM administration demonstrating that at gestational day 15 MAM treatment causes a decrease in hippocampal NPY positive cells (Zoli et al. 1990) is in line with our results. In humans affected by neuropsychiatric disorders NPY has been supposed to be altered in the brain and cerebrospinal fluid (Wettstein et al. 2000; Minthon et al. 1997), with a decrease in the temporal cortex but not in the hypothalamus (Frederiksen et al. 1991). We discussed that since NPY is known to be regulated by neurotrophic factors (Croll et al. 1994) disrupted levels of both NGF and BDNF could have been contributed in down-regulating NPY.

Results of Paper 5

The findings of the present study show that prenatal MAM exposure at GD17 produces long-lasting effects on NGF and BDNF levels of the rat brain. Indeed the main effects were found in 9 months old rats. In the parietal cortex NGF and BDNF were evidenced to be significantly lower in MAM rats than controls. This is in line with studies on MAM administered at GD15 showing decreased cortical neurotrophin presence associated with atrophy of some cholinergic nuclei of the basal forebrain (Cimino et al. 1996). In the hippocampus both NGF and BDNF were significantly higher in 9 month old MAM rats and NGF was also elevated in the entorhinal cortex of young MAM animals. Similar results were found with MAM administered at GD11 and GD12 (Paper 4). Since neurotrophins are produced in neurons and glial cells (Lewin and Barde 1996), the observed potentation in levels in selected structures of the limbic system may be indicative of a sustained overproduction of neurotrophins in an attempt to restore the mitotoxin-induced impaired development in these regions (Sofronew et al. 2001). The data on the striatum and hypothalamus are novel and seem to represent a specific effect of MAM.

We show that the experimental disruption of the limbic cortex during differing stages of development could give additional information to analyze the interactions between abnormal ontogenesis, neurotrophins and neuropsychiatric disorders. Indeed several neuropsychiatric disorders, including schizophrenia, have their origin based in a neurodevelopmental pathogenesis of limbic cortex (Grace 2000; Arnold et al. 2000) with important key roles for neurotrophins (Connor and Dragunow 1998; Thome et al. 1998). The present MAM model may in several respects exhibits both anatomical and behavioral characteristics that appear to provide an additional representative model of this disorder (Moore et al. 2001), and

which also exhibits some alterations in neurotrophins that are in line with those observed in schizophrenic patients (Nawa et al. 2000; Takahashi et al. 2000). There are other studies that provide evidence for a temporal limbic cortical involvement in the pathophysiology of schizophrenia (see Egan and Weinberger 1997). Rats with postnatal lesions of the entorhinal cortex exhibit elevated concentrations of dopamine in the amygdala, and methamphetamine-induced dopamine release in the amygdala (Kurachi et al. 2003). It should be also noted that the changes in BDNF and NGF seen after MAM exposure at GD17 show temporal changes in the adult rat with differences at 3 versus 9 months.

It is also interesting to note the data showing an opposite trend of NGF concentrations in the hippocampus between young adult rats 3 months old and adult pre-senescent rats 9 months old, with the significantly higher levels of NGF observed in MAM rats at 9 months due primarily to a failure to show the normal NGF reduction observed in controls at that age. This elevation could participate to increase progenitor brain cells that are normally present in the aged animal and are known to be regulated by neurotrophins (Alvarez-Buylla and Garcia-Verdugo 2002; Fiore et al. 2003; Tirassa et al. 2003). Indeed recent data on neurogenesis (Ciaroni et al. 2002 a,b) shows that treatment with MAM reduced neural precursor cells in adult rat hippocampus and enhanced the survival of new granule cells in the dentate gyrus, thus allowing the recovery of proper cell layer structures and decreased dentate granule cell death. Thus, in the view of the crucial role played by neurotrophins in neuronal survival and differentiation, it may be hypothesized that the data on hippocampal recovery following prenatal MAM exposure (Ciaroni et al. 2002 a,b) might be due to the augmented levels of neurotrophins in the hippocampus as suggested by the results of the present study.

PART V: GENERAL DISCUSSION

Neurotrophins and the Development of Schizophrenia

Several hypotheses have been proposed to explain the pathophysiology of schizophrenia, however, no single theory seems to account for all the physiopathological manifestations of the disorder. Each hypothesis explains some of the phenomena associated with schizophrenia and it is likely that many of the variables described interact to produce the disorder. Post mortem studies have shown that certain limbic or associative brain areas of schizophrenic subjects are characterized by decreased cell numbers and volume (Brown et al. 1986, Falkai and Bogerts 1986), disorganized cytoarchitecture and reduced content of certain microtubule associated proteins (Arnold et al. 1991b). These data have indicated a failure of both setting and migration of young neurons into their appropriate cortical target sites, particularly in the hippocampus and entorhinal cortex (Braak and Braak 1992; Weinberger 1999).

The entorhinal cortex is a brain structure contained in the limbic allocortex of the medial temporal lobe having a key role in processing associational information (Cho and Kesner 1996) and is connected to the hippocampus and other subcortical structures (Witter et al. 1989). It has also been shown that memory and learning processing as well as memory consolidation are regulated in the hippocampus and parahippocampal regions, including the entorhinal cortex (Eichenbaum et al. 1994).

Neurotrophic factors play important roles in the development and maintenance of hippocampal and cortical structures. More specifically NGF participates in the neuroregulation of the mechanisms leading to the consolidation of hippocampal spatial memory through cholinergic pathways while BDNF promotes actions on neurons of hippocampal, nigral and cortical structures (Alderson et al. 1990; Ghosh et al. 1994; Hyman et al. 1991; Lindholm et al. 1996). Thus it has been proposed the neurotrophic factor hypothesis of schizophrenia (Thome et al. 1998) explaining the changes in the brain of schizophrenic patients as the result of disturbances of developing processes involving trophic factors. Neurotransmitter deficits are thereby considered as epiphenomena of underlying neurotrophic factor disorganization. This hypothesis is supported by the MAM studies of the present thesis showing that NGF and BDNF are abnormally regulated in the CNS, by evidence that schizophrenic patients have low circulating levels of NGF (Bersani et al. 1999; Parikh et al. 2003) and by the fact that schizophrenics are characterized by reduced BDNF in the serum (Toyooka et al. 2002). A link between neurotrophins and schizophrenia is potentiated by the evidence that neuronal development of embryonic brain tissue derived from schizophrenic women shows neurite-growth deficits (Freedman et al. 1992) and the polymorphism gene of neurotrophin-3 resulted to be associated with schizophrenia (Hattori et al. 2002;

Nanko et al. 1994). However it should be noted that the hypothesis of a genetic origin of schizophrenia is still debated (Neves-Pereira et al. 2002; Sklar et al. 2002; Virgos et al. 2001). Findings obtained with studies on human post-mortem tissues have also evidenced a significant increase in BDNF concentrations in cortical areas and a decrease in this neurotrophin in the hippocampus of patients when compared with controls (Durany et al. 2001) whereas other authors have found that BDNF protein is elevated in the anterior cingulate cortex and hippocampus of schizophrenic patients (Takahashi et al. 2000) or reduced in the prefrontal cortex (Weickert et al. 2003). In addition, analysis of post mortem brain tissues of schizophrenic patients revealed decreased mesopontine ChAT levels in schizophrenia which were correlated with the cognitive impairments evidenced by schizophrenics (Karson et al. 1996) findings in line with the data of the paper 1, 3 and 4 of the present thesis.

Other data on schizophrenia and NGF showed that never medicated first episode patients (Parikh et al. 2003) had NGF levels lower than medicated chronic schizophrenic patients. Among the medicated patients, higher plasma NGF levels were in patients treated with atypical antipsychotics compared to treated with typical antipsychotics. The findings in patients treated with typicals are in agreement with earlier reports (Aloe et al. 1997; Bersani et al. 1999). In an other paper it has been shown that chronic cannabis abuse raises nerve growth factor serum concentrations in drug-naive schizophrenic patients (Jockers-Scherubl et al 2003). However, reduced plasma NGF levels may represent a deficiency of this neurotrophic factor in never medicated first episode patients, since these patients were well matched with normals. It is likely that the NGF levels in never medicated first episode patients may be even overestimates since physical and emotional stresses, drug abuse, hormonal changes and smoking can increase NGF levels. However, no drug abuse was found and very few patients sparingly smoked cigarettes. It is premature to attribute a role for NGF deficiency to abnormal neurodevelopment. Higher NGF levels in chronic schizophrenic patients treated for several years with antipsychotics as compared to never-medicated schizophrenic patients may suggest the effect of antipsychotic treatment. Furthermore, higher levels of NGF were found in patients treated with only atypical antipsychotics (e.g. risperidone, clozapine and olanzapine) and not with typicals (e.g. haloperidol and fluphenazine). Rather, NGF levels in patients treated with typicals were similar to levels in never medicated first episode, further supporting that elevated levels were related to treatment with atypicals and not to any other factors. This is very important since treatment with atypicals have been found to improve psychopathology, particularly cognitive performance and negative symptoms compared to typicals. These clinical differences may be related to the differential antipsychotic neuroprotective effects, which include induction of trophic factors

(e.g. NGF) and protection of neuronal systems such as cholinergic in the brain and improved functional performance (Mahadik et al. 2001a,b; 2002). The source of plasma NGF is the peripheral target tissues, such as neuromuscular, salivary glands and lymphocytes, and the cerebrospinal fluid. NGF has been found to increase the survival and function of CNS cholinergic neurons (Korsching et al. 1985; Shelton and Reichardt, 1986; Levi-Montalcini, 1987; Kromer, 1987) and improve the cognitive deficits (Mohammed et al. 1990). NGF also plays a vital regulatory role in stress and coping in animals and humans (Alleva et al. 1996).

All together these findings suggest that neurotrophic abnormalities are associated with the corticolimbic structures of schizophrenic patients providing the molecular substrate for pathological manifestations of the disease. Moreover these data were discussed as further evidence to the neurotrophin hypothesis of schizophrenic psychoses proposing that alterations in the expression of neurotrophins might participate in the neural maldevelopment and in the disturbed neural plasticity as important event in the etiopathogenesis of schizophrenia.

It is known that antipsychotic drugs as haloperidol and risperidone are widely used to reduce or overcome the clinical manifestation of schizophrenia. Haloperidol mainly acts on the dopamine D_2 and D_3 receptors (Guillin et al. 2001). The latter receptor is also susceptible to the action of BDNF. Risperidone binds to both DA and serotonin (5HT) receptors, particularly in the neurons of striatal and limbic structures. Findings revealing a link between antipsychotic administration and synthesis and release of neurotrophins and some neurotransmitters regulated by NGF and BDNF have been recently reported (Angelucci et al. 2000a,b,c).

The amount of research supporting a role for neurodevelopmental abnormalities resulting in a predisposition to schizophrenia involves other than the present MAM models also studies on animal models and BDNF (Lipska, et al. 2001; Ashe et al. 1999; Ashe, 2000). Rat pups were lesioned bilaterally in the ventral hippocampus using ibotenic acid, sham lesioned animals were injected with artificial cerebrospinal fluid and the results demonstrate that animals with hippocampal lesions express significantly lower basal levels of BDNF mRNA in the prefrontal cortex than sham animals. This suggests that animals with neurodevelopmental lesions may demonstrate increased susceptibility to neuronal damage. In addition, both lesioned and sham animals demonstrate a significant upregulation of BDNF mRNA following exposure to stress but higher in lesioned rats. This indicates that lesioned animals are hypersensitive to stress and require greater resources to protect from the neuronal damaging effects of stress. Investigation of cell death demonstrates that even in the presence of increased BDNF there is an increase in cell death in lesioned animals exposed to stress as compared to sham lesioned animals exposed to stress (Ashe 2000). While this increase was small, over extended periods of time, significant functional impairments could result.

Perspectives

Although still debated, the evidence so far available both in humans and animal models suggest that schizophrenia might be the result of developmental cytoarchitectural alterations of the hippocampal–entorhinal axis (Akil and Lewis 1997; Arnold et al. 1991a,b; Harrison 1999; Weinberger and Lipska 1995). However, basic studies on animal models have raised the question as to whether neurotrophins, particularly NGF and BDNF, play a role in regulating behavior and in neurodevelopmental disorders (Bersani et al. 1999; 2000; Nawa et al. 2000; Takahashi et al. 2000; Alleva et al. 1993). Supportive evidence related to these hypotheses come from studies on human brain neurotrophin levels showing that neurotrophins' levels undergo significant changes following neuropsychiatric diseases. Schizophrenic patients showed changes in neurotrophins' levels in both the PNS and CNS (Takahashi et al. 2000; Nawa et al. 2000) suggesting that changes in the expression of neurotrophins might contribute in the neural maldevelopment and in the disturbed neural plasticity as important event in the etiopathogenesis of schizophrenia. Indeed, schizophrenic patients showed low circulating levels of NGF and BDNF (Bersani et al. 1999; Toyooka et al. 2002), elevated BDNF protein in the cortex and reduced BDNF in the hippocampus (Durany et al. 2001; Takahashi et al. 2000) though a recent work did not find differences in plasma BDNF between drug naive schizophrenics and controls (Shimizu et al. 2003). We speculated in the papers 1, 3 and 4 that some deficits in cognitive processes observed in schizophrenic patients (Gold and Weinberger 1995) associated with decreased brain ChAT immunopresence (Karson et al. 1996) and the altered immunopositivity of ChAT and NPY found in MAM exposed rats may be related to the disrupted availability of NGF and/or BDNF in the hippocampal/entorhinal axis. Morphological changes in NPY-positive fiber in the hippocampal formation of schizophrenic patients were also found (Iritani et al. 2000). The working hypothesis of the present thesis is that the deficiency of one or both of these neurotrophins may contribute to the maldevelopment of the hippocampal/entorhinal axis and participate to the development of some specific neuropsychiatric manifestations. A wide variety of pharmacological trials has been tested to check whether or not they might increase cognitive performance in the treatment of disorders such as anxiety, memory dysfunction and neuropsychiatric diseases (Friedman et al. 1999). Based on the above observation seems reasonable to prospect that the use of drugs influencing the synthesis and release of cholinergic neurotransmitters might be helpful in potentiating cognition in healthy subjects and suggests that cholinomimetics may be important factors for treating cognitive deficits (Tandon and Greden 1989). Indeed cognitive deficits and core negative symptoms are fundamental features of the psychopathology of

schizophrenia (Tollefson 1996). Cognitive deficits in schizophrenics are reported to be negatively correlated with ChAT activity (Powchik et al. 1998). Moreover, cortical ChAT activity has been shown to correlate with clinical dementia rating scores (Haroutunian et al. 1994) and treatment with cholinomimetics augment cognitive performance in schizophrenics by increasing cortical cholinergic activity (Friedman et al. 1999).

Consequently, NGF and BDNF, which play key roles in regulating of ChAT and NPY activity, could assemble a potential therapeutic action in the treatment of neuropsychiatric diseases. In the framework of this hypothesis the prenatal MAM model could be considered a useful tool to study some specific pathogenic mechanisms involved in human brain maldevelopment. Indeed, the experimental disruption of limbic cortex development may present a good approach to investigate the relationship between abnormal ontogenesis and schizophrenia. Similar strategies might be considered to study other neuropsychiatric disorders with a presumed neurodevelopmental pathogenesis. Nonetheless, it should be noted that antipsychotic agents as Haloperidol and Risperidone commonly used by schizophrenics are known to reduce hippocampal neurotrophic factor expression (Angelucci et al. 2000b). These findings apparently do not fit with our working hypothesis of a reduced neurotrophic support during development. However, it should be considered that treatment with antipsychotics reduces dendritic spines in brain regions (Kelley et al. 1997), decreases hippocampal and striatal ChAT activity (Mahadik et al. 1988) leading to strong impairments in cognitive abilities (Gallhofer et al. 1996). Thus an unexpected secondary effect of chronic antipsychotic administration is to disrupt cognition throughout the inhibition of neurotrophins' production. All together these data highly potentiate the debate on the real levels of the side effects of antipsychotics in schizophrenia (Gallhofer et al. 1999; Krausz 2002; Tandon and Halbreich 2003). Based on the brain mechanisms of atypical antipsychotics investigated in animals and their clinical effects in schizophrenic patients, plasma or brain NGF levels may have implications for treatment outcome. However, atypical antipsychotics have been also reported to improve the neurocognitive performance and negative symptoms in schizophrenic patients (Sharma and Mockler 1998; Keefe et al. 1999; Meltzer and McGurk 1999; Green and Braff 2001), whereas typical antipsychotics were found to improve cognitive function and sometimes to impair some aspects of cognition such as memory and fine motor function (Lee et al. 1999). Recently, it has been reported that acute treatment with either typical or atypical antipsychotics may improve negative symptoms and may reduce cognitive deficits in first episode schizophrenia (Schuepbach et al. 2002). In addition, in the case of schizophrenia the discrepancy between antipsychotics and animal models could also be due to the fact that antipsychotics do not really affect the course of schizophrenia. Essentially, the

drugs only reduce the positive and not so much the negative symptoms and also reduce the anxiety that often is associated with the disorder. In schizophrenia one might argue that because of some cerebral cortical abnormalities the mesolimbic dopamine system becomes dysregulated, thus causing some symptoms. So either by repairing the cortical deficit (which is at the present time an illusion) or by reducing dopamine activity antipsychotic effects may be obtained.

On the whole the data on NGF and BDNF in humans affected by neuropsychiatric diseases and in animal models suggest that modulation of neurotrophic function may affect therapeutic interventions in depression and schizophrenia and indicate that this approach may be a new way to promote limbic system neuroplasticity.

A secondary recent perspective of the present thesis that could lead to future lines of research is that fetal MAM administration may play a role in the investigations of brain progenitor cells activation throughout neurotrophins elevation as indicated by the data of Paper 5. Studies published previously revealed that the brain of adult and aged mammalians stores cells with the capability of self-renewal if receive appropriate chemical or environmental stimuli (Cameron and McKay 1998; Gage 2000; Gage 2002). Proliferating cells have been found in human and rodent brains mainly localized in the subventricular zone and in the subgranular zone of the dentate gyrus of the hippocampal formation (Alvarez-Buylla and Garcia-Verdugo 2002; Gage 2002). However, the *in vivo* environmental factors that influence proliferation and/or differentiation of these cells have not been clearly identified. Works *in vitro* and *in vivo* have produced a substantial body of knowledge showing that brain immature cells require growth factors, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) for cell growth and differentiation (Giardino et al. 2000; Fiore et al. 2003; Ohmiya et al. 2002; Zigova et al. 1998).

Specifically for MAM it has been shown that MAM disrupted in the adult rat hippocampus neural precursor cells but increased the survival of new granule cells in the dentate gyrus, thus allowing the recovery of proper hippocampal cell layer structures and decreased dentate granule cell death (Ciaroni et al. 2002a, b). We proposed in the paper 5 of the present thesis that since fetal MAM exposure is able to elevate NGF/BDNF in the hippocampus and since neurotrophins play a crucial role in neuronal survival and stem cell differentiation, it may be hypothesized that the data on hippocampal recovery of the Ciaroni's group (Ciaroni et al. 2002 a,b) following MAM is due to the neurotrophin elevation induced by MAM. Furthermore elevation of neurotrophins in the brain following stress, physical activity, selected behavioral parameters or throughout exogenous administration has been shown to increase the number of brain stem cells which resulted to be associated with a major increase in ChAT expression or ChAT immunopositivity

(Fiore et al. 2003; Tirassa et al. 2003) These findings imply that some brain areas as the hippocampus and the subventricular zone remain a strong reservoir of cholinergic cells (or of other neuropeptides) opening new perspectives for understanding the role of NGF or BDNF during neuropsychiatric disorders and the MAM models of the present thesis could be an effective device to investigate elected aspects of these questions.

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SUMMARY

Damage during prenatal development of certain brain regions of the limbic system as the entorhinal cortex and hippocampus are possibly involved in the development of psychiatric disorders associated with stress, anxiety and impairments in sociality and learning and memory abilities as schizophrenia. Because neurotrophins, particularly both NGF and BDNF, play a crucial role in brain neurogenesis, control some aspects of brain disorders and participate in the regulation of the fine tuning of behavior and cognition we used a rat model of entorhinal cortex and hippocampus maldevelopment to study neurotrophins and behavior. We injected in pregnant rats an antimitotic compound, the methylazoxymethanol (MAM) at different days of gestation and brain development (GD11, 12 or 17) and we analyzed the effects in their progeny when rats were young, adult and aged. Analysis of the forebrain in adult animals showed reduction of the entorhinal cortex size and this effect shifts from lateral to medial divisions of the entorhinal cortex with later administration of MAM, following a known developmental gradient. Morphological consequences of MAM administration appear to be largely confined to the hippocampal-entorhinal axis although slight reductions of the frontal and occipital neocortex were also observed. MAM treatment on GD 12 revealed relatively more widespread damage, as reflected among other in a small decrease in brain weight. Prenatally treated MAM (GD11 or 12) rats had impairments in memory, displacement behaviors, locomotion, attentional capabilities and pain sensitivity. No effects were found in selected parameters of sociality. In particular, young rats showed a retardation in passive avoidance acquisition. The alterations in learning and memory processes were limited to the acquisition phase, since no effects were found during the retention phase, which took place 1 day later. This leads to a disruption in the associative mechanisms serving the learning and memory processes, active during acquisition. Young MAM rats had also increased analgesia and abnormalities in locomotion and rearing observed in the open-field and under novel environmental situations which may reflect impaired capability to cope with stressful conditions. In adult MAM rats we found in the social interaction test increased locomotion, in the open-field locomotion and exploratory activity were unaffected while displacement behaviors were elevated. Aged animals were tested for permanent changes in learning and memory. It was shown that prenatal MAM administration induces marked deficits in a Morris maze test as a higher latency response during both acquisition and reversal phases without changes in the swimming speed or the total distance moved by the animals in the pool suggesting MAM rats were unable to cope with the new spatial information for forgetting or for motivational

impairments due to the new environmental situation. MAM rats could be also more "confused" in learning tests which require more complex spatial processing as retrieving the platform position.

These behavioral findings were associated with disrupted development of both entorhinal cortex and hippocampus and with changes in NGF/BDNF production of these two brain areas. In the entorhinal cortex both NGF and BDNF were during the life of MAM rats constantly higher compared to controls, data that were discussed as an attempt of the maldeveloped brain tissue to restore brain damage. Enhanced neurotrophin content was also found in the hippocampus of young and adult MAM rats that underwent to a dramatic decrease in the aged MAM rats. We speculated these findings as a long-lasting effect of prenatal MAM exposure leading to impairments in rat spatial memory capabilities that were also associated with disrupted brain ChAT and NPY distribution in brain areas as the septum and the Meynert's nuclei.

When MAM was administered later (GD17) adult rats showed small-to-moderate reductions in the thickness of limbic and paralimbic cortices, had significant deficits in cognitive tasks that depend on prefrontal-and hippocampal-striatal circuits and displayed behaviors similar to rats with frontal lesions. We found that neurotrophins decreased in adult rats in the parietal cortex, however, NGF and BDNF were elevated in the hippocampus leading to a role of endogenous progenitor cells, known to be regulated by neurotrophins.

The present MAM models may in several respects exhibits both anatomical and behavioral characteristics that appear to provide an additional representative model of schizophrenia and which also exhibits some alterations in neurotrophins that are in line with those observed in schizophrenic patients. Accordingly, NGF and BDNF, which play key roles in regulating of ChAT and NPY activity, could assemble a potential therapeutic action in the treatment of some neuropsychiatric diseases. Thus, this study suggests that the animal model based on prenatal MAM administration at specific time points of brain neurogenesis can be used to investigate anxiety, attentional and emotional disorders, including some aspects of human neuropsychiatric disorders.

SAMENVATTING VOOR DE LEEK (DUTCH SUMMARY)

Schade van bepaalde hersengebieden, met name van het limbische systeem, tijdens de foetale ontwikkeling zou de oorzaak zijn van het ontstaan van een aantal psychiatrische aandoeningen en cerebrale disfuncties. Vooral een minder goede aanleg van de hippocampus en de entorhinale cortex zouden zijn betrokken bij stoornissen waarbij stress een belangrijke rol speelt, zoals depressieve dstoornissen en angststoornissen, en bij cognitieve disfuncties en onsociaal gedrag zoals bij schizofrenie vaak wordt waargenomen. In de normale ontwikkeling van de hersenen spelen de zogenaamde neurotrofinen een belangrijke rol. Neurotrofinen zijn kleine eiwitten (peptiden) die essentieel zijn voor de uitgroei van zenuwcellen tijdens de ontwikkeling van het zenuwstelsel en waarvan de permanente aanwezigheid noodzakelijk is om eenmaal aangelegde verbindingen in stand te houden. In dit proefschrift ligt de nadruk op de peptiden NGF (nerve growth factor) en BDNF (brain derived neurotrophic factor).

We ontwierpen een dierexperimenteel model om tijdens de foetale ontwikkeling beperkte en selectieve schade aan de hersenen toe te brengen. Zwangere ratten kregen een injectie van een remmer van de celdeling, het MAM (methylazoxymethanol), die gedurende ongeveer 48 uur werkt. Indien MAM gegeven wordt op een bepaalde dag tijdens de foetale ontwikkeling zullen vooral die hersengebieden worden getroffen die op die dag worden aangelegd. MAM werd toegediend op dag 11, 12 of 17 (GD11, 12 of 17) na conceptie. We analyseerden de gevolgen van deze behandeling bij jongvolwassen en bij oudere mannelijke ratten.

Het bleek dat de ontwikkeling van met name de entorhinale cortex verstoord was: dit hersengebied was kleiner bij de door MAM- beschadigde ratten. Voorts bleek dat er een gradiënt optrad tussen de meer laterale (zijdelingse) en de mediale (meer in het midden) delen van de entorhinale cortex. Aldus was bij de op GD11/12 geïntoxikeerde ratten vooral het laterale gebied aangedaan, terwijl bij de GD17 ratten de mediale entorhinale cortex kleiner was, hetgeen was te verwachten gezien de wetenschappelijke literatuur hierover. Ook andere hersengebieden werden bestudeerd. Het bleek niet alleen dat de entorhinale cortex kleiner was, maar ook dat de hippocampus en enkele prefrontale hersengebieden waren aangetast. Behandeling met MAM op GD12 gaf een meer algemene schade, zoals onder andere bleek uit kleinere hersengewichten.

Prenatale behandeling met MAM op GD11 of 12 geeft ook gedragsveranderingen en verder afwijkingen bij geheugen testen, van motorisch gedrag, aandacht en pijngevoeligheid. Geen effecten werden gevonden in sociaal gedrag. In de jongere ratten werd vooral een verstoring van het passieve

vermijdingsgedrag waargenomen. De veranderingen in het leergedrag beperkten zich tot aanleren, omdat we waarnamen dat een dag later er geen geheugendefecten meer werden gevonden.

De jongere ratten die met MAM waren behandeld hadden een verhoogde pijngevoeligheid en verder abnormaal motorisch, exploratief en opricht gedrag, onder omstandigheden waarbij de dieren waren blootgesteld aan een nieuwe omgeving. Deze waarnemingen wijzen erop dat vooral tijdens stress allerlei functies kunnen zijn verstoord. In volwassen MAM - behandelde ratten vonden we in een sociale interactietest verhoogde locomotie, terwijl in een open veld opstelling het motorisch en exploratief gedrag niet was beïnvloed, maar nu wel het ontwijkgedrag.

Oudere MAM – behandelde ratten werden ook getest naar blijvende veranderingen in leer- en geheugenfuncties. Verslechterde functies werden aangetoond met de Morris maze test (vertraagd aanleren en inspelen op veranderde posities van het platform), terwijl geen verschillen werden gevonden in de snelheid van zwemmen en de afstand die de dieren aflegden tijdens de testen. Deze observaties suggereren dat de MAM-ratten niet goed in staat zijn in te springen op nieuwe ruimtelijke informatie. Anders gesteld: MAM ratten zijn gemakkelijker in de war te brengen (of af te leiden) in leertesten in een complexe omgeving.

Deze gedragsstudies werden in verband gebracht met de morfologische en biochemische analyses van de diverse hersengebieden, zoals die post mortem werden vastgesteld. In de entorhinale cortex en de hippocampus waren zowel de NGF als ook de BDNF gehaltes verhoogd, ongeacht de ouderdom van de ratten. Deze observaties zouden kunnen wijzen op een poging van de hersenen om de prenataal opgedane schade te compenseren door extra aanmaak van de groeifactoren. Verhoogde gehaltes van de neurotrofines werden verder gevonden in de hippocampus van jonge en volwassen MAM - behandelde ratten, maar niet bij oudere ratten. We stelden de hypothese voor om het verlies van geheugencapaciteit bij oudere ratten toe te schrijven aan een verstoorde acetylcholine neurotransmissie en mogelijk aan veranderde neuropeptide Y in onder andere het septum en de nucleus van Meynert.

Bij de volwassen ratten die op GD17 waren blootgesteld aan MAM werden bescheiden maar wel significante verminderingen van de dikte van de limbische en paralimbische cortices gevonden. Deze ratten hadden defecten in cognitieve taken waarbij vooral neuronale circuits waren betrokken die de prefrontale, de hippocampale en de striatale hersengebieden verbinden. Bij deze ratten leken de gedragsveranderingen op die welke geassocieerd zijn met letsels van de frontale cortex. We vonden dat de neurotrofinen waren verlaagd in de volwassen ratten in de pariëtale cortex, terwijl - tot onze verrassing - de BDNF en de NGF gehaltes alleen waren verhoogd in de hippocampus.

De studies van dit proefschrift laten zien dat prenatale blootstelling aan neurotoxische stoffen, die de celdeling gedurende een korte periode verstoren, langdurige gevolgen kan hebben voor het functioneren van de hersenen. Deze veranderingen zijn terug te vinden in het gedrag van het proefdier, in de morfologie van de hersenen en in de cerebrale gehaltes van neurotrofe eiwitten. Een aantal van de veranderingen die wij waarnamen in volwassen ratten is in overeenstemming met waarnemingen gedaan in hersenen van overleden schizofrene patiënten. Dus de veranderingen, gezien met de analyses van NGF en BDNF, wijzen op een veranderd functioneren van zenuwcellen die acetylcholine en/of het peptide NPY bevatten.

Onze aanpak kan eventueel worden toegepast voor het ontwikkelen van geneesmiddelen die specifiek de neuroplasticiteit van het zenuwweefsel vergroten, om daarmee prenataal veroorzaakte hersenschade te compenseren.

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