BRAIN NEUROTROPHIN LEVELS AND MOUSE BEHAVIOUR:

RELATIONSHIP TO ENVIRONMENTAL INFLUENCES

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To my parents, with love

献给我挚爱的父母

"The road to wisdom?
Well, it's plain and simple to express:
Err and err and err again
But less and less and less"

-- Piet Hein (1905-1996) Danish poet and scientist

ABSTRACT

All normal functions over the lifetime of an animal are based on a dynamic balance of at least three fundamental elements: brain, environment and behaviour. This thesis investigated different aspects of these three elements and revealed novel interactions of brain neurotrophins, environmental influences and emotional behaviour in mice.

In the first two studies, we used C57BL6 mice housed in standard or enriched conditions to investigate anxiety- and fear-related behaviour and neurotrophin levels in different brain regions. We discovered: (a) The normal (steady state) levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) protein are differently distributed in dorsal and ventral parts of hippocampus in both male and female mice, with the dorsal hippocampal levels being consistently higher than those in ventral hippocampus. (b) Exposure to a series of behavioural tests induced complex changes on neurotrophin levels in selected brain regions. (c) Differential housing exerted significant influences on anxiety-related behaviour and brain neurotrophins; these changes were gender and brain region dependent. (d) Significant correlations between behavioural measures and postmortem brain regional neurotrophic factors contents were related: whereby the magnitude of anxiety-like behaviour in the elevated plus maze was positively related to dorsal hippocampal BDNF levels, but negatively related to NGF levels in dorsal hippocampus and in the amygdala, while the expression of conditioned fear is positively related to amygdala BDNF and NGF levels, and to dorsal hippocampus NGF levels.

The third and fourth studies further explored the interrelationship of environmental conditions, behaviour and brain neurotrophins, using BALB/c mice and BDNF knockout mice. The studies involved exposure to intermittent individual housing with or without access to physical exercise in anxious BALB/c mice and environmental enrichment (EE) in BDNF mutant mice. The results showed that: (a) Intermittent exposure to individual housing induced anxiety-like behaviours with significantly enhanced motor activity, (b) Alternate social isolation caused down regulation of NGF and BDNF levels in frontal cortex, while up regulation of BDNF protein content in the amygdala and BDNF protein and mRNA levels in the hippocampus. (c) Access to running wheels for intermittently isolated mice normalized motor activity. Besides increased cerebellar BDNF and hippocampal NGF and BDNF protein levels, physical exercise did not attenuate down regulation of cortical NGF and BDNF protein levels induced by intermittent social isolation. These results demonstrated for the first time that alternate housing had significant impact on behaviour and neurotrophin levels in selected brain regions in mice, which can be partially altered by voluntary physical exercise. It also suggested that substantial changes induced by intermittent social isolation are different from previous findings caused by sustained social isolation on behaviour and brain neurotrophin. (d) EE increased numbers of dendritic spines in the hippocampal dentate gyrus neurons in wild-type (WT) mice, whereas less impact was found in this brain region in the BDNF mutant mice. (e) Behavioural results showed that in enriched WT mice, there was increased exploration and faster habituation, while this effect was abolished or attenuated in BDNF mutant mice. These findings provided evidence for the involvement of BDNF in regulating emotional behaviour and neural plasticity associated with EE.

LIST OF PUBLICATIONS

The thesis is based on the following four (I-IV) publications, which are referred to in the text by their Roman numbers:

I. **Shun-Wei Zhu**, Benjamin K. Yee, Myriel Nyffeler, Bengt Winblad, Joram Feldon, Abdul H. Mohammed.
Influence of differential housing on emotional behaviour and neurotrophin levels in mice.
Behavioural Brain Research 2006; 169:10-20

II. Benjamin K. Yee *, **Shun-Wei Zhu ***, Abdul H. Mohammed, Joram Feldon.
Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice.
Journal of Neural Transmission 2006; In press

* These authors contributed equally to this work.

III. **Shun-Wei Zhu**, Therese Pham, Elin Åberg, Stefan Brené, Bengt Winblad, Abdul H. Mohammed, Vera Baumans.
Neurotrophin levels and behaviour in BALB/c mice: impact of intermittent exposure to individual housing and wheel running.
Behavioural Brain Research 2006; 167:1-8

IV. **Shun-Wei Zhu**, Alina Codita, Jens Hjerling-Leffler, Patrik Ernfors, Nenad Bogdanovic, Bengt Winblad, David W Dickins, Abdul H Mohammed.
Analysis of exploratory behaviour and hippocampal dendritic spines in BDNF knockout mice: influence of environmental manipulation.
Manuscript

V. Appendix Abdul H Mohammed, **Shun-Wei Zhu**, Sanja Darmopil, Jens Hjerling-Leffler, Patrik Ernfors, Bengt Winblad, Maria C. Diamond, Peter S. Eriksson, Nenad Bogdanovic.
Environmental Enrichment and the Brain.
Progress in Brain Research 2002;138:109-133.

Contents

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DG	Dentate gyrus
EC	Enriched housing condition
EE	Environmental enrichment
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated Plus-Maze
GR	Glucocorticoid receptors
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
IC	Impoverished housing condition
ITI	Intertrial interval
LABORAS	Laboratory Animal Behaviour Observation Registration and Analysis System
LTP	Long-term potentiation
NGF	Nerve growth factor
NT-3	Neurotrophin-3
RT	Room temperature
SC	Standard housing condition
ST	Standard house
TrB	Tyrosine kinase receptor B
WT	Wild type

1 INTRODUCTION

1.1 Neurotrophic Factors

Neurotrophic factors are special endogenous signalling proteins that play important roles in promoting the growth, differentiation, maintenance and plasticity of neurons in the central and peripheral nervous systems under physiological and pathological conditions (Barde, 1994; Thoenen, 1995). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are three structurally related members of the neurotrophin family and exert certain trophic functions in selected neuronal population. For instance, within the central nervous system (CNS), NGF mainly regulates the basal forebrain cholinergic neurons which send topographically organized projections to the hippocampus and cerebral cortex; BDNF is more widely distributed in the CNS (Barde, 1990, 1994; Thoenen, 1995) and has survival-promoting actions on a variety of CNS neuronal cells including cholinergic, serotonergic and nigral dopaminergic neurons; whereas NT-3 mainly serves motor neurons and sympathetic neurons.

Neurotrophins have been implicated as key molecules in the fine-tuning of behaviour (Alleva, 1993; Smith, 1996) and in the physiological regulation of cognitive processes during both development and aging (Rylett & Williams, 1994; Thoenen, 2000). In the brain, neurotrophins are abundantly expressed at mRNA and protein levels in the hippocampus (Ernfors et al., 1990), where they are critically involved in activity-dependent synaptic plasticity. NGF and BDNF levels in the hippocampus have been linked to spatial learning in rats (Falkenberg et al., 1992; Henriksson et al., 1992; Mizuno et al., 2003; Scaccianoce et al., 2003), and long-term potentiation (LTP), the electrophysiological model of learning (Kang and Schuman, 1995).

1.1.1 BDNF and Emotion

Throughout the brain BDNF is extensively distributed and abundantly expressed in the hippocampus. Recent studies have linked BDNF to hippocampal function and indicate that BDNF is required for maintenance of hippocampus-related plasticity phenomena such as LTP (Gooney et al., 2002; Kovalchuk et al., 2002; Ernfors and Bramham, 2003; Lu, 2003), spatial learning and kindling (reviewed in Schuman, 1999; Ernfors and Branham, 2003). Thus administration of BDNF in the hippocampus

enhances learning, while injection of BDNF antibodies impairs learning (Johnston et al., 1999).

While a large body of data supports the role of BDNF in learning and memory, the involvement of this neurotrophin in other types of behaviour such as anxiety and depression remains unclear. Recent studies have reported that BDNF also has an important role in regulating emotional behaviours. For example, BDNF-deficient mice develop enhanced inter-male aggressiveness (Lyons et al., 1999). Abnormal levels of BDNF have been observed in psychiatric and neurodegenerative conditions, such as Alzheimer's disease (AD), bipolar disorders, depression and schizophrenia (Hoffmann et al., 1990; Takahashi et al., 2000; Garzon et al., 2002), as well as drug addiction (Castren, 2004). Since depression, a mood disorder, coexists with AD, this suggests a possible common link through BDNF. Dysfunction in neurotrophin-mediated signalling mechanisms has been implicated in the etiology and response to drug treatment of a number of psychiatric disorders including psychosis, depression, mania, eating disorders and obsessive compulsive disorder (Russo-Neustadt, 2003). It is therefore reasonable to hypothesize that BDNF could be involved in behaviour related to these disorders such as anxiety and fear.

It is commonly believed that the neurotrophins, especially BDNF and NGF, play an important role in the regulation and expression of emotional behaviours and stress. Increasing evidence shows that BDNF and its receptor, tyrosine kinase receptor B (TrkB), play a critical role in activity-dependent synaptic plasticity and have been implicated as mediators of hippocampal-dependent learning and memory. Also they have an essential maintenance function in the regulation of fear and anxiety-related behaviour. BDNF is involved in amygdala-dependent learning and amygdala BDNF levels are positively correlated to Pavlovian fear conditioning (for review, see Rattiner et al., 2005). BDNF signaling through TrkB receptors in the amygdala is required for the acquisition of conditioned fear (Rattiner LM et al., 2004). Rios et al. (2001) reported that mice with conditional deletion of BDNF were hyperactive after exposure to stressors and had higher levels of anxiety when evaluated in the light/dark exploration test. With regard to depression, it is found that BDNF in hippocampus is reduced in mice whose number of glucocorticoid receptors (GR) is manipulated to be only a fraction of that of a normal mouse. And the lack of GR leads to the manifestation of depression (Ridder et al., 2005). However, chronic reduction of BDNF is not sufficient to induce

neurochemical or behavioural alterations that are reminiscent of depressive symptoms in human (Chourbaji et al., 2004). It is well-known that exposure to learning procedures often leads to upregulation of brain neurotrophins. Many studies in stress have demonstrated that exposure to stress often results in decreases of BDNF and NGF levels. For instance, plasma NGF is reduced when the stress level is lower (Kimata, 2004), while BDNF is also found to be negatively related to the stress level in human (Kimata, 2003). In rats, acute (1-2 hrs) restraint stress transiently reduces BDNF mRNA expression in the hippocampus, a region important in the memory and in hypothalamic-pituitary-adrenal (HPA) regulation; and also down regulates BDNF expression in the basolateral amygdala, a region important for fear consolidation and emotional memory (Pisarro et al., 2004). Interestingly, in another study, Marmigere et al, (2003) discovered that short-time stress application induced a significant increase (at 60 min) in BDNF mRNA levels in the whole rat hippocampus, suggesting that rapid changes in BDNF expression may be part of a compensatory response to preserve hippocampal homeostasis or a form of neuronal plasticity to cope with new stimuli.

1.1.2 BDNF and Environment

Different environmental factors can induce alterations in gene expression and protein levels of hippocampal BDNF. Important among these factors are environmental complexity, physical exercise and dietary restriction. Housing adult and even middle aged rats in enriched environment increases hippocampal BDNF mRNA expression and protein levels (Falkenberg et al., 1992; Young et al., 1999; Ickes et al., 2000, Mohammed et al., 2002; Gobbo et al., 2004). These increased neurotrophin levels are associated with enhanced cognitive functions. BDNF has been shown to be produced in an activity dependent manner, indicative of a role in neural plasticity (Kang and Schunman, 1995). Thus rats with access to running wheels (that can, therefore, engage in voluntary physical exercise) have increased expression of hippocampal BDNF mRNA and protein levels (Neeper et al., 1996; Cotman and Berchtold, 2002; Adlard et al., 2004) and show enhanced spatial learning (van Praag et al., 1999). Likewise, dietary restriction, which is known to extend life span in rodents (Sohal and Weindruch, 1996) and improve cognitive function (Ingram et al., 1987), has been shown to result in increased expression of BDNF when compared to ad lib feeding (Duan et al., 2001; Lee et al., 2000; 2002).

1.2 Environmental Manipulations

Environmental stimulation can induce profound neurochemical, neuroanatomical and behavioural changes in many animal species (for reviews see Mohammed et al., 2002; van Praag et al.,1999).

1.2.1 Environmental Enrichment

It has become a truism that the environment has a major impact on brain and behaviour. Studies initiated in the 1960s provided convincing illustration that environmental enrichment (EE) leads to significant changes in brain biochemistry and anatomy (Bennet at al., 1964, 1969). These changes include increased brain weight and numbers of glial cells, dendritic branching and spines, as well as synaptic density. While most of these aspects were reported in the visual cortex, the rest of the neocortex also showed anatomical changes as well. Further research showed that the hippocampus responds to EE by increasing neurogenesis, the number of dendritic spines in the dentate gyrus (Walsh et al., 1969; Walsh and Cummins, 1979) and the levels of NGF, BDNF and NT-3 neurotrophins (Falkenberg et al., 1992; Mohammed et al., 1993; Torasdotter et al., 1996, 1998). These environmentally induced brain changes are accompanied by alterations in cognitive function and emotional behaviour (Mohammed et al., 1986, 1990; Galani et al., 1998; Pham et al., 1999; Renner and Rosenzweig, 1987). The effects of enrichment have been observed in young, adult and aged rodents (Kempermann et al., 1997, 1998; Grennough et al., 1986; Nilsson et al., 1999; Diamond et al., 1985a). Thus, in elderly individuals EE appears to result in increased neural reserve that can be beneficial and is compatible with the 'use it or lose it' notion. EE facilitates recovery of function in genetically manipulated mice and in animals with brain lesions (e.g. Will et al., 1977; Young et al., 1999). Initially observed in rodents, the impact of EE on brain and behaviour appears to be an universal phenomenon - from flies to philosophers, as it has been demonstrated in a variety of species including fruit flies, fishes, bears and primates (e.g. Lomassese et al., 2000; Sandeman and Sandeman, 2000; Diamond et al., 1985b).

We have previously demonstrated that enrichment condition (EC) enhances spatial learning in adult and aged rats as compared to standard housing condition (SC) (Falkernberg et al., 1992; Mohammed et al., 1986; 1990; Pham et al., 1999). While the major focus of previous research work has been on cognitive function including learning and memory, not much is known about the impact of environmental factors on emotional

behaviours, and the literature on this topic has not always been consistent. Increasing evidence indicates that EC and impoverished housing condition (IC) animals do differ in emotional behaviour (Larsson et al., 2002; Mohammed et al., 1993; Nikolaev et al., 2002; Roy et al., 2001). We have also previously reported that EC animals cope better in a mildly stressful situation than impoverished animals (Larsson et al., 2002). Mild stress was found to enhance learning in the water maze in EC but not IC rats, while severe stress impairs spatial learning regardless of the housing conditions (Larsson et al., 2002). Furthermore, other groups have found that EC animals appear to be less anxious as shown by behaviour in anxiety and open-field tests (Chapillon et al., 1999; Fernandez-Teruel et al., 2002). Recently it was found that EC reduced anxiety-like behaviour in mice tested in the elevated plus-maze (Benaroya-Milshtein et al., 2004) or the O-maze (Wolfer et al., 2004).

1.2.2 Physical Exercise

In studies investigating the effects of environmental factors on brain and behaviour in laboratory rodents, running-wheel is often included as an enrichment device. It is known that regular exercise is beneficial for cardiovascular system, improves the stress response mechanism, and can be beneficial to the treatment of depression. Wheel running was found to attenuate depression-like behaviour in a rat model of depression (Bjornebekk A, 2004). However, the neurochemical bases of the motivation to run, as well as the influence on behavioural changes are not well understood, although there are numerous theories for the motivation to initiate wheel running (for review see Sherwin, 1998). In addition to its effects on neurotrophins, wheel running and EE share other neural and behavioural effects in rodents. Both of them are known to increase the number of neurons in the hippocampus (Kempermann et al., 1997; van Praag et al., 1999b; Mohammed et al., 2002; Bjornebekk, 2004; Holmes et al., 2004), to enhance LTP (van Praag et al., 1999a; Duffy et al., 2001), to promote learning and memory (Mohammed et al., 1986; Mohammed et al., 1990; van Praag et al., 1999a) and to influence recovery of function following brain damage (for review see: Will et al., 2004). Indeed, the similarity in effects of enrichment and wheel-running inspired investigators to explore whether the enrichment effects are predominantly due to the animals´ propensity to exercise in the EC cages. However, some studies have shown that environment-induced changes in hippocampal morphology are caused more by environmental novelty rather than physical exercise (Faherty et al., 2003; Will et al.,

2004). Wheel running can induce neurochemical alterations in different brain regions involved in learning, memory and motivational drive, still a better understanding of the use of running wheel as an enrichment device is required.

1.2.3 Intermittent Individual Housing

Long-term exposure to individual housing in rodents is frequently used to analyze particular aspects of psychiatric disorders, like schizophrenia (Geyer et al., 1993; Wilkinson et al., 1994) and depression (Ehlers et al., 1993; Jaffe et al., 1993). Social isolation implies animals are isolated from their conspecifics in terms of visual, auditory, olfactory and tactile stimuli. Individual housing refers to animals being singly housed but able to see, smell and/or hear other animals. Our previous studies in rats have shown that social isolation, in the form of non-physical contact with other conspecifics, can induce changes on behaviour and the expression of brain neurotrophic factors (Ickes et al., 2000; Pham et al., 1999). Other studies report that long lasting social isolation affects a number of physiological variables, including plasma corticosterone concentration (Brain & Benton, 1979), brain corticotrophin releasing hormone (Stanton et al., 1988) and brain opiate systems (Iglesias et al., 1992). In spite of these findings, little is known about the impact of intermittent exposure to individual housing on behaviour and neurobiology.

1.2.4 Other Concerns about Environment

With regard to the environmental conditions, it should be pointed out that terms as "environmental enrichment" and "environmental enhancement" are not synonymous. "Environmental enrichment" is often used in the field of neuroscience, to refer mainly to social housing in a large, complex cage comprising different toys that are changed frequently in order to induce changes in the brain and behaviour. "Environmental enhancement", on the other hand, focuses on specific needs of the animals such as nest building, hiding and gnawing in order to improve the well-being of the animals (for reviews see Baumans, 2005; Benefiel et al., 2005).

The levels of brain neurotrophins might also be influenced by other determinants that can be categorized as "state" and technical factors. One such significant state factor is the large battery of behavioural tests that animals are subjected to, which can be considered as a kind of environmental manipulation that may lead to changes in brain neurotrophin levels. To determine whether behavioural testing itself contribute to

any observed difference in neurotrophin content between EC and SC subjects, behaviourally naïve (i.e. non-tested) groups should be included.

1.3 Dissociation of Hippocampus Functions

Many considerable evidences indicate that the hippocampus is greatly susceptible to environmental influences, and that one of the key effects of these environmentally induced changes in the hippocampus is on learning. However, the hippocampus is known to be involved in mnemonic as well as emotional processing (Gray, 1982; Gray and McNaughton, 2000). Recent studies suggest that the dorsal and ventral hippocampus can subserve different functions, as the hippocampus may not constitute a functionally uniform structure, and this functional diversity may segregate along its septotemporal axis. In a series of experiments, Moser et al. (1993, 1995) compared the effects of dorsal against ventral lesions, of various sizes and locations, by means of the water maze test. Whereas lesions confined to the dorsal pole of the hippocampus led to a significant spatial learning deficit, ventral lesions of equivalent size did not. Pothuizen et al. (2004) further confirmed the view of a dorso-ventral dissociation in the rat's hippocampus with respect to spatial reference and working memory using a four-baited/four-unbaited eight-arm radial maze. Furthermore, other studies showed that the two poles of hippocampus differ in their involvement in emotional behaviour (Kjelstrup et al., 2002; Bannerman et al., 2004; McHugh et al., 2004). While the dorsal hippocampus has been found to be involved in spatial memory, the ventral hippocampus seems to be more closely involved in emotional information processing. Ventral, but not dorsal, lesions attenuate fear response in a number of anxiogenic paradigms including light/dark exploration, hyponeophagia, open-field exploration and the elevated plus-maze (Bannerman et al., 2002, 2003; Kjelstup et al., 2002). In addition, ventral lesion preferentially affected conditioned fear (Richmond et al., 1999). Hence, a double dissociation of the dorsal and ventral hippocampus seems feasible (Moser and Moser, 1998), supported by the evidence of a differentiation in function along the septotemporal axis of the hippocampus. However, these studies did not fully take into consideration the functional significance of different brain neurotrophic factor levels, specifically BDNF and NGF, in the dorsal and ventral hippocampus.

2 AIMS

The studies presented in this thesis sought to comprehensively investigate the influences of different housing conditions and environmental factors on brain neurotrophin levels and their involvement on anxiety-related behaviour in laboratory mice. This project also aimed to further explore the association between different neurotrophin levels in the dorsal and ventral hippocampus and behaviour.

The specific objectives of the studies were:

- To define the effect of environmental enrichment on emotional behaviour and brain neurotrophin levels in male and female mice. (Study I)
- To examine the impact of extensive behavioural tests on variation of neurotrophin levels in selected brain regions. (Study I)
- To investigate the correlation pattern of regional specific variations in neurotrophic factors with anxiety related behaviour. (Study II)
- To study the effects of intermittent individual housing on behaviour and brain neurotrophins; and further investigate how physical exercise influences the effects induced by alternate social-isolation. (Study III)
- To separately analyze the levels of neurotrophins in the dorsal and ventral halves of the hippocampus. (Studies I, II and III)
- To characterize the influence of enriched environment on the number of hippocampal dendritic spines and on exploratory behaviour in male BDNF knock-out mice. (Study IV)

3 MATERIALS AND METHODS

3.1 Animals

Pathogenic free C57BL6/J male and female mice bred at the laboratory of Behavioural Neurobiology, ETH, Schwerzenbach, Switzerland, were used in study I and II; Female BALB/c/BKI mice purchased from a commercial breeder, (B&K Universal AB, Sweden) were used in study III; In study IV; Male BDNF heterozygous mice (BDNF +/-) for a targeted deletion in the BDNF gene (Ernfors et al., 1994) and their wild type (WT) littermates were used.

All animals used in these studies were kept at the institutional vivarium. The animal room maintained at 21°C with a reversed 12/12 hr light-dark cycle, for study I & II lights were on at 20:00-08:00 hrs, for study III & IV lights were on at 6:00-18:00 hrs. The subjects were housed in standard Macrolon cages with bedding materials, food and water provided on ad lib. The cages and bedding materials were changed twice per week. All experimental procedures and housing conditions followed the guidelines and recommendations of the Swiss or Swedish animal protection legislation and were approved by the animal Ethics Committee. Ethical number 153/2002 (Paper I, II); N39/03 (Paper III); S 220-04 (Paper IV).

3.2 Animal Housing Conditions

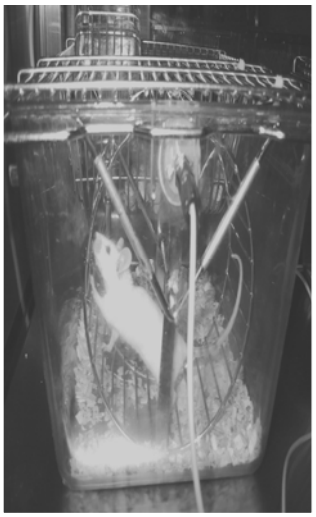
For all studies included in this thesis, different animal housing environmental conditions were manipulated as follows:

Enhanced Social Cage

Enhanced Social Cage

Standard Social Cage

Running-Wheel Cage



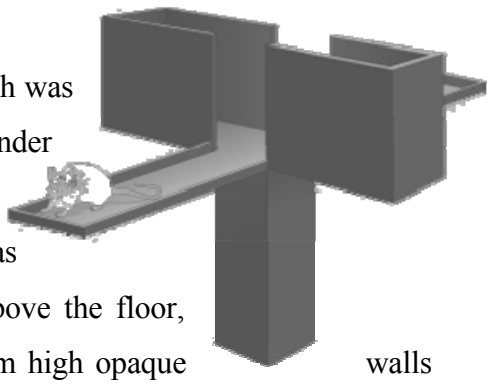
(Photographs made by Margot Meijer and Therese Pham)

- Standard and social housing condition (SC; S-control): The mice were group housed 6 or 4 per cage in standard cages with only sawdust bedding [measuring (26.5 × 47.5 × 21 cm) in studies I, II; (25.5 × 40.5 ×14.5 cm) in studies III, IV].
- Enriched housing condition (EC): In study I and II. The EC consisted of housing 6 to 14 mice in large cages that permitted social interaction, stimulation of exploratory behaviour with objects such as wooden blocks, shelves, ropes, ceramic figures, ladders, tunnels, as well as a running-wheel for exercise. The objects were changed twice a week.
- Enhanced social condition (E-control): In study III, the E-control animals were housed in Macrolon cages with a carton shelter (Shepherd Shack), two Kleenex® tissues and two aspen wood gnawing sticks in contrast to the S-control housing condition.
- Intermittent exposure to individual cage or wheel running condition: In study III, (S-control and E-control) mice were respectively exposed to individual cages (Macrolon type II, 25.5 x 19.5 x 13.5 cm) either with or without a running wheel on alternate days for 24 hours.

3.3 Apparatus, Behavioural Testing Procedure

All behavioural tests were performed in quiet experimental rooms between 10:00-16:00 hrs. During experimental days, the animals were brought to the laboratory holding room for one hour of habituation before being subjected to any behavioural test.

3.3.1 Elevated Plus-Maze [Paper I & II]

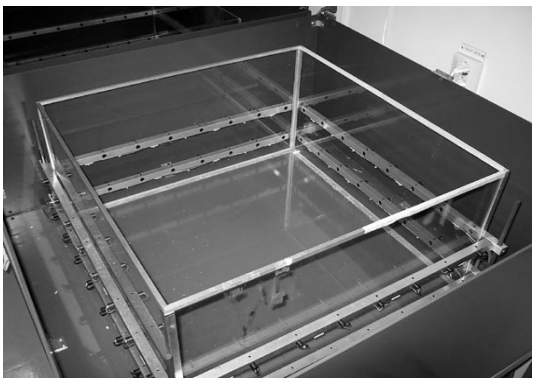


The EPM was set up under a digital camera, which was connected to a video recorder and a computer under control of the EthoVision (Noldus, Wageningen, the Netherlands) tracking system. The maze was constructed of black Plexiglas, and raised 70 cm above the floor, consisting of two opposite enclosed arms with 14 cm high opaque walls and two opposite open arms of the same size (30 x 5 cm). Testing was carried out under diffuse dim lighting. Each mouse was placed in the central platform (5 x 5 cm) facing an open arm, and was observed for 5 min. The maze was cleaned with tap water and dried after each trial to eliminate possible odour cues left by previous subjects.

The following measures were recorded: The number of entries into both the open and enclosed arms (an entry was registered when the animal put the four paws in one arm); the time spent in those two areas; and the frequency of center head scanning

(exploration of 4 angles with the head, while the body was crouching over the center area). Anxiety level was measured by the amount of exploration devoted to the open arms relative to that to the enclosed arms. This was quantified by two indices: (i) percentage of time spent in the open arms = (time $_{\text{open arms}}$ / time $_{\text{open and enclosed arms}}$) × 100%, and (ii) percentage of entries into in the open arms (entries $_{\text{open arms}}$ / entries $_{\text{open and enclosed arms}}$) × 100%. In addition, the number of head scan was taken as a more ethologically relevant measure of anxiety-like behaviour. The total number of arm entries was taken as a measure of general motor activity (File, 2001).

3.3.2 Open-field Test [Paper I & IV]



The apparatus consisted of 4 identical open field arenas (Paper I 40 x 40 x 49 cm, Paper IV 35 x 35 x 18 cm). In paper I, they were made of wood and painted white and were set up under a digital camera, which was connected to a video recorder and a computer using EthoVision software tracking program (Noldus, Wageningen, the Netherlands) tracking system. The distance moved in the arena was recorded in 1-min bins for 60 minutes. In paper IV, the open-field consisted of a Plexiglas box with a low and a higher row of infrared sensitive photocells that are connected to a computer that registers the interruptions of these cells which correspond to the horizontal and vertical activities of the animals. Four mice with arena position counterbalanced across the different housing groups were tested simultaneously. Overall locomotion and rearing behaviour in the open-field arena were measured in 10-min bins for 60 minutes.

3.3.3 Novel Objects Exploration Test [Paper I]

This test commenced immediately following the conclusion of open-field testing. At the end of the 60 min in open-field arena, the mice were placed in a set of waiting cages for 5 min, during which time the experimenter placed two pairs of distinct objects (comprising 2 plastic funnels and 2 metal hinges) into every arena. The two pairs of objects were arranged at the middle on opposite side near one of the four arena walls. The animals were returned to the same arenas where they were before, and left to explore for another 6 min. After every session the four arenas were cleaned with wet paper towels and dried. The session was recorded on videotape, and the duration and

frequency of object exploration were subsequently manually recorded by the experimenters under blind experimental conditions.

3.3.4 Food Neophobia test [Paper I]

Food neophobia can be induced either by the novelty of the food presented and/or the place where it is presented, and the latency to consume the food is typically taken as the dependent measure of food neophobia (hyponeophagia).

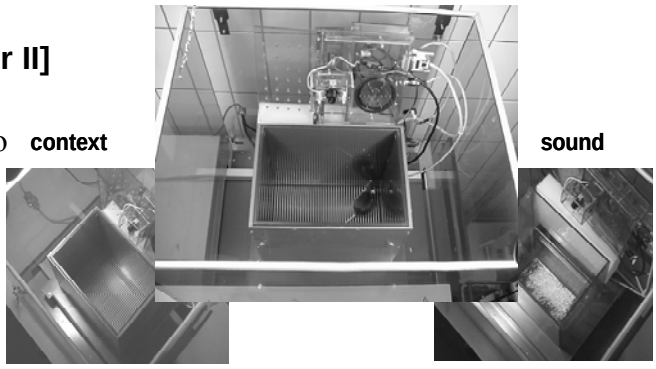
To assess neophobia, all animals were maintained on a restricted diet with 1-h access to food per day, which lasted for 7 days. The animals' body weight was monitored daily. Those mice which lost more than 10% of their ad lib weight were excluded from the experiment. The test included two novel food items: (i) dried banana and (ii) pasta, and two test contexts: Context A = a rectangular plastic area (26 x 41.5 x 21 cm) placed under dim lighting inside a fume cupboard with the ventilation system providing a constant background noise, Context B = a cylindrical Plexiglas enclosure measuring 29cm in diameter and 40cm high, with its floor covered with fresh sawdust, under normal lighting condition.

The animals were tested twice: one in each of the two test contexts. On the first test, a novel food item was presented in conjunction with familiar laboratory food chow. On the second test, the novel food item featured in the first test was presented against another novel food item. Half of the animals had banana, and the other half had pasta in the first test. All animals were confronted with banana and pasta in the second test. At the same time, half of the animals were tested in Context A (the first test) and then in Context B (the second test), and the other half vice versa.

To begin a test trial, the animal was placed facing the two food items, and the latency to consume the novel food was recorded. A maximum of 5 min was allowed.

3.3.5 Conditioned Freezing [Paper II]

Two sets of chambers were used to provide two distinct contexts. For the detail description of the chambers condition see methodological section in Paper III.



On day 1, all animals were given three separate paired stimuli, The conditioned stimulus 8CS) tone, and unconditioned stimulus (US) shock were CS-US (tone-shock) pairings, presented at 3-min intervals, in context A. In each pairing, the 1-sec US followed immediately the 30-sec tone CS. On the day of conditioning, the amount of freezing during the three occasions of tone presentation provided a measure for the evaluation of the acquisition of conditioning.

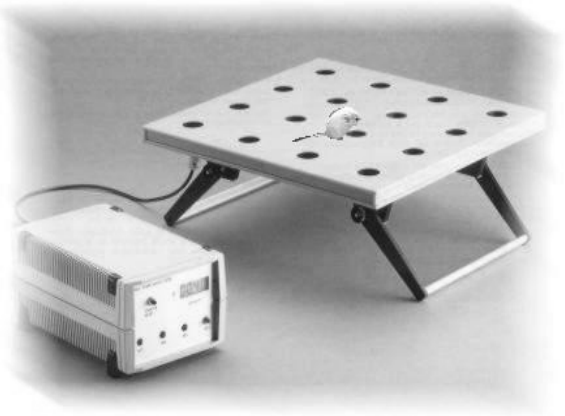
On day 2, the animals were returned to context A. They were placed in the test chamber for a period of 8 min. This served as a test of context freezing. The expression of context freezing was indexed as percent time freezing across the 8 min period.

On days 3 to 5, CS-freezing to the tone stimulus was assessed in context B. The tone stimulus was administered 3 min after the animals were placed into the test chamber. The tone remained on for a period of 8 min, to parallel the test period of context freezing.

3.3.6 LABORAS [Paper III]

A behavioural profile of the mice was assessed in the automated behaviour registration system LABORAS (Laboratory Animal Behaviour Observation, Registration and Analysis System; Metris, The Netherlands). With this system, the following behaviours were assessed: immobility, locomotion, grooming and climbing together with distance travelled and velocity. Four mice from different groups were tested simultaneously on 4 vibration sensing platforms for 30 min. Each mouse was placed individually in a clean Macrolon type II cage with the wood chips bedding, food and water as in their home cages. Each cage was put on a sensing platform. The animals' movements were transformed via the measurement platforms, which convert the movements of the animal into electrical signals. Each movement has its own unique frequency, amplitude and pattern, which can be separated into behavioural categories and automatically registered by a computer. Movement signals not recognized by the system are registered as 'undefined' behaviour.

3.3.7 Hole-Board Exploration [Paper IV]



This test takes advantages of the tendency of mice to poke their noses into a hole in the floor (Rao et al., 1999). The hole-board apparatus consisted of Perspex board (Ugo Basle, 6652, Comerio VA. Italy), 40 × 40 × 27 cm, with 16 round holes uniformly spaced on the floor, each 3 cm in diameter. Each mouse was run on an elevated hole-board for two 9-minute episodes 24 hours apart.

Three behavioural dependent variables were logged in this situation.

1. Hole-probing. An automatic device counted beam breaks recorded by photocells in each hole. Any and each probing by the mouse's snout to that depth which interrupted a beam would add a single count to the total score on a combined counter visible to the experimenter who recorded the scores for each mouse for the 9-min period in the apparatus at 3-, 6- and 9- min.

The mouse's behaviour on the hole-board was also filmed by a VHS video camera from above the board, and the resulting VHS tapes were digitized for observational analysis using "The Observer 5.0" software (Noldus Information Technology bv).

The analysis was confined to locomotory movement around the apparatus.

2. Hole visits. Movements within the boundaries of the board were logged by recording the times when the mouse moved near a specific hole from somewhere else, whether or not it probed the hole or stopped near it or passed by.
3. Peering. This measure was added to include periods when the mouse extended its head and neck beyond an edge of the board. The edges were being labelled North, South, East and West.

3.4 Neurotrophins Determination [Paper I - III]

All brain tissue samples were homogenized in ice-cold lysis buffer, containing 137 mM NaCl, 20 mM Tris HCL (pH 8.0), 1% NP40 10% glycerol, 1 mM PMSF 10 µg/ml aprotinin, 1 µg/ml leupetin, 0.5 mM sodium vanadate, and the total protein and neurotrophin levels were measured using the supernatants of the tissue homogenate solutions.

3.4.1 Brain Sample Preparation and Protein Assay

At the end of the experiment, the animals were killed by decapitation; brains were taken out and dissected for different brain regions according to each paper's design: Dissected brain tissues were stored in 1.5 ml Eppendorf tubes at -70 °C until time of neurotrophins protein analyses.

The total protein level was measured by using the Peirce BCA Protein Assay Reagent kit (working range = 20-2,000 μg/ml). In brief, 25 μl of each protein-standard solution and 4% diluted (with distilled water) supernatant of tissue homogenate solution was added in flat-bottom plate. Then 200 μl of working reagent [a mixture of solution A and B (50:1)] was rapidly added to plate and incubated at 37 °C for 30 min. After the plate had cooled to room temperature (RT), the absorbance of every well was measured at 565 nm in a plate reader. The total protein values were evaluated by comparison with the regression line of the standard curve.

3.4.2 Enzyme Immunoassay for BDNF, NGF and NT-3

BDNF, NGF and NT-3 levels were assessed in selected brain regions using the Promega ELISA assay kit. Briefly, Standard 96-well flat-bottom NUNC-Immuno maxisorp ELISA plates were incubated overnight at 4 °C, with the corresponding captured antibody, which binds the neurotrophin of interest. The next day the plates were blocked by incubation for 1hr at room temperature (RT) with a 1 × Block & Sample buffer. Serial dilutions of known amount of BDNF, NGF or NT-3 ranging from 500- or 300- 0 pg/ml were performed in duplicate for the standard curve. Wells containing the standard curves and supernatants of brain tissue homogenates were incubated at RT for 6 or 2hrs, as specified by the protocol. They were then incubated with secondary specific antibody overnight at 4 ºC or for 2 hrs at RT, as specified by the protocol. Next, a species-specific antibody conjugated to horseradish peroxidase (HRP) was used as a tertiary reactant for 1-2.5 hrs at RT. 'TMB One Solution' was used to develop color in the wells. This reaction was terminated with 1N hydrochloric acid at a specific time (10 to 15 min) at RT, and the absorbance was then recorded at 450 nm in a plate reader within 30 min of stopping the reaction. The neurotrophin values were evaluated by comparison with the regression line for each proposed neurotrophin standard. Using these kits, NGF and BDNF can be quantified in the range of 7.8–500 pg/ml, and NT-3 can be quantified in the range of 4.7-300 pg/ml. For each assay kit, the cross-reactivity with other trophic proteins is ≤2-3%.

3.5 In Situ Hybridization for BDNF mRNA [Paper III]

Coronal brain sections (30 μm) were cut on a cryostat at -20 ºC and thawed on glass slides. The hybridization cocktail contained 50% formamide, 4 x SSC (1 x SSC is, in M, NaCl, 0.15; sodium citrate, 0.015, pH 7.0), 1 x Denhardt's solution, 1% Sarcosyl, 0.02 M Na_3PO_4, pH 7.0, 10% dextransulphate, 0.06 M dithiothreitol and 0.1 mg/ml sheared salmon sperm DNA. Single-stranded oligonucleotide 48-mer DNA probes specific for BDNF (250-298) (Leibrock et al., 1989) mRNA were used. The probes were 3´-end labeled with α-^{33} P-dATP (Dupont NEN, Wilmington, DE) using terminal deoxynucleotidyl transferase (Gibco) to a specific activity of approximately 1 x 10^9 c.p.m./mg. Hybridization was performed for 18 hrs in a humidified chamber at 42 °C. Following hybridization, the sections were rinsed 4 x 20 min each in 1 x SSC at 60 °C. Finally, the sections were rinsed in autoclaved water for 10 sec, dehydrated in alcohol and air-dried. Thereafter, the slides were exposed to film (Kodak Biomax MR Film, Kodak, Rochester, NY) for 4 days and developed. Films were scanned and optical density values were quantified using appropriate software (NIH image analysis program, version 1,62). A ^{14}C step standard (Amersham, Buckinghamshire, UK) was included to calibrate optical density readings and convert measured values into nCi/g.

3.6 Brain Morphology (Golgi staining) [Paper IV]

The mice were deeply anaesthetized and then decapitated, the brains were removed in the midsagital direction. The left hemisphere was further cut in a coronal plane into 3 blocks of tissue and processed for Golgi Cox staining. The granule cells in the dentate gyrus and pyramidal cells in the CA1 hippocampal region were detected and observed on microphotographs. For the detailed description see methodological section in Paper IV.

3.7 Statistic Analyses

All behavioural and neurotrophins results were analysed using StatView (version 5.01) software. All measures were subjected to parametric analysis of variance (ANOVA). Fisher's post-hoc tests were carried out to assist data interpretation when appropriate. The level of significance was set at $p < 0.05$ for all tests.

4 RESULTS AND DISCUSSION

4.1 Paper I

Enriched environment condition (EC) induces profound behavioural, neurochemical and neuroanatomical changes (for reviews, see Mohammed et al., 2002; van Praag at al., 2000). Increasing evidence has shown that the hippocampus, which is implicated in a range of cognitive functions, including learning and memory, is one of the most susceptible brain areas to the effects of enriched rearing. Recent work also suggests that the hippocampus is functionally segregated; lesion studies have shown that the dorsal hippocampus is important for spatial learning, whereas the ventral part is critical in emotional behaviour in rats. In this study, we investigated the effects of differential housing environments on anxiety related behaviour and neurotrophin levels in dorsal and ventral hippocampus, and other brain regions in mice. The main findings can be summarized as follows:

- **Environmental enrichment has impact on emotional behaviour and neurotrophin levels in relevant brain regions regulating emotionality in mice. These changes show a different pattern in males and females.**

The variety of behavioural tests we used to assess emotional behaviour in the present study included elevated plus-maze (EPM) test, open-field test, neophobia and novel object exploration, all of which involved some elements of stress. (a) In the EPM test, the results clearly showed that both male and female EC mice made proportionally fewer entries into, and spent less time in, the open arms of the EPM; (b) In the open-field test, our results showed that enriched animals were less reactive than the SC mice as shown by the distance traveled, and both male and female SC mice showed reduced locomotor activity. This effect is often interpreted as enhanced habituation in the enriched animals. Surprisingly, when we introduced an additional manipulation of food-deprivation in half of the animals, this locomotor activity difference between EC and SC disappeared in males, while in females this difference was still retained. This effect of food deprivation might indicate enhanced sensitivity to hunger drive in the enriched animals. However, it is unclear why such an effect was apparent only in the males; (c) The data of novel objects exploration showed that enriched and control mice exhibited an equivalent level of object exploration; (d) In the test of food neophobia, there was a clear indication that EC mice exhibited a significantly longer latency to consume the novel food as compared to SC mice.

We can exclude the possibility that this could be accounted for by exploration as such, because no difference between EC and SC appeared in the previous test of novel objects exploration. Thus the results of both the EPM and the food neophobia tests might be taken to reflect increased anxious-like behaviour in the EC mice. Other interpretations of this finding of increased anxiety in EC animals relate to the apparatus used to test anxiety. The EPM test as a measure of anxiety is open to debate, as it can be contended that the index of anxiety used (such as open arms entries) and "risk assessment" cannot be adequately dissociated from simple changes in locomotor activity and exploratory behaviour (see for example Weiss et al., 1998). The general locomotor activity that may occur during the EPM test that we used to assess anxiety may be due to nonspecific hyperactivity. The fact that the SC mice showed more entries into the open arms than the EC animals, may also reflect a hyperactivity on their part, as shown in the open-field test rather than being less anxious to visit the open arms.

Because there are few reports comparing sex differences in response to environmental enrichment, this study also investigated the influences of gender on the effect of EC on behaviour and neurotrophins. In behavioural tests, female mice were less fearful than male mice in the elevated plus-maze test; and female mice showed enhanced locomotor activity during the first 10 min of the open-field session as compared with male mice; both EC and SC male mice spent longer time on novel objects exploration; but no difference in food neophobia was found between male and female mice. The results of neurotrophin analysis showed less pronounced gender difference in NGF levels, whereas in BDNF levels, male mice generally had higher levels of BDNF in amygdala and ventral hippocampus than female mice. In summary, our results demonstrate gender differences in mice anxious-like behaviour and brain neurotrophin levels; and that rearing environments exert a differential impact on behavioural and neurochemical parameters in male and female mice. The SC mice had higher levels of neurotrophins than the EC mice. However, it should also be mentioned that higher brain neurotrophin levels do not necessarily lead to improved brain function, as there is evidence of higher levels of brain NGF to impaired learning in aged rats (Albeck et al., 2003; Hellweg et all., 1994), and there are also studies indicating higher expression of BDNF in brains of Alzheimer's disease patients compared to controls (Fahnestock et al., 2001; Peng et al., 2004). Whether these variations in neurotrophin levels in neurodegenerative diseases can in some cases be related to environmental

factors such as education, environmental toxins, and diet is currently a topic of increased interest and investigation (see for example Karp et al., 2004; Mattson, 2003).

- **Exposure to a series of behavioural tests induces alterations in levels of neurotrophins in different brain regions. The alterations of neurotrophin levels show different patterns in males and females.**

Compared with the untested animals, the animals experiencing behavioural tests in the present study showed diverse changes in levels of BDNF and NGF protein in selected brain regions, such as frontal cortex, amygdala, cerebellum and hippocampus.

Exposure to behavioural tests caused significant reduction of NGF levels in all tested animals; and also suppression of BDNF levels in amygdala and ventral hippocampus in males, but increased the BDNF levels in the dorsal hippocampus and cerebellum in the females housed in standard and enriched condition. Thus brain neurotrophin levels appear exquisitely sensitive to behavioural testing stimuli. The behavioural tests employed here involved some forms of stress or cognitive demand that would likely impinge on neurotrophin levels, as some studies have shown that stress can reduce BDNF and NGF gene expression in the hippocampus (Smith, 1996), and aggression can cause increased release of NGF (Falkenberg et al., 1992; Fiore et al., 2003).

In the non-tested animals, the enriched male mice had higher levels of BDNF than the standard housed males in both the dorsal and the ventral hippocampus. This group difference as a result of differential housing was also evident in the non-tested female mice, but only in the ventral hippocampus in which the levels were also lower than those of males. The differential housing effects were less evident on NGF levels, although there were some differences, in the dorsal and ventral hippocampi in male tested mice in which the enriched had higher levels of NGF than the non-enriched animals. The hippocampus of enriched mice is known to show increased dendritic spines in the CA1 (dorsal part of the hippocampus) (Rampon et al., 2000) and in the dentate gyrus (Mohammed et al., 2002), which could in part be attributed to the increased release of BDNF in these regions. When animals were exposed to different behavioural tests, the effects in males were still present for ventral hippocampus, but the levels were reduced with respect to non-tested animals.

- **Different baseline levels of BDNF and NGF are observed in dorsal and ventral hippocampus in both male and female mice. This is more pronounced with respect to BDNF levels. The levels of these neurotrophins are higher (about 25-30%) in dorsal than in ventral half of the hippocampus.**

Accumulating evidence has suggested that the hippocampus is functionally segregated: the dorsal and ventral parts subserving different functions in behavioural performances (Bannerman 2002, 2003 and 2004). Our results further support this hypothesis with new biological evidence in intact hippocampus, and suggest the difference in neurotrophins levels between the two hippocampal sub-regions may be related to the functional distinction between the dorsal and ventral half of the hippocampus. However, from results of this study we cannot relate the changes in neurotrophin levels in the hippocampus (and amygdala) to solely anxious-like behaviour in the plus-maze, since the animals experienced several other behavioural tests before the biochemical analysis.

- **The brain BDNF levels are different in male and female mice in most of the regions analysed: frontal cortex, amygdala, dorsal and ventral hippocampus. Biochemical analyses in these regions indicated that male mice have higher levels of BDNF as compared with female mice.**

4.2 Paper II

Recent findings indicate that amygdala and hippocampal activation is affected by fear conditioned stimuli differently in normal individuals and in those afflicted with social phobia (Veltman et al., 2004; Schienle et al., 2005; Russo-Neustadt, 2003). While the level in both regions is decreased in the former, a significant increase in the latter is observed (Knight et al., 2004; Birbaumer et al., 2005). The role of neurotrophic factors is suggested as decisive in the different outcomes. Unlike schizophrenia and depression, the opportunity for post-mortem analysis of anxiety in humans is limited by the lack of suitable post-mortem materials. No studies to date have investigated possible relationship between anxiety and brain neurotrophic factors in human (Weissman et al., 1993; Cosoff and Hafner, 1998; Rapaport, 2001). And as mentioned in paper I, we can not relate the changes in neurotrophin levels in the hippocampus (and amygdala) to anxious like behaviour in the plus-maze, since the animals experienced several other behavioural tests before the biochemical analysis. In this paper, we investigate the association between postmortem contents of neurotrophic factors, particularly BDNF, in the amygdala and the hippocampus, and fear- and anxiety-related behaviour in mice. To detect and quantify anxiety-like behaviour in mice, we used the standard EPM test of anxiety and the Pavlovian tone-shock conditioning paradigm for learned fear. The dorsal and ventral halves of the hippocampus were analyzed separately to study their

involvement in emotional behaviour respectively as recent studies suggest such differences (Bannerman 2002, 2003 and 2004) and the evidence presented in Paper I.

- **Sexual difference is not apparent in the levels of BDNF and NT-3. NGF levels in the amygdala show significant differences in male and female mice.**

The results showed that there was no sex difference in anxiety behaviour but some difference in the conditioning phase of the Pavlovian experiment. The levels of BDNF did not differ between male and female mice, or between ventral and dorsal hippocampal regions. NT-3 content was relatively low in comparison to that of BDNF and NGF, and there was no sex difference in NT-3 levels either. A pronounced sex difference in the NGF content was seen in the amygdala, demonstrating a robust sexual dimorphism. The sex difference in dorsal hippocampal NGF levels showed no significant correlation to the EPM test, but attained significance in the Pavlovian conditioning experiment with an increased sample size.

- **BDNF is associated positively with anxiety-like behaviour in the EPM and conditioned fear. However, enriched rearing has no influence on the BDNF levels in conditioned freezing.**

With regards to behavioural correlates of BDNF, there was a positive correlation between dorsal hippocampal BDNF level and anxiety-like behaviour in the EPM, and another positive correlation was indicated between amygdala BDNF level and the acquisition of conditioned fear to a discrete tone. Hence, BDNF signaling may be related to fear expression in unconditioned (or ethological) as well as conditioned tests of anxiety, and it may do so via multiple brain structures with distinct functional relevance to fear and anxiety. Particularly, our finding indicates that higher amygdala BDNF content is associated with the development or expression of a stronger CS-US association. Another indication is the significance of housing in enriched environment. In study I, hippocampal BDNF level can be enhanced in mice housed in enriched environment; these mice also displayed increased anxiety-like behaviour in the EPM test. However, for comparison, rearing in enriched environment exerted little influence on the acquisition of conditioned freezing and furthermore, there is no correlation between dorsal hippocampal BDNF levels and conditioned freezing.

- **Dorsal and ventral hippocampal BDNF levels have different associations with anxiety-like behaviour, and NGF levels are related both to anxiety/fear behaviour and the sex of the animals.**

We found that the dorsal hippocampal BDNF level was clearly related to anxiety-like behaviour in the EPM while BDNF level in the ventral hippocampus appeared to be

inconsequential. There was not an association between the dorsal hippocampal BDNF level and locomotor activity. On the other hand, amygdala NGF level correlated with anxiety indices as well as locomotor activity in the EPM. This pointed to the possibility that the apparent association between amygdala NGF and EPM anxiety behaviour might be mediated via non-specific locomotor effects.

As for behavioural correlates of NGF, the NGF levels in the amygdala and in the dorsal hippocampus are not only related to anxiety/fear behaviour in both experiments, but are also sex dependent with a comparable magnitude of correlation. However, the correlation found in the two experiments is of different directions. While the NGF levels have a negative correlation with expressed anxiety in the EPM test, they have a positive correlation with conditioned fear in the Pavlovian conditioning test.

The cholinergic neurotransmission is influenced by NGF positively according to previous studies (Moises et al., 1995; Fusco et al., 1989). Consequently, the correlation between dorsal hippocampal NGF and EPM anxiety might be anticipated, as the hippocampal cholinergic system is involved in the modulation of anxiety behaviour. Anxiolytic effects accompany enhancement of hippocampal cholinergic transmission, whilst blockade of cholinergic (muscarinic M1) receptors has anxiogenic properties (File et al., 1998; Degroot et al., 2001; Degroot and Treit, 2002, 2003).

NGF content in the amygdala may also vary with differences in cholinergic transmission. Studies showing that amygdala NGF levels correlated with locomotor activity are in agreement with an inter-strains comparison in mice identifying a positive effect of muscarinic M1 receptors activation on general activity (Yilmazer-Hanke et al., 2003). The fact that the NGF levels in the dorsal but not the ventral hippocampus are affected indicates that the two poles of the hippocampus have different functionalities in fear/anxiety related behaviour. As some lesion studies show that the ventral instead of the dorsal region is involved, further research is needed to test the hypothesis that altered fear/anxiety related behaviour after the lesion of the ventral region is a consequence of altered dorsal hippocampal functions.

The positive relationship between NGF and tone-freezing cannot possibly be accounted for by the link between NGF and hippocampal acetylcholine neurotransmission. On the other hand, the muscarinic cholinergic receptor antagonist, scopolamine, if administered systemically, has been shown to disrupt Pavlovian conditioned freezing (Anagnostaras et al., 1995, 1999). The effect is the same if it is

administered directly into the hippocampus (Wallenstein and Vago, 2001; Gale et al., 2001; Rogers and Kesner, 2004). From this study, however, any relationship between context freezing and brain neurotrophin levels cannot be established.

4.3 Paper III

Long-term exposure to individual housing in rodents is frequently used to analyze particular aspects of psychiatric disorders. Many studies report that long lasting social isolation affects a number of physiological variables, including plasma corticosterone concentration (Brain et al., 1979) brain corticotrophin releasing hormone (Stanton et al., 1988) and brain opiate systems (Iglesias et al., 1992). In spite of these findings, little is known about the impact of intermittent exposure to individual housing on behaviour and neurobiology. This study assessed the effects of intermittent individual housing on behaviour and brain neurotrophins, and whether physical exercise could ameliorate these consequent effects.

Five-weeks old BALB/c mice were either housed in enhanced social (E) or standard social (S) housing conditions for two weeks. Thereafter they were divided into 6 groups and for 6 weeks remained in the following experimental conditions: Control groups remained in their respective housing conditions (E-control, S-control); enhanced individual (E-individual) and standard individual (S-individual) groups were exposed every other day to individual cages without running-wheels; enhanced running-wheel (E-wheel) and standard running-wheel (S-wheel) groups were put on alternate days in individual running-wheel cages. Animals were assessed for activity in an automated individual cage system (LABORAS) and brain neurotrophins analysed.

Intermittent individual housing can induce increased locomotion in open-field similar to what is observed following long-term isolation, and physical exercise can counteract this behavioural change.

The behavioural test results in this study clearly showed that intermittent individual housing had a strong impact on motor activity in BALB/c mice. Regardless of the housing condition (E or S), both E-individual and S-individual groups demonstrated significantly increased motor activity as compared to the control groups maintained in stable social conditions and the groups exposed to intermittent individual housing with running-wheels. Moreover, the increased activity was more pronounced in E-individual mice than in S-individual mice, as shown by total distance traveled, velocity and locomotion duration, indicating that E individual animals might be more alert or more aroused, possibly due to the more pronounced impact of the change in housing

conditions from enhanced social group to individual house. This phenomenon could be counteracted, however, by providing a running wheel in the single cage.

Intermittent individual housing causes downregulated NGF and BDNF protein levels in the frontal cortex, voluntary exercise can not attenuate these neurochemical changes in this region.

This effect is comparable to that reported in an earlier study (Ickes et al., 2000), that showed a similar decrease in brain neurotrophins levels following long-term individual housing in rats. It further demonstrates that intermittent individual housing experience can also cause neurotrophin down regulations in the frontal cortex, a brain region found to play a key role in locomotor responses in an unfamiliar environment (Broersen et al., 1999). It is possible that the observed locomotor changes of E-individual and S-individual mice may be a consequence of altered neurotrophin levels in this critical brain region. Moreover voluntary exercise could not attenuate the neurochemical changes in this region, although it could counteract increased motor activity and affect neurotrophin levels in other brain regions like amygdala.

Mice exposed to intermittent individual housing have increased hippocampal BDNF protein and mRNA levels, and the changes are unlike those observed after long-term isolation.

In line with the increased levels of BDNF in hippocampus there was also an increased cell proliferation in the sub granular zone of the dentate gyrus in the groups of animals that were subjected to intermittent individual housing (Bjornebekk et al., 2005). Thus, together these findings indicate a possible increased neuronal activity in hippocampus, which may be elicited by frequent alteration in housing conditions. Unlike in long-term social isolation, animals exposed to intermittent social deprivation had the chance to interact with their cage-mates during certain periods. Consequently a neurobiological compensatory pathway might be provoked by the intermittent social and physical contacts, resulting in enhanced neurotrophin secretion. Compared with other brain regions, the hippocampus is known to be a sensitive region and responsive to a variety of internal and external stimuli (Kampermann et al., 1997). Interestingly, the effect on BDNF upregulation was specifically found in the hippocampus.

Higher BDNF levels are detected in hippocampus and cerebellum of running-wheel groups as compared to control groups.

The involvement of cerebellum in regulating motor activity and learning is well established (for review see Boyden et al., 2004), and our finding of increased BDNF affecting runners suggests a possible involvement of cerebellar neurotrophins in motor

learning. We also found that the running-wheel groups had higher NGF and BDNF levels in hippocampus as compared to control groups. The relocation from the home cages to individual cages with running-wheels providing voluntary exercise induced higher hippocampal levels of NGF and BDNF in the E-wheel and S-wheel mice.

This study demonstrates that alternate individual housing has significant impact on behaviour and brain neurotrophin levels in mice, which can be partially altered by voluntary physical exercise. Our results also suggest that some changes in neurotrophin levels induced by intermittent individual housing are not similar to those caused by continuous individual housing.

4.4 Paper IV

While a wealth of data supports the view that BDNF is critically involved in learning and memory, the role of BDNF in regulating emotional behaviour such as anxiety is less clear. BDNF administration in rats causes improvement in behavioural responses as observed following treatment with antidepressants (Shirayama et al., 2002), no such effects are seen in transgenic mice with reduced BDNF expression or signalling (Saarelainen et al., 2003). In this study mice lacking the BDNF gene were used to: further understand the role of BDNF in exploratory behaviour and anxiety-like behaviour; and to dissect the role of BDNF in enriched-induced neuroanatomical changes in the hippocampus.

We employed automatic recording, direct observation and video analysis to study the behaviour of differentially housed BDNF mutant (BDNF+/-) mice and their wild type (WT) controls in situations that produce moderate levels of anxiety and exploration. In the open-field test, EC reduced the locomotor counts of WT mice but not BDNF+/- mice. BDNF+/- mice housed both in enriched and standard environment were hyperactive, showing higher locomotion counts than the WT controls. However, on the second day of testing only the EC mutants displayed reduced locomotor counts, indicating habituation to the testing environment, while the SC mutants continued to show increased locomotor counts compared to the other groups. A similar pattern of habituation on the second day of testing emerged from the rearing counts with the EC mutants showing less rearing than the SC mutants. This suggests that the deletion of the BDNF gene can prevent some of the behavioural effects of EC such as the reduction of locomotor activity in the open-field; while EC effects on habituation learning persist even in mutant mice. In the hole-board test, the automatic measurement showed that the

enriched WT animals probed the holes more than the other groups on the first day of testing. We interpreted this as evidence of enhanced exploratory behaviour in the WT animals; since this effect was absent in the enriched BDNF+/- mice. However, both the WT and BDNF+/- housed in EC paid more visits to the holes than the non-EC groups. Similarly EC animals displayed more instances of peering behaviour than the non-EC animals, suggesting that both the enriched BDNF+/- and WT mice showed enhanced exploratory behaviour than their non-EC counterparts. However, it is noteworthy that BDNF+/- mice had low counts of hole-probing which could be due to the effects of BDNF on behaviours related to motivation and anxiety. Another measure known to reflect fearfulness and emotionality in the open-field and the hole-board is the number of fecal boli deposited. We found that both EC- and SC- BDNF+/- animals deposited significantly more fecal boli in the open-filed on day 2, and in the hole-board on day 1 (BDNF+/- EC) than did the WT controls. In summary: mice lacking the BDNF gene displayed a phenotype of heightened emotionality and anxiety as characterized by the higher locomotion and rearing counts, more edge-peering, fewer hole-probings and larger numbers of fecal boli. Thus the deletion of BDNF gene caused changes in exploration and emotionality, and was compatible with clinical findings implicating the pathophysiological roles of BDNF in conditions such as depression and anxiety.

In the present study we also found that the EC BDNF+/- mice had more dendritic spines than non-EC mutants in hippocampal CA1 region and dentate gyrus, which could conceivably facilitate habituation learning in the open-field. It is also reasonable to suggest that the changes observed in peering behaviour and hole-visits by EC BDNF+/- and WT mice are at least in part orchestrated by mechanisms involving subtle structural changes (involving dendritic spine density) in hippocampal CA1 region and dentate gyrus, these area have been implicated in learning and exploratory behaviour (eg. Moser et al., 1994). Interestingly, even in EC housed BDNF+/- mice, an increase in the number of dendritic spines was documented in these hippocampal regions, albeit not to the same extent as in WT mice. Our results indicate that knocking out the BDNF gene does not completely abolish the EC effects on hippocampal morphology or on behaviour, such as the open-field behaviour.

In conclusion, these findings point to an involvement of BDNF in regulating behavioural and neural plasticity associated with EE. They are also compatible with suggestions that BDNF plays an important role in mechanisms mediating anxiety.

5 CONCLUSIONS

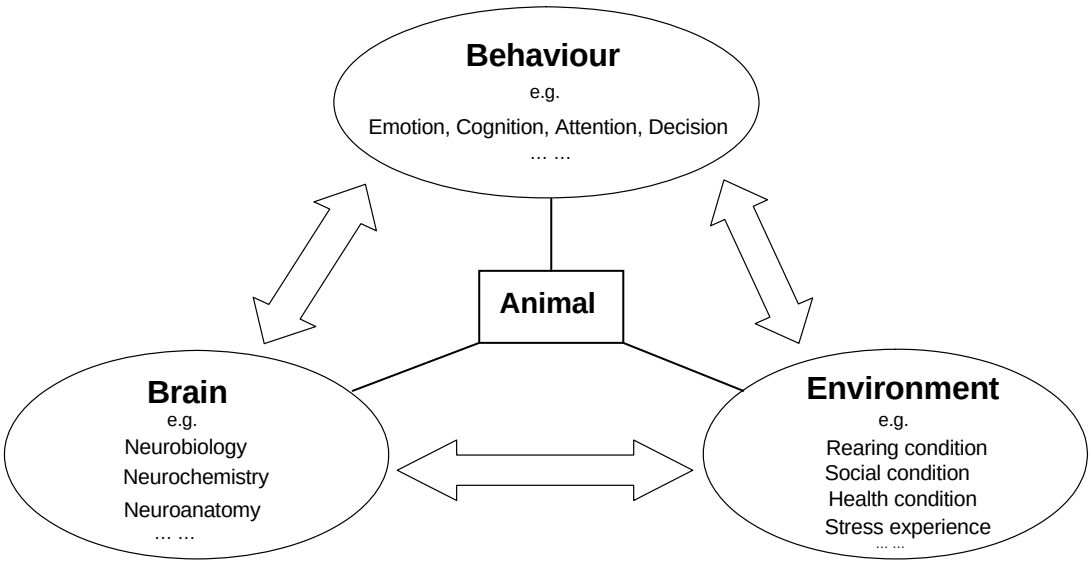
This thesis has focused on how different environmental interventions facilitate emotional and accompanying neurochemical changes in mice. The objective of this work was to find out the evidence of involvement of brain neurotrophins, particularly BDNF, in anxiety-related behaviour of animals, housed under different environmental complexities.

The major findings of this thesis are:

- The normal state levels of brain neurotrophins are different between dorsal and ventral halves of hippocampus, with a higher concentration of BDNF and NGF in the dorsal hippocampus.
- The anxiety-like behaviour is positively related to dorsal hippocampal BDNF levels, and negatively related to NGF levels in both dorsal hippocampus and amygdala.
- Expression of conditioned fear is positively related to amygdala BDNF and NGF levels as well as dorsal hippocampus NGF levels.
- Enriched environment has been shown to be efficacious in alleviating some of the pathological hallmarks of neurodegenerative diseases in genetic mice models. Housing in enriched environment results in more aroused and active animals. These effects could at least in part be mediated by neurotrophins such as BDNF which are elevated following exposure to enriched environment and physical exercise.
- Intermittent social isolation causes anxiety-like behaviours with increased motor activity, which is manifested physiologically in the form of reduced NGF and BDNF in frontal cortex and increased BDNF in amygdala and hippocampus.
- The excessive motor activity induced by social isolation can be counteracted by voluntary physical exercise while the BDNF and NGF levels in the brain remain elevated.
- BDNF mutant mice are less responsive to environmental changes, providing suggestive evidence for a role of BDNF in regulating emotional behavior. Further work is needed in order to provide conclusive evidence.

Any normal function that contributes to the wellbeing of the animal might depend on the dynamically balanced interaction of at least three fundamental elements: brain,

environment and behaviour. They maintain manifold links and could directly or indirectly act and interact on each other via diverse internal and external stimuli. Our studies investigated different aspects of these three elements of the dynamic balance, and revealed novel interactions of brain neurotrophins, environmental influences and anxiety-related behaviour in mice. Collectively the results in this report should contribute to further elucidate the complex interaction involving the brain, behaviour and environment, providing insight into the neurobiochemical bases of emotional behaviour in mammals.



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