From the Group of Behavioural Biology, Institute of Psychiatry
Central Institute of Mental Health (Director Prof. Dr. Dr. Fritz A. Henn)
University of Heidelberg
(PD Dr. Peter Gass)

Coping with stress:
The Impact of the Hypothalamus Pituitary Adrenal (HPA) System and
Neurotrophic Circuits in the Learned Helplessness Model of
Depression

Doctoral Thesis
Ruprecht-Karls-University of Heidelberg
presented by Sabine Chourbaji
from Heidelberg
2005
Index:

ABSTRACT ............................................................................................................................. 5

1 INTRODUCTION ............................................................................................................. 6

1.1 DEPRESSION ............................................................................................................ 6
  1.1.1 THEORETICAL CONCEPTS OF PATHOPHYSIOLOGY AND TREATMENT OF DEPRESSION 7
  1.1.2 THE MONOAMINE HYPOTHESIS ........................................................................... 8
  1.1.3 THE NEUROTROPHIN HYPOTHESIS ....................................................................... 9
  1.1.4 THE STRESS HYPOTHESIS .................................................................................. 11
  1.1.5 NEUROGENESIS .................................................................................................. 14
  1.1.6 THE MACROPHAGE THEORY OF DEPRESSION .................................................. 14

1.2 ANIMAL MODELS OF DEPRESSION ........................................................................ 15
  1.2.1 LESION MODELS ................................................................................................. 16
  1.2.2 CHRONIC MILD STRESS ...................................................................................... 17
  1.2.3 LEARNED HELPLESSNESS .................................................................................. 17
  1.2.4 GENETIC APPROACHES ...................................................................................... 18
    1.2.4.1 BDNF Heterozygous Mice (BDNF²⁺⁻) ................................................................. 18
    1.2.4.2 CREB Ser142A Mutant Mice ............................................................................ 19
    1.2.4.3 Glucocorticoid Receptor Mutant Mice and Current Implications .................. 19
    1.2.4.4 Interleukin-6 Receptor Mutant Mice (IL-6⁻/⁻) .................................................... 22
    1.2.4.5 Endothelial Nitric Oxide Deficient Mice (NOS III⁻⁻) ........................................ 22

1.3 CURRENT TREATMENT CONCEPTS OF DEPRESSION ........................................... 23

1.4 CONCEPTUAL CONSIDERATIONS ............................................................................ 24

1.5 AIM OF THIS THESIS ............................................................................................ 26

2 MATERIAL AND METHODS .......................................................................................... 28

2.1 ANIMALS .................................................................................................................. 28
  2.1.1 HOUSING CONDITIONS ....................................................................................... 28

2.2 GENETIC APPROACHES .......................................................................................... 29
  2.2.1 BDNF HETEROZYGOUS ANIMALS (BDNF²⁺⁻) ...................................................... 29
  2.2.2 CREB Ser142A MUTANT ANIMALS ..................................................................... 30
  2.2.3 GLUCOCORTICOID HETEROZYGOUS ANIMALS (GR⁻⁻) ..................................... 30
  2.2.4 GLUCOCORTICOID OVEREXPRESSING ANIMALS (YGR⁻⁻) .............................. 30
  2.2.5 INTERLEUKIN-6 KNOCK-OUT ANIMALS (IL-6⁻⁻) .............................................. 30
  2.2.6 ENDOTHELIAL NITRIC OXIDE SYNTHASE KNOCK-OUT ANIMALS (NOS III⁻⁻) ....... 31

2.3 BEHAVIOURAL ANALYSIS ....................................................................................... 31
  2.3.1 BASAL BEHAVIOUR ............................................................................................ 31
    2.3.1.1 Motoric Abilities .............................................................................................. 31
    2.3.1.2 Openfield ......................................................................................................... 31
    2.3.1.3 Novel Cage ....................................................................................................... 32
    2.3.1.4 Barrier Test ...................................................................................................... 33
    2.3.1.5 Dark-Light Box (DLB) .................................................................................... 33
    2.3.1.6 O-Maze ........................................................................................................... 34
    2.3.1.7 Marble-Burying (Defensive Burying) ............................................................... 34
2.3.2 LEARNING .......................................................... 34
2.3.2.1 Fear Conditioning ........................................... 34
2.3.2.2 T-Maze ......................................................... 35
2.3.3 DEPRESSION-LINKED BEHAVIOUR .................. 36
2.3.3.1 Porsolt Forced Swim Test ................................. 36
2.3.3.2Sucrose Consumption ...................................... 37
2.3.4 NOVEL OBJECT TEST ...................................... 37
2.3.4.1 Learned Helplessness ................................. 38

2.4 STRESS ENDOCRINOLOGY ................................ 39
2.4.1 CORTICOSTERONE DETERMINATION .................. 39
2.4.2 DEXAMETHASONE TEST (DEX-TEST) AND DEX/CRH-TEST ........................................ 40
2.4.3 ACTH DETERMINATION .................................... 41

2.5 NEUROTROPHINS AND MONOAMINES ................. 41
2.5.1 BRAIN–DERIVED NEUROTROPIC FACTOR AND NERVE GROWTH FACTOR .......... 42
2.5.2 TIME COURSE EXPERIMENT .............................. 42
2.5.3 MONOAMINES AND CHOLINE ACETYLTRANSFERASE ................................. 42

2.6 INTERLEUKIN-6 (IL-6) ........................................ 43

2.7 PHARMACOLOGICAL EXPERIMENTS ..................... 43
2.7.1 PHARMACOLOGICAL VALIDATION ..................... 43

2.8 STATISTICAL EVALUATION .................................. 44

3 RESULTS ...................................................................... 45

3.1 ESTABLISHMENT OF THE LEARNED HELPLESSNESS PARADIGM .................. 45
3.1.1 PROTOCOLS ...................................................... 45
3.1.2 FINAL PROTOCOL FOR THE LEARNED HELPLESSNESS: ...................... 47
3.1.3 VALIDATION .................................................. 48
3.1.4 NEUROTROPHIN TIMECOURSE ............................ 48
3.1.5 ASSOCIATED PARAMETERS ................................. 49
3.1.6 CHRONIC STRESS AND HOUSING CONDITIONS .................. 49
3.1.6.1 Group Housing Affects Exploratory Behaviour but not Locomotion ............ 49
3.1.6.2 Group Housing and Enrichment Evoke Reduced Anxiety-like Behaviour ....... 50
3.1.6.3 Housing Conditions Affect the Coping Behaviour in the Learned Helplessness Paradigm .............................................................................................................. 52
3.1.6.4 Hotplate: Housing Conditions do not Affect Pain Sensitivity ................... 53

3.2 GENETIC APPROACH .......................................... 53
3.2.1 BDNF+/− ANIMALS DO NOT REPRESENT AN ANIMAL MODEL OF DEPRESSION .... 53
3.2.1.1 Basal Behaviour .......................................... 54
3.2.1.2 Depressive-Linked Behaviour ............................ 55
3.2.1.3 Stress Endocrinology ..................................... 56
3.2.1.4 Neurotrophins and Monoamines .................................. 57
3.2.2 CREB5142A ANIMALS DO NOT REPRESENT AN ANIMAL MODEL OF DEPRESSION .... 58
3.2.2.1 Basal Behaviour .......................................... 59
3.2.2.2 Depressive-Linked Behaviour ............................ 59
3.2.2.3 Stress Endocrinology ..................................... 60
3.2.3 GR+/− ANIMALS DISPLAY A DEPRESSIVE-LIKE PHENOTYPE WITH CHARACTERISTIC STRESSPHYSIOLOGY IN THE DEX/CRH TEST AND REDUCTION OF NEUROTROPHINS .............................................................................................................. 61
3.2.3.1 Basal Behaviour ........................................................................................................ 62
3.2.3.2 Depressive-Linked Behaviour ................................................................................... 63
3.2.3.3 Stress Endocrinology .............................................................................................. 63
3.2.3.4 Neurotrophins .......................................................................................................... 65
3.2.4 YGR MUTANT ANIMALS DEMONSTRATE AN ANTI-DEPRESSIVE PHENOTYPE ...... 66
3.2.4.1 Basal Behaviour ....................................................................................................... 66
3.2.4.2 Depressive-Linked Behaviour ................................................................................... 66
3.2.4.3 Stress Endocrinology .............................................................................................. 67
3.2.4.4 Neurotrophins .......................................................................................................... 68
3.2.5 IL-6 DEFICIENT ANIMALS DEMONSTRATE A STRESS RESISTANCE ............. 69
3.2.5.1 Basal Behaviour ....................................................................................................... 70
3.2.5.2 Depressive-Linked Behaviour ................................................................................... 70
3.2.6 NOS III (-/-) MUTANT ANIMALS ............................................................................. 71
3.2.6.1 Basal Behaviour ....................................................................................................... 71
3.2.6.2 Depressive-Linked Behaviour ................................................................................... 71

4 DISCUSSION ..................................................................................................................... 73

4.1 GENERAL DISCUSSION ......................................................................................... 73
4.2 THE LEARNED HELPlessness AS A MODEL OF DEPRESSION ......................... 74
4.3 ROLE OF NEUROTROPHIC CIRCUITS ................................................................. 77
4.3.1 BRAIN-DERIVED NEUROTROPHIC FACTOR ..................................................... 77
4.3.2 CREB ......................................................................................................................... 79
4.4 ROLE OF THE HPA SYSTEM ................................................................................. 81
4.4.1 GLUCOCORTICOID RECEPTOR ............................................................................. 81
4.5 ROLE OF INTERLEUKIN-6 ....................................................................................... 84
4.6 ROLE OF NOS III ........................................................................................................ 85
4.7 GENERAL CONCLUSIONS ..................................................................................... 86
4.8 PERSPECTIVES ......................................................................................................... 88

5 OWN PUBLICATIONS ................................................................................................ 89

6 LITERATURE ................................................................................................................. 90

7 APPENDIX .................................................................................................................... 111

8 CURRICULUM VITAE .................................................................................................. 134

9 ACKNOWLEDGEMENTS ............................................................................................. 135
Abstract

Animal models currently represent a viable route for gaining further insights into the mechanisms involved in the pathogenesis of particular diseases. Depression, in this respect, constitutes a major challenge since the characterization of disease-specific traits is complicated due to the multifactorial nature of the disorder. The understanding of diverse factors, *e.g.* neurotrophic circuits and the role of the HPA axis, which have to be considered in the pathophysiology of the disease represent a major target of behavioural animal models of depression. Working on a model such as Learned Helplessness, consequently requires careful consideration of modulating aspects to ensure representative results. This work aims at elucidating the role of recently postulated target genes of depression as well as the impact of potential distorting factors, such as housing conditions of the experimental animals. To guarantee a specific readout, which permits concrete statements regarding the role of particular target genes like BDNF, CREB, and GR, we compared both, the effects of different social and as environmental factors with regard to general and Helplessness-specific effects on behaviour. Furthermore, we confirmed the model by a pharmacological validation, simultaneously monitoring effects of the obligatory handling procedure. In studies of depression and emotionality it is important to establish standardized protocols, involving the animal’s environment, to be able to precisely assess potential sources of stress and exclude artefacts. The design and modification of animal models like the Learned Helplessness subsequently bears the advantage of not only detecting potential genetic aspects by investigating mice carrying mutations of particular target genes, *e.g.* the glucocorticoid receptor, in which significant differences with regard to helpless behaviour and further depressive-like parameters became evident, but also to exploit fundamental causes of depressive-like phenotypes such as stress effects.

The detailed evaluation of the Learned Helplessness in mice as a model of depression suggests it as a valuable instrument to investigate mouse models for depression, like GR heterozygous animals, in which the behavioural phenotype was associated with depressive-like characteristics such as a decrease of BDNF protein and relevant physiological parameters which mimick stress, *i.e.* a depression-typical Dex/CRH Test and elevated corticosterone levels after restraint stress.
1 Introduction

Affective disorders represent a serious, nevertheless still often underestimated disease with a course that has always been difficult to define clearly and precisely as documented by the remarkable breadth the research on this topic during the last decades. Distinct characteristics of this illness as well as co-morbidities are still relatively ill defined and the interrelations are not yet fully understood. This most certainly results from the heterogeneity and multi-factorial pathogenesis of this disease. Presumably it will be impossible to formulate a single mechanism describing the exact causes and symptoms of depression, however, target-orientated hypothesis-conducted research on structural, molecular, and behavioural level ought to lead to some classification about single elements involved in the pathophysiology and treatment of this disease. Since genetic vulnerability as well as environmental factors, such as stress, are postulated to play a predominant role, it appears essential to combine the possibilities of genetic manipulation and determination with the standardized examination of environmentally induced effects. The development of an interdisciplinary interest in this field seems to be one of the most relevant steps to elucidate pathogenesis-related mechanisms of depression. Since genetic as well as environmental factors are likely to influence each other, this fact has to be considered in the experimental design. Consequently, an aim of this doctoral thesis comprises the merging of insights from a stress-induced model of depression, the Learned Helplessness paradigm, with findings of genetically engineered mouse models, which have been postulated to reflect depressive-like changes according to particular hypotheses of depression, i.e. the Neurotrophin- and/or Stress Hypothesis of Depression. After the detailed description of the materials and methods employed in this thesis, the published as well as unpublished results of the experiments are presented and subsequently utilized to point to future ideas and suggestions concerning research in this area.

1.1 Depression

Depression is a devastating illness, affecting approximately 12-17% of the population at some point in life (121). Worldwide the occurrence of depression is such that this disorder represents a major health problem. Despite this overwhelming impact there is still a lack of knowledge concerning the underlying aetiology and pathophysiology. Certainly antidepressants are commonly prescribed for depression as well as other types of affective disorders, even though the molecular and cellular mechanisms, by which these agents exert their therapeutic effects, are not yet fully explainable. The appearance of mood disorders likely arises from the complex interaction of multiple vulnerability genes and environmental factors. Pre-clinical and clinical studies have focused on the interactions
between stress and depression and their effects on particular brain regions (63, 146). Loss of subjective control over stressors as well as their unpredictability seem to be very important factors for the development of behavioural depression (237). The phenotypic expression of this disorder includes not only episodic and often profound mood disturbances, but also a distinct constellation of cognitive, psychomotor, autonomic, and endocrine abnormalities. Most hypotheses regarding depressive disorders are based on the dysregulation of the HPA-axis and the malfunction of the hippocampus and implicate corticotropin-releasing factor, glucocorticoids, brain-derived neurotrophic factor, and the transcription factor CREB (54, 223). The hippocampus on one hand is an important candidate area as an anatomic localization of emotional behaviours; on the other hand it is an essential region with regard to concentration- and memory processes, which are also regularly affected in severe depressive episodes. Recently, other brain regions, that are thought to be involved in depression, have also been investigated. The nucleus accumbens, amygdala, and certain hypothalamic nuclei are critical in regulating motivation, eating, sleeping, energy level, circadian rhythm, and response to rewarding and aversive stimuli, which are all abnormal in depressed patients.

For better understanding of the neurobiology of depression, it is also essential to identify the genes according to distinct hypotheses, e.g. the Stress- or Neurotrophin Hypothesis, which modulate the predisposition of individuals to be vulnerable or resistant to the syndrome (61, 223).

1.1.1 Theoretical Concepts of Pathophysiology and Treatment of Depression

Depression represents a multifactorial disease, which is not defined by one distinct clear course or pathogenesis. So far it remains unclear which features or combination of factors are responsible for the origin of a depressive state, which makes it extremely difficult to model this disease. By means of behavioural, pharmacological, as well as genetic tools it is nevertheless feasible to mimic some characteristics of depression and to investigate the occurrence of symptomatic correlates. This offers options to look for the interrelations between genetic and environmental aspects in this disease, aiming at illuminating the problem of cause and consequence, i.e. by vulnerability-, or gene expression studies (Fig. 1). On the other hand it is nowadays possible to target points of action of antidepressants on a molecular level and look for potential behavioural effects, whereby one has to consider the still unknown cause for a resistance to antidepressive treatment occurring in up to 30 % of depressed patients as well as the fairly long period to initiation of efficacy.
Introduction

Figure 1: The Interaction of Monoamines, Neurotrophins, Stress, and Neurogenesis in Depression.
Different mechanisms regarding the pathogenesis of depression are postulated. It is, however, thinkable that the disturbed balance of these postulated mechanisms might occur in diverse variations, always dependent on timing and combination of physiological, environmental, and genetic factors. The primary regulation is hypothesized to follow general rules and interact up to a distinct point, which can be used for the design of experiments, though theoretic and practical conditions may diverge from the assumption.

1.1.2 The Monoamine Hypothesis
The monoaminergic hypothesis predicts that depression is associated with an impairment of neurotransmission by serotonin, norepinephrine, and most likely also dopamine (22, 30, 189). This insufficiency can result from several mechanisms: i) decreased synthesis or increased degradation of neurotransmitters, ii) altered expression or function of the respective neurotransmitter receptors, iii) impairment of the signal transduction systems activated by post-synaptic receptors. Almost all antidepressant drugs act primarily via the first mechanism, which is supposed to improve monoaminergic transmission by increasing the presence of neurotransmitters inside the synaptic cleft. This can be caused by an inhibition of neurotransmitter re-uptake (e.g. by tricyclic or selective serotonin or norepinephrine re-uptake inhibitors, TCAs, SSRI, NARIs) or by reducing the degradation (monoamine oxidase inhibitors, MAOIs). Progress has also been made with regard to the
selectivity of antidepressants and their number and intensity of side effects. Some of these compounds have additional effects on pre- or postsynaptic receptors.

Yet it remains unclear how the various antidepressants with their different modes of action finally induce emotional and behavioural improvement and recovery. The common feature of all classes of antidepressants is, that their onset of action can take up to three weeks. Therefore, a key biological mechanism must account for the fact that chronic administration has delayed mood-elevating effects in patients, while enhancement of serotonergic or noradrenergic neurotransmission is already enhanced within minutes after the drug reaches the brain. Recent concepts for pathogenesis and therapy focus on slowly developing plasticity changes induced by chronic alterations in monoaminergic neurotransmission. In this respect, two biological systems have attracted attention: i) the stress-responsive hypothalamic-pituitary-adrenal (HPA) system, which is disinhibited in many patients with major depressive episodes (86, 106, 154, 165) and ii) the neurotrophin "brain-derived neurotrophic factor" (BDNF), which has been implicated in hippocampal maladaptation processes related to depressive episodes (59, 78). Interestingly, regulatory mechanisms of stress hormones and the mechanisms that control hippocampal BDNF expression are linked to each other. This has led to the so-called "Neurotrophin Hypothesis" of depression.

1.1.3 The Neurotrophin Hypothesis

The "Neurotrophin Hypothesis" of depression predicts that depressive disorders in humans coincide with a decreased activity and/or expression of brain-derived neurotrophic factor (BDNF) in the brain (3, 61). Recent basic and clinical studies have provided evidence for this hypothesis of depression and antidepressive treatment, postulating that plasticity-related changes, such as hippocampal atrophy in depressed patients, are related to a decreased expression or function of brain-derived neurotrophic factor (BDNF) and/or its high-affinity receptor TrkB (3, 61, 155, 224, 247) (Fig. 2). This theory is supported by the fact that BDNF is found in high concentrations in the hippocampus and cerebral cortex, brain areas known to play a role in depression (85, 130, 140). Furthermore, BDNF expression in these areas is decreased by stress exposure, which is currently the only behavioural measure to induce depression-like states in rodents (201, 202, 220). Moreover, chronic, but not acute treatment with antidepressants as well as electroconvulsive therapy induce increased levels of BDNF mRNA and protein, mainly in the hippocampus (64, 186, 253). Local cerebral administration of BDNF itself is reported to exert antidepressant-like effects in animal models of depression (197, 199). The hypothesis has been put forward that mice with compromised BDNF-TrkB signalling pathways could provide a genetic murine model of depression, which would be reflected by neurochemical and neuroendocrinological alterations in specific brain regions as well as by characteristic changes of
emotional behaviours (137, 187). Therefore it is interesting to investigate, whether i.e. mice with a reduced BDNF or TrkB expression due to a heterozygous gene disruption represent potential mouse models for depression-like neurochemical changes or behavioural symptoms. Thus, a chronic reduction of BDNF protein content in adult mice could induce neurochemical or behavioural alterations modelling depressive symptoms in humans. It seems furthermore promising to examine, whether mice with a heterozygous BDNF knock-out exhibit differences in monoamine levels in various forebrain areas, because the “Monoamine Hypothesis of Depression” predicts that depression is related to an impairment of neurotransmission by serotonin (5-HT), norepinephrine (NE), and most likely also dopamine (DA) (22, 25, 189). Disinhibition of the hypothalamic-pituitary-adrenal- (HPA) system is regarded as a hallmark neuroendocrinological correlate for major depressive episodes in human patients (10, 154) The analysis of the HPA-system of BDNF-heterozygous mice under baseline conditions and following stress exposure could shed light on the interrelationship between these systems. Furthermore, identification of alterations in a test battery for emotional behaviours, analysing locomotion and exploration as well as anxiety- and depression-related behaviours in these mice, is obligatory. For better understanding of the neurobiology of depression it is also essential to identify the genes, which make individuals vulnerable or resistant to the syndrome. With the help of the Cre-LoxP technique, transgenic mice that lack specific genes in the brain or even in defined brain regions can be designed and studied according to current hypotheses. Appropriate behavioural analyses serve to investigate, if the mutagenesis of these genes leads to behavioural changes within the animal models of depression (222). These advances will fundamentally improve the treatment and prevention of depressive disorders.
Figure 2: The Neurotrophin Hypothesis of Depression. It is stated that a downregulation of brain derived neurotrophic factor (BDNF), its tyrosine kinase receptor TrkB as well as the transcription factor CREB is associated with a depressive state, which results in a lack of synaptic neurotransmitters. In the course of depression external stress may block the expression of BDNF mRNA via activation of glucocorticoid receptors, which exert their effects by binding to negative glucocorticoid receptor binding elements (nGRE). The reduction of neurotrophins can be reverted by antidepressants that enhance the circuit followed by an increase of monoamines in the synaptic cleft.

1.1.4 The Stress Hypothesis

Dysregulations and dysfunctions of corticosteroid receptors have been implicated in the pathogenesis of stress-related psychiatric disorders such as depression and posttraumatic stress disorder (7, 17, 23, 50, 105, 250). It is still uncertain whether these corticosteroid receptor disturbances are cause or consequence of affective disorders. Clinical studies have convincingly shown a hyperactivity of the hypothalamic-pituitary-adrenal (HPA) system with elevated plasma cortisol levels in many patients with major depression (103, 106, 254). In these patients, diminished corticosteroid receptor expression or functioning have been postulated as a causative factor for a deficient feedback of cortisol and may explain their increased HPA activity and stress sensitivity (Fig. 3).
An alternative hypothesis claims that the cause of the HPA dysregulation is due to a primary upregulation of hypothalamic corticotropin releasing hormone (CRH), which secondarily leads to corticosteroid receptor downregulation in the limbic system and the hypothalamus which perpetuates the disease state (7, 105). According to the latter concept, the changes of the HPA system may result from a primary disturbance of monoaminergic systems and their widespread connections to higher brain centres including cortex, limbic system, and hypothalamus (30).

Molecular studies have so far identified two corticosteroid receptor subtypes: type 1 or mineralocorticoid receptor (MR) and type 2 or glucocorticoid receptor (GR) (9, 104). Both receptors function as ligand-binding transcription factors that belong to the nuclear hormone receptor superfamily (13, 217). They modulate a wide range of neural functions including stress responsiveness and cognition (50, 105, 181). According to current concepts, particularly GR mediated functions are disturbed in patients with severe depressive episodes. However, since studies of the GR in the human brain are not feasible in vivo, such evidence mainly derives from pharmacological challenge tests with GR agonists, like the dexamethasone suppression Test, which indirectly measures GR function in the pituitary and maybe in part also in the central nervous system (96). Therefore, the analysis of direct effects of GR dysfunction and its potential role for “emotional behaviour” are currently restricted to animal models. For this purpose, targeted mutagenesis in mice seems to be particularly promising, since it allows the genetical manipulation of both GR expression and specific GR functions (81).

Several mouse strains with impaired GR expression or function have been generated: i) mice with a GR point mutation (GR$^{dim}$), which prevents GR dimerization and binding to its cognate element GRE (176); ii) a strain with a brain-specific GR knock-out (GR$^{NesCre}$) (216); and iii) a GR-antisense model with reduced expression in brain and some peripheral tissues (171). While GR$^{dim}$ mice do not reveal any alterations in emotional behaviour, both brain-specific GR knock-out mice as well as GR-antisense mice surprisingly exhibit reduced depression-like behaviours. However, with respect to human (psycho-) pathology, the genetic defects generated in these mouse strains are very unlikely to occur in man. To test the “GR-Hypothesis of depression” we decided to work with mice that over- or underexpress GR, which may – according to the concepts outlined above - mimic the situation of patients with affective disorders more closely. Such mice have been generated by a knock-out strategy (GR$^{+/}$ mice; (217)) and by a transgenic approach using a yeast artificial chromosome (YGR mice; (179)). So far, these strains have not been characterized behaviourally. According to the theory, one would expect mice, which underexpress GR to have a predisposition for developing depression-like behaviours after stress. Furthermore, they should show a depression-like response in the Dex/CRH Test. In this test, the synthetic glucocorticoid dexamethasone is administrated to challenge the negative feedback function of the HPA system (11). On the other hand, the Glucocorticoid Hypothesis of Depression would predict that mice overexpressing GR are more resistant to develop depression-
like features than wildtype mice. To test these hypotheses, we subjected GR\(^{+/−}\) and YGR mice to a large test battery for emotional behaviours, including tests for depression-like signs such as despair and Helplessness. Furthermore, we analysed the HPA system under baseline conditions, after stress, and following a Dex/CRH challenge.

**Figure 3: The Stress Hypothesis of Depression.** This hypothesis declares that stress via an activation of glucocorticoid receptors by glucocorticoids leads to a decrease of neurotrophins (red arrows), as predicted by the Neurotrophin Hypothesis of Depression. Moreover an activation of those receptors challenges the hypothalamus pituitary adrenal axis (HPA) with increased levels of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and corticosterone (red arrows), which are regulated by a negative feedback system. In view of the fact that these hormones are dysregulated in depressive patients but normalized by antidepressive treatment (green arrows) the HPA axis is suggested to play a crucial role for the pathophysiology of depression which may be linked to the dysregulation in the neurotrophin circuit.
1.1.5 Neurogenesis

The proliferation of so-called progenitor cells and their differentiation (neurogenesis), which is controversially discussed to be a major factor in the pathogenesis of depression (101) takes place in the hippocampus, a part of the limbic system, and in the olfactory bulb. Since neurogenesis represents an important biological correlate for plasticity in the nervous system (88), a new hypothesis arose, which assumed that genesis of neurons is involved in the pathogenesis of affective disorders (23, 111). This is indicated by the fact, that depression-evoking aspects, such as stress and an elevation of glucocorticoids, cause a reduction of neurogenesis in the hippocampi of mammals and a decrease of serotonin levels, which may be transmitted by an activation of glucocorticoid receptors (GR). Conversely, the rate of neurogenesis in the rat is increased by antidepressive therapy, which causes an elevation of the serotonin concentration in the synaptic cleft (30). An alternative method to increase neurogenesis is the behavioural stimulation of animals by means of physical activity (running wheels) or housing in an enriched environment. Mouse models for depressive diseases open the possibility to mimic the molecular mechanisms of neurogenesis by means of targeted mutation of genes to investigate their respective relevance for affective disorders. First, several genes suspected to be involved in pathogenetic mechanisms and responsible for changes in neurogenesis: BDNF (brain-derived neurotrophic factor), TrkB, its tyrosine kinase receptor and the type I and II glucocorticoid receptors GR (glucocorticoid receptor) and MR (mineralocorticoid receptor) as well as the recently described endothelial NOS III (nitric oxide synthase).

1.1.6 The Macrophage Theory of Depression

An interaction between immune system and mood disorders has been hypothesized for more than 70 years, however, it is still unclear whether immune system changes are just a reflection of the stress-induced pathological state or if they are primarily responsible for the modifications in central neurotransmission that induce behavioural alterations (128). A variety of immunological aspects have been established as valuable markers of depression (128). Moreover, the links between HPA axis and immune system are numerous and have led to the so-called macrophage theory of depression (203). In particular, cytokines are considered as potential triggers of major depression (5, 28, 124). The relationship between mood disorders and immune system are evidenced on three levels (5): i) Administration of cytokines or activation of the immune system induces depression and physiological alterations similar to those observed in depression (39, 47). The role of cytokines in psychopathologies has been discovered by clinicians, who treated cancer patients with cytokines to enhance their immune system. After several weeks of treatment these patients exhibited a variety of psychiatric disorders, including depression (28). Consistently, cytokine antagonists have been shown to prevent the
development of Learned Helplessness in rats. ii) Major depression is accompanied by an immune response with an increased activation of several components of the immune system. It is hypothesized that increased monocyctic production of interleukins in severe depression may constitute key phenomena underlying the various aspects of the immune and "acute" phase response, while contributing to hypothalamic-pituitary-adrenal (HPA) axis hyperactivity, disorders in the serotonin metabolism, and to the vegetative symptoms (*i.e.* the sickness behaviour) of severe depression (138). iii) Successful antidepressant treatment normalizes the immune system activation in humans (190, 200), and attenuates the behavioural and neuroendocrine effects of immune activation in rodents (251). However, it is not known whether the effect of antidepressants on immune function contributes to the efficacy of the treatment or is just an epiphenomenon.

1.2 Animal Models of Depression

At present animal models represent the unique possibility to gain insights into the pathophysiology of depression because the examination of stress effects and accordingly the inspection of brain tissue may not be done in patients. Several animal models of depression have been described (for review see (238)), but only a small fraction is capable to fulfil predictive, face, and construct validity, according to their quality of mimicking the situation of depressive patients. Some of the traditional models and tests methods were rejected as invalid; the models and/or respective tests with the highest overall validity are the intracranial self-stimulation, chronic stress, Learned Helplessness in rats, and primate separation models (238). An ideal model for human mental disorders is thought to be applicable if it resembles the condition it models in its aetiology, biochemistry, symptomatology, and treatment (148), however it has to be noted that validating criteria themselves may be subject of intense research and speculation. Therefore face validity describes the phenomenological similarities between the model and the condition modelled, predictive validity describes the success of predictions made from the model, and construct validity reflects its theoretical rationale. The following paragraphs introduce five approaches by which depression could be modelled and illustrates examples for each possibility to induce a depressive state. Signs of depression described in the human “Diagnostic Statistical Manual IV” that can so far be modelled by current animal analyses are listed in the catalogue below:
1.2.1 Lesion Models

As an example for lesion models, the bilateral destruction of the olfactory bulbs creates a chronically altered brain state with complex changes of behavioural, neurochemical, neuroendocrinological, and neuroimmunological parameters, many of which are comparable to those seen in patients with major depression (46, 112, 135, 227). Since such a lesion does not naturally occur in humans, the face validity of this model is not very strong, nevertheless it can be used to induce a depressive-like state that can be reversed by chronic antidepressive treatment and can therefore serve to deal with basic questions regarding the pathophysiological background, bearing the advantage of being a high-throughput model of depression. Thus, the olfactory bulbectomy (OBX) in rodents has been proposed to represent a model for chronic psychomotoric agitated depression, which also has a high predictive validity (45, 46, 95). The major behavioural change in this model is a hyperactive response in a brightly illuminated Openfield arena (256), which is reversed almost exclusively by chronic, but not acute, antidepressant treatment (117, 129). Furthermore, in rats bulbectomy leads to different signs of anhedonia, combined with deficits in spatial learning, avoidance learning, conditioned taste aversion, and food-motivated behaviours (95, 117). While the olfactory bulbectomy (OBX) model has been intensely investigated in rats, fewer studies were conducted with mice. Indeed it has been cautioned to overextrapolate the data obtained in rats to mice (46). Apart from the characteristic hyperlocomotion, in mice OBX induces deficits in active and passive avoidance learning (93, 108, 164, 213, 256) and in spatial memory (19, 20, 256).
1.2.2 Chronic Mild Stress

The model of “chronic mild stress”, a model of depression with good face, predictive, and construct validity, states that stress, which cannot be controlled, may lead to a depressive state (240). Besides its time-consuming experimental protocols, however, the problem persists that the model cannot constantly be reproduced adequately, not even by the same experimentators, which reduces its reliability, and restricts its possible applications in studies that require high throughput. The chronic stress model involves mild stressors that are applied for several weeks, e.g. wet chow, changes in their social constellation, short periods of food deprivation or altered illumination during activity-and resting periods, respectively (48). Anhedonia, which is the major readout of this concept, is assessed by means of measuring the preference for a 2 % sugar solution versus water in stressed and control animals. In addition to the anhedonic state, decreases in locomotion, body weight, libido, as well as disturbances in the sleep structure are reported, which are comparable to symptoms in human depression (241). Those changes can be reverted by chronic antidepressive treatment, which makes this model an excellent tool for an adequately naturalistic induction of a depressive state, which is however restricted by the fact that it is endangered by artefacts and therefore not always reliable.

In this thesis, one category of chronic mild stress and its effects on the development of a depressive-like phenotype in the Learned Helplessness paradigm was investigated by analysing the influence of different, commonly applied housing conditions that could represent potential stressors regarding this paradigm.

1.2.3 Learned Helplessness

In 1976 the concept of the Learned Helplessness was created by Seligman and Meier, who hypothesized that exposure to unpredictable and uncontrollable stress may lead to a depressive state (191). This hypothesis has initiated an epoch of florid discussions regarding the aetiology of depression, since it is true for animals as well as humans, that only a part of the individuals exposed to stress develop depressive features or Learned Helplessness. Hence a hypothesis was postulated assuming that humans with a stable style of attribution regarding negative experiences are vulnerable for depression (1). In the original version of the model, rats are exposed to uncontrollable footshocks and are tested the following day concerning their ability to terminate the shocks by pressing a lever. Animals without preceding stress exposure learn promptly how to avoid the aversive stimuli, while stressed animals show a passive coping style demonstrating less motivation to stop the aversive stimulus (66). Like the chronic mild stress, the model of Learned Helplessness produces signs characteristic for a depressive disease, such as cognitive deficits, loss of appetite, libido, and locomotion, as well as alterations in sleep structure (2). Those changes are reversed by chronic
antidepressive treatment with substances from different classes as well as by electroconvulsive therapy (ECT), while other psychopharmaceuticals like antipsychotics or benzodiazepines, are not effective (196). It is assumed that corticosterone plays a major role in this model. This is supported by the fact that helpless animals present typical endocrinological changes, e.g. decreased suppression of corticosterone after application of dexamethasone (91). Moreover it is concluded, that secretion from the adrenal cortex is necessary for the incorporation of a learned response after stress and that a dysregulation of the hypothalamic-pituitary-adrenal axis seems to be involved in helpless behaviour (65, 100). Furthermore several neurobiological alterations were identified, for example a stable increase of β-adrenergic and serotonergic receptors and the serotonin reuptake transporter (SERT) in the hippocampus (142). These changes may be converted by antidepressants (100). While there are numerous studies on the subject of Learned Helplessness and its mechanisms in rats, in mice this procedure remains rather unexploited.

1.2.4 Genetic Approaches

The genetic approach permits to study the role of target genes, which are postulated to play a role in the pathogenesis or treatment of depression, in vivo. The deletion and overexpression of such genes of interest is especially attractive with regard to the search for the pathophysiological genetic fundaments of the vulnerability to depression and how this can be manipulated by environmental factors. Hereby transgenic mice represent the major tool since currently the mouse is the main specimen in which transgenic techniques are practicable. In the following section mutations of relevance concerning pathophysiological background of depression will be described.

1.2.4.1 BDNF Heterozygous Mice (BDNF+/−)

Recent studies propose the CREB-BDNF-TrkB pathway to be relevant regarding pathogenesis and therapy of depression (3, 223). While stress compromises the activity of this signalling cascade (158), antidepressants activate it (31, 57, 123, 158, 159). Likewise, BDNF-infusion into the hippocampus exerts antidepressive effects (197, 199). A conventional BDNF knock-out leads to severe developmental defects that are incompatible with survival to adulthood (40). Behavioural experiments are therefore restricted to heterozygous mice (BDNF+/−) or mice with a conditional knock-out.
1.2.4.2  CREB \textit{Ser}^{142A} Mutant Mice

Expression and activation of CREB (cAMP response element-binding protein) have been implicated in the molecular mechanisms of pathogenesis and therapy of affective disorders (155, 212). Thus, human \textit{postmortem} studies demonstrate decreased CREB levels in temporal lobes of untreated depressive patients (29, 57, 187). In animal models chronic antidepressive treatment increases hippocampal expression/activation of CREB (159, 215). Furthermore, virus-mediated hippocampal CREB overexpression produces antidepressant effects in Forced Swim Test and Learned Helplessness (31). CREB is activated by cellular signalling pathways acting via cAMP- or Ca\textsuperscript{2+}-activated kinases or tyrosine kinases. This activation is achieved by phosphorylation of CREB at two different serine residues (\textit{Ser}^{133} (152) and \textit{Ser}^{142} (144)), resulting in CRE-mediated transcription of genes, \textit{i.e.} brain-derived neurotrophic factor (BDNF). Transgenic studies showed that CREB activation by \textit{Ser}^{142} phosphorylation reflects a mechanism regarding synchronization of day-night cycle and circadian rhythm (83). Indeed, CREB is involved in light-induced entrainment of the circadian clock in the suprachiasmatic nucleus (SCN) and is proposed to draw a molecular interface between neuronal and endocrine cues resetting this clock (55, 232). This is especially relevant concerning the role of CREB, since disrupted biological rhythms represent a hallmark of depression (155, 212). Thus, depressive patients show disturbances of their sleep/wake cycle and/or circadian rhythms, \textit{e.g.} body temperature, cortisol secretion etc. \textit{Vice versa}, sleep deprivation, phase advance of circadian rhythm and light-therapy represent therapeutics alleviating depressive symptoms (115, 244). In seasonal affective disorder (SAD) (168), the efficacy of light-treatment and the seasonality of symptoms suggest a circadian pacemaker. This implies that CREB \textit{Ser}^{142} phosphorylation could be involved in the development of depressive symptoms.

1.2.4.3  Glucocorticoid Receptor Mutant Mice and Current Implications

Glucocorticoid receptors (GR, or type II corticosteroid receptors) and mineralocorticoid receptors (MR, or type I corticosteroid receptors) are ligand-dependent transcription factors. They belong to a superfamily of nuclear hormone receptors. The GR is expressed ubiquitously in the brain. In the HPA system, GR plays a key role in the feedback regulation of glucocorticoid hormones. Its absence is expected to lead to hypercortisolism, mimicking the HPA system disturbances observed in humans with severe depression. Several mouse lines with an altered expression of the GR have been generated: i) two different conventional knock-outs, ii) mice overexpressing an antisense of the GR mRNA, iii) nervous system-specific knock-outs, iv) mice in which the GR dimerization has been disrupted, v) mice overexpressing GR, and vi) mice with conditional overexpression of the GR. HPA system
dysregulation and behavioural symptoms in mice with targeted mutations of GR and MR have been reviewed earlier (81, 153, 223).

**GR-antisense:** Chronic stress leads to a desensitisation of the GR, impairing the negative feedback loop of the HPA axis, which, by a vicious circle results in hypercortisolemia in humans. Reduction of GR expression in mutant mice by overexpressing an antisense GR gene leads to similar dysfunctions (170). GR antisense mice show a reduced CRF expression in the hypothalamus but no changes in ACTH and corticosterone levels at various time points of the circadian rhythm (11, 53, 113). The expected up-regulation of the HPA system in GR antisense mice only becomes apparent under stressful conditions (170, 171) and is reversed by antidepressant treatment (10, 151). Moreover, GR antisense mice fail to respond adequately to the dexamethasone suppression Test, which is in agreement with observations in depressed patients (11). On the behavioural level, GR antisense mice present several cognitive deficits (151, 185). Surprisingly, they demonstrate less anxiety-like behaviour in the Elevated Plus-Maze (151, 182, 211) and following intense psychological stress (*i.e.* rat exposure) (132). Moreover, they display locomotor hyperactivity when placed into a novel environment (14) and they exhibit enhanced responses to novelty and increased conditioned approach responses, again in contrast to the predictions of the theory (207). Furthermore, these mice are less immobile than wildtype control mice in the Forced Swim Test (151), which also suggests a decreased depression-like behaviour. Taken together, GR antisense mice display only few of the neuroendocrine and behavioural features observed in depression. However, since many of the deficits observed in these mice are reversed by antidepressant treatment, this line may be a valuable tool for the discovery of new antidepressants (151).

**GR\textsuperscript{NesCre}:** Since conventional GR knock-out mice do not survive to adulthood, nervous system specific knock-out mice have been generated using the Cre/loxP recombination system under the control of the rat nestin promoter (216). The so-called GR\textsuperscript{NesCre} mice are viable and lack the GR in neurons and glial cells. As one would expect, their HPA system is drastically over-activated (216) but is still responsive to acute immobilization stress, resulting in increased levels of both circulating ACTH and corticosterone. These mice thus display neuroendocrinological alterations that closely resemble those of patients with a major depression. At the behavioural level, however, GR\textsuperscript{NesCre} mice enter the aversive compartments of both the Dark-Light Box and the elevated O-Maze more often, thereby displaying less anxiety-related behaviour (216). Additionally, they do not develop despair-like behaviour in the Forced Swim Test, contrarily to what would be expected in a depression model (216). This paradoxical finding can be explained by the fact that the neurons of GR\textsuperscript{NesCre} mice do not express
the GR. Consequently the hypercortisolism cannot affect these neurons and cause subsequent changes in behaviour. Thus, despite their hypercortisolism, these mice most likely represent a genetic model of resistance to depression. To test this hypothesis, they have to be subjected to stress-induced behavioural depression paradigms.

\textbf{GR}^{\text{dim}}: \text{GR} \text{ regulates transcription by two major mechanisms: i) as dimer, binding to positive and negative GREs in the promoter of target genes; ii) as monomer, modulating the activity of other transcription factors via protein-protein interactions (176). The two modes of action can be dissected by introducing a point mutation (A458T) into one of the dimerization domains of the GR (97). Using a knock-in strategy to replace the endogenous GR gene, this mutation was generated in mice (176). These so-called GR^{\text{dim}} mice express GR molecules that cannot dimerize, but still act as monomers. Consequently, GR^{\text{dim}} mice cannot activate GRE driven genes, but are still able to modulate other transcription factors, \textit{e.g.} AP-1 and NF-\kappaB (176, 218). In contrast to mice carrying disrupted alleles of GR, GR^{\text{dim}} mice are viable and can be used to study physiology and behaviour in adulthood (176). While CRF levels in the hypothalamus are normal in these mutants, ACTH and corticosterone plasma levels are markedly elevated compared to control mice, indicating that the mechanism of protein-protein interactions are important for the negative feedback at the level of the hypothalamus (176). Behaviourally GR^{\text{dim}} mice do not differ from controls regarding locomotion and exploration, but display spatial memory deficits in the Morris Watermaze (162). Neither anxiety-related behaviours nor immobility in the Forced Swim Test are affected in this line (162). This result indicates that this approach, even if it can be used to dissect the modes of action of GR, does not represent a suitable model of depression.

\textbf{GR}^{\text{+/−}}: \text{The GR is essential for survival: its disruption leads to death immediately after birth due to severe atelectasis of the lungs (216). A conventional knock-out of the receptor, obtained by insertion of a neomycin cassette into exon 2 of the GR gene, also results in an impaired lung development and causes death in more than 90 \% of the newborns because of respiratory failure (37). The founders of GR heterozygous mice (GR^{\text{+/−}}) were developed by using homologous recombination in embryonic stem cells. Homozygous knock-out mice show enhanced transcription of both CRH in the hypothalamus and proopiomelanocortin (POMC) in the anterior lobe of the pituitary, as well as elevated corticosterone plasma levels. Even though the HPA axis dysregulation in these mutants is similar to the alterations seen in depressed patients, it is difficult to speculate about their potential role as a model of depression, since the surviving individuals have not yet been tested behaviourally.}
YGR: Overexpressing a gene is another strategy to study its role in pathophysiological mechanisms. GR overexpression in mice has been achieved by insertion of two additional copies of the GR gene using a yeast artificial chromosome (YGR) (178). These mice overexpress GR mRNA by 20 to 25 %, and GR protein by 50 %, and display a strong suppression of the HPA system (178). Overexpression of GR can also be obtained conditionally by putting the gene under the control of the forebrain specific promoter calcium calmodulin dependent kinase II (CaMKII) (236). In this line, the HPA system is not affected and the animals show normal locomotor activity, while displaying more anxiety in the Dark-Light Box Test.

1.2.4.4 Interleukin-6 Receptor Mutant Mice (IL-6-/-)

According to the macrophage theory, IL-6 knock-out mice (125) represent an interesting animal model of depression. They display an increased locomotion in the Openfield (26) and spend less time in the open arms of the Elevated Plus-Maze (8, 26). IL-6-knock-out female mice show a blunted corticosterone response to restraint stress. For males however, the stress-induced corticosterone response is comparable to wildtype mice. This result suggests that the dimorphic HPA axis response to stress may involve IL-6 activation (16).

1.2.4.5 Endothelial Nitric Oxide Deficient Mice (NOS III)

The generation of new neurons in the adult brain has been increasingly within the last years (118). On the functional level adult neurogenesis (119) has been postulated to be involved in the pathogenesis of depressive disorders and/or mediation action of antidepressants (188). So far, little is known about the mechanisms that regulate neurogenesis. Stress, age, physical exercise, as well as a variety of messenger molecules, transcription-, and growth factors have been implicated (62). The gaseous messenger molecule nitric oxide (NO) is synthesized from L-arginine by a family of three NO synthases (NOS), nominated NOS I, II, and the endothelial NOS III. NO exerts multiple actions in the central nervous system and is involved in behavioural processes such as learning and memory (204), which makes it an interesting factor to be investigated regarding neurogenesis. The neuroprotective role of NO was demonstrated by the finding that an inhibition of NOS I increased cell death in the dentate gyrus after adrenalectomy (167). In the human as well as in the rodent dentate gyrus, the main site of adult neurogenesis, NOS I and NOS III have been detected and NOS III is expressed in endothelial cells and probably in CA 1 pyramidal cells (161, 166). Since NOS III therefore
might have the spatial potential to regulate adult neurogenesis, it seemed interesting to analyse the behaviour in these mice as well (180).

1.3 Current Treatment Concepts of Depression
Antidepressants were discovered by serendipity half a century ago, when it was observed in 1954 that some tuberculosis therapies exerted a beneficial effect for the well-being of the treated patients (18, 193). According to these results, iproniazid, a member of the monoamine oxidase inhibitor (MAO-I family), became the first antidepressant (134, 252). At the same time, imipramine was found to exert positive effects in depression, exposing a completely new approach: the monoamine theory of depression (25, 42, 189). However, the effectiveness of antidepressants cannot be explained sufficiently by neurotransmission theories. Molecular-pharmacological studies demonstrate that, besides the effects in synaptic neurotransmission, antidepressive treatment influences signal transduction cascades and gene transcription mechanisms, thereby regulating the expression of particular target genes (157). Those genes control molecules, which are responsible for synaptic plasticity, i.e. chronic, but not acute treatment with antidepressants influences the cAMP messenger system and elevates the expression of neurotrophic factors, e.g. BDNF, which is known to play an important role in neuronal plasticity, survival and function. Decreased BDNF expression as a consequence of stress may play a role in stress-induced neuronal damage, which is accompanied by neuronal atrophy or apoptosis in hippocampal CA3 neurons (146). Besides an increase of BDNF, an elevation of its tyrosine kinase receptor TrkB can be observed within the hippocampus. Moreover, several classes of antidepressants (selective norepinephrine and serotonin reuptake inhibitors, non-selective tricyclic monoamine re-uptake inhibitors and MAO inhibitors) as well as electroconvulsive therapy up-regulate the expression of CREB mRNA in the hippocampus (159). This suggests the transcription factor CREB, that is down-regulated in the cortex of depressed patients (57) to serve as a common target for antidepressant treatment with very different primary sites of action. It is induced after approximately 10 days of treatment, which is in line with the onset of effectiveness of antidepressive therapy (159). Support for the hypothesis that enhanced cAMP signalling may be induced by antidepressants, derives from studies of the phosphodiesterase inhibitor rolipram, which has been reported to have antidepressive effects in clinical trials (224). Furthermore, antidepressive treatment can enhance neurogenesis in the hippocampus (214). Stress represents an important risk factor with regard to psychiatric disorders but often exerts opposite effects. Antidepressants, which affect on glucocorticoid receptor functioning, affect not only the peripheral stress hormone system which is normalized by antidepressive pharmacotherapy; therefore current strategies for developing
antidepressants with high efficacy and good compatibility depend on an improved knowledge about the molecular, cellular, and behavioural effects of stress (210, 214).

The chronic application of antidepressants causes a decrease in ACTH and corticosterone under basal and stress conditions. When analysed in a time course, an alteration of GR and MR functioning first became detectable after one week of treatment (169). According to the suppressive activity of the MR on the stress hormone system, which was characterized by MR antagonists and antisense studies, the elevation of MR appears to be the first step towards the inhibition of CRH in the hypothalamus. Subsequently an enhanced capacity of GR transcription can be observed during antidepressive therapy (105).

**Figure 4: Point of action in antidepressive therapy.** Antidepressants exert their mood-elevating effects by different mechanisms, *i.e.* inhibition of serotonin and norepinephrine re-uptake (SSRI/NRI), inhibition of degradation by blocking MAO A activity or increasing the neurotransmitters in the synaptic cleft. Chronic treatment with antidepressants causes an elevation of CREB, thereby regulating all factors of the neurotrophic pathway (*i.e.* BDNF and TrkB), as well as a CREB-enhanced synaptic function and release of monoamines.

### 1.4 Conceptual Considerations

Since there is an enormous demand on behavioural phenotyping of transgenic mice carrying mutations of target genes relevant for research of human diseases, it is important to improve tools for proper behavioural analysis. The adequate design of animal models, also considering limitations and distinctiveness of other types of approaches, represents a cornerstone for the clarification of postulated aspects in depression. A first goal was therefore the optimisation and adaptation of the “Learned Helplessness” paradigm to mice (in which genetic manipulations may be studied) and to embed it in a
standardized behavioural test battery for associated parameters at the behavioural and neurochemical level. Despite the need of animal models such as the Learned Helplessness, which represent valid tools for research, this model, demonstrating excellent validity, was rarely utilized during the last decades. Optimal connections between the different disciplines consequently open promising possibilities for a complex and detailed investigation of the pathophysiological and therapeutic pathways.

A major goal of generating animals by targeted mutagenesis is to elucidate the role of the genes that might be changed in (patho)physiological processes. Mutant mice are powerful tools to study specific functions of molecules such as neurotransmitters or their receptors. Apart from these fundamental objectives, mutant animals can also constitute disease models. With respect to depression, many lines of mice have been generated according to the molecular hypotheses underlying affective disorders to explore the biological mechanisms thought to be involved in the pathogenesis and therapy of depression. Using this approach successfully, two possible outcomes may be surveyed theoretically: inactivation of a gene, whose product is usually blocked by antidepressants, should lead to models of resistance to depression; vice versa inactivation of a gene, whose product is induced or activated by antidepressants, e.g. BDNF, should result in overt "endogenous depression" or in a model of susceptibility to depression. Among the lines generated, only very few may represent models of endogenous depression. A few lines most likely constitute susceptibility models of depression, i.e. they do not present depression-like symptoms under baseline conditions but are more "depressed" following environmental stress, like in the Learned Helplessness model or chronic (mild) stress (e.g. CREB over-expressing mice). Like any model, also the transgenic approach has limitations. Classical transgenic and knock-out mice carry their mutations from the very first stages of development. Thus, the phenotype observed in adulthood could be due to developmental defects or adaptations of the organism (92). Hidden side effects of a mutation may also entail differences in the animals' social behaviour, affecting the whole progeny. If mutagenesis affects maternal care behaviour, a phenotype observed in the offspring may rather reflect this environmental factor than the mutation (73). Another level of complexity is added by the fact that many behavioural findings are only present in a specific genetic background (e.g. C57BL/6) but missing when this background is lost (245, 246). In light of these deficiencies, mutant mice often serve to "confirm" hypotheses derived from pharmacological studies or clinical observations, but may also falsify such concepts.
1.5 **Aim of this Thesis**

To elucidate the role and interrelation of genetic and environmental factors, the investigations of all mice with targeted stress-related and neurotrophic candidate genes in this thesis are investigated hypotheses-conducted in essential paradigms for depression-like behaviour. Coupling behavioural with neurochemical markers of depressive behaviour serve to enhance confidence regarding the results obtained (79), which is applied for the investigation of the aspects involved in the Learned Helplessness (see Figure below).

![Figure 5: Regulation of Learned Helplessness: Potential Factors and Systems.](image)

The regulation of Learned Helplessness is driven by stressful experiences causing this phenotype, which can be reverted by antidepressive therapy. Stress, as well as antidepressants, moreover exerts controversial effects regarding neurotrophic circuits, glucocorticoid receptors, and Interleukin-6.

It is known that Learned Helplessness and depression, respectively, are influenced by different systems, which are stated in the common hypotheses of depression. To gain further insight into the relations and prove the relevance of particular factors in these systems, the subsequent questions were addressed:

- Is the Learned Helplessness paradigm of depression a stable, reproducible model regarding a depressive-like phenotype in mice, and may this be proven pharmacologically? How does this model affect BDNF and NGF protein levels when examined in a time course experiment?
Introduction

• How and to what extent does “chronic stress”, caused by a particular housing condition affect the results in the Learned Helplessness model?

• Does a heterozygous mutation of the BDNF gene lead to a depressive-like phenotype and is this phenotype accompanied by disease-specific alterations at the monoaminergic and stress-physiological level?

• Does a point-mutation of CREB, which prevents phosphorylation at this particular site, cause a depressive-like phenotype (besides the observed phase shifts as potential indicators), which is associated with changes in stress-hormone release?

• To what extent does a heterozygous mutation of the glucocorticoid receptor affect depressive-like behaviour in the Learned Helplessness paradigm, stress endocrinology, and neurotrophin levels and mimic depression-like features in the DEX/CRH Test?

• In how far can the expected changes be replicated by the over-expression of the glucocorticoid receptor?

• Is a knock-out of IL-6 capable of inducing depression-typical behavioural and stress-endocrinological parameters in the Learned Helplessness paradigm, and does the Learned Helplessness paradigm itself cause changes in IL-6?

• How does a knock-out of endothelial nitric oxide synthase (NOS III), which is suggested to be involved in the reduction in neuronal progenitor cell proliferation in the dentate gyrus, affect the behaviour in the Learned Helplessness?
2 Material and Methods

2.1 Animals
The experimental animals were generally housed individual in standard macrolon cages (type II) with bedding material and were acclimatized for 2 weeks to a reversed 12-hour dark-light cycle (light: 18:00 - 6:00) at 22±1°C. Animals received a standard pellet diet and water ad libitum. The numbers of mutant animals tested in each experiment were dependent on the availability of mice of approximately the same age. All animal experiments were approved by the animal welfare office of the Regierungspräsidium Karlsruhe, Germany.

2.1.1 Housing Conditions
All behavioural tests were conducted in 4-months old male C57BL6/N mice. The animals were purchased from Charles River (Sulzfeld, Germany) at an age of 8 weeks and were then housed under specific conditions in type II cages for single and type III cages for group rearing, for 7 weeks. Four cohorts of mice were investigated: 1.) single housed, impoverished conditions (n=16), 2.) single housed, enriched conditions (n=16), 3.) group housed, impoverished conditions (n=16), 4.) group housed, enriched conditions (n=16). Enrichment consisted of red, transparent plastic mouse igloos and tunnels (EMSICON Jung GmbH, Forstinning, Germany) that were cleaned weekly, when the cage was changed. Additionally, nesting material (tissue) was provided. Impoverished cages simply contained bedding material. All mice were kept in the same room, in a 12h:12h reversed dark-light cycle, lights on at 6.00 p.m, water and food pellets were available ad libitum. Body weight was assessed once a week when the cages were changed. All experiments were approved by German animal welfare authorities. After 7 weeks of housing under the aforementioned conditions, all animals were subjected to tests for locomotion, exploration and anxiety, followed by the Learned Helplessness procedure. Thus, following earlier recommendations for repetitive behavioural testing, animals were initially tested in the experiments ranked as least stressful (147, 226). Considering that the determination of the pain threshold by the Hotplate Test could have a direct influence on helpless behaviour, we assessed the effect of housing conditions on pain sensitivity in a different group of mice reared under identical housing conditions. Test procedures were essentially performed as described earlier (33, 71, 180). Between the individual tests was a pause of at least 24 h. Prior to each test, mice were acclimatized to the experimental room for at least 30 min. All behavioural tests were conducted during the dark cycle, i.e. during the animals' active phase.
Material and Methods

Figure 6: Housing conditions. Mice are housed in different conditions implying an enriched environment and different social constellations for seven weeks prior to the behavioural assessment of housing effects.

2.2 Genetic Approaches

Animals carrying diverse mutations of target genes, which are postulated to be involved in pathological mechanisms of depression, were monitored with regard to depressive-like behavioural and physiological features to verify their compatibility to represent an animal model for depression.

2.2.1 BDNF Heterozygous Animals (BDNF+/−)

BDNF+/− male mice on a mixed C57BL/6 x SV129 background were supplied by the Jackson laboratories (Bar Harbor, Maine, USA) and used for breeding. They were crossed to C57BL/6N female mice obtained from Charles River (Sulzfeld, Germany). All experiments were performed with offspring of this breeding. For all experiments only female BDNF+/− mice were used, and BDNF+/+ female littermates served as controls. The age of the experimental animals was between 3 and 5 months. For genotyping, 3 mm tail segments were taken for polymerase chain reaction (PCR) at about
Material and Methods

4 - 6 weeks of age. A conventional tail prep kit (DNeasy tissue Kit, Qiagen GmbH, Hilden, Germany) was used to prepare the DNA for PCR, which was conducted according to the reference provided by the Jackson Laboratories.

2.2.2 CREB Ser142A Mutant Animals

CREB<sup>S142A</sup> mice were generated and bred as described elsewhere (83). For the present study animals were backcrossed for more than 10 generations into the C57BL/6N background. 3-6 months old male CREB<sup>S142A</sup> mice were purchased from the German Cancer Research Centre (dkfz) in Heidelberg. Genotypes were determined by PCR as described earlier (83).

2.2.3 Glucocorticoid Heterozygous Animals (GR<sup>+/−</sup>)

The founders of GR heterozygous mice (GR<sup>+/−</sup>) were developed by using homologous recombination in embryonic stem cells as described elsewhere (216). For all experiments three months old male mice, provided by the German Cancer Research Centre (dkfz) in Heidelberg, were used. Mice were bred as F1 hybrids from two commonly used inbred strains, C57BL/6 and FVB/N (backcrossed for more than 10 generations), to ensure the so-called hybrid vigour.

2.2.4 Glucocorticoid Overexpressing Animals (YGRs)

Furthermore, so-called YGR mice were investigated, which carry two additional copies of the GR, generated by a transgenic approach using a yeast artificial chromosome (177). In order to obtain an F1 hybrid background in YGR mice, double transgenic male YGR founders on a pure FVB/N background were mated with female C57BL/6N wildtype mice. All animals were between 3-6 months of age when exposed to the tests.

2.2.5 Interleukin-6 Knock-out Animals (IL-6<sup>−/−</sup>)

The IL-6-deficient mice (125) had been back-crossed on a C57BL/6 genetic background for more than 10 generations. For genotyping, 3-mm tail segments were taken for tissue samples for polymerase chain reaction (PCR) at about six weeks of age. A conventional tail prep kit (DNeasy Tissue Kit, Quiagen GmbH Hilden, Germany) was used to prepare the DNA for the PCR, which was conducted according to the recent publication of Butterweck <i>et al.</i> (26).
2.2.6 Endothelial Nitric Oxide Synthase Knock-out Animals (NOS III⁻/⁻)

Male 3-5 months-old homozygous NOS III knock-out mice (n = 10) with the same genetic background (for review see (109)) and wildtype controls (n = 10), obtained from the Molecular and Clinical Psychobiology, University of Würzburg, were investigated.

2.3 Behavioural Analysis

2.3.1 Basal Behaviour

All behavioural tests were conducted during the dark cycle, *i.e.* in the animals' active phase. Prior to each test, mice were acclimatized to the experimental room for at least 15 min. Test procedures were essentially performed as described earlier (33, 71). The animals were subjected to several basal tests of locomotion, exploration, and anxiety as well as depression-relevant paradigms such as the Porsolt Forced Swim Test, Fear Conditioning, and Learned Helplessness. The order of the tests followed earlier recommendations ranking the tests from least stressful to more stressful (147, 226). Between individual tests was a pause of at least 24 h. In all experiments, the investigator was blind to the genotype of the mice during behavioural testing.

2.3.1.1 Motoric Abilities

The rotarod Test assesses motoric abilities of mice placed onto a rotating rod by evaluation of the latency to fall within maximal 300 s when the velocity of the rotation is increasing.

![Figure 7: Rotarod Test](assessment of locomotor abilities on a rotating rod)

2.3.1.2 Openfield

The Openfield Test examines the locomotor- and explorative features of an animal placed into an unknown arena. Activity monitoring was conducted in a square, white Openfield, measuring 50x50...
Material and Methods

cm\(^2\) and illuminated from above by 25 Lux. Mice were placed individually into the arena and monitored for 15 min by a Video camera (Sony CCD IRIS). The resulting data were analysed using the image processing system EthoVision 2.3 (Noldus Information Technology, Wageningen, the Netherlands). For each sample, the system recorded position, object area and the status of defined events. Parameters assessed for the present study were total distance moved, velocity, and time in centre, which was defined as the area 10 cm distant from the walls.

![Figure 8: Openfield Test](image)

**Figure 8: Openfield Test**

Videotracked assessment of locomotor and exploratory features of mice placed in an unknown Openfield arena

### 2.3.1.3 Novel Cage

The Novel Cage Test is used to investigate exploratory behaviour in a new environment by measuring vertical activity. Animals were placed in a new macrolon cage, with a thin layer of bedding material as described earlier (180). Rearings were counted for 300 s under dimmed redlight conditions.

![Figure 9: Novel Cage Test](image)

**Figure 9: Novel Cage Test**

Assessment of exploratory features of mice placed in an unknown new cage, indicated by number of rearings
2.3.1.4 Barrier Test

The Barrier Test represents an alternative measure for the exploratory drive of mice. This test was performed in a plastic macrolon cage type II inside the animals’ housing rooms. The cage was equipped with a 1 cm high plastic barrier separating the cage into two equal-sized compartments. The mice were placed into the centre of one section following a random order. The latency (max. 300 s) to climb over the barrier was interpreted as a measure of exploration.

![Figure 10: Barrier Test](image)

Assessment of exploratory features of mice by assessing the latency until the mouse crosses a hurdle to get into the other section.

2.3.1.5 Dark-Light Box (DLB)

In the Dark-Light Box, animals can be investigated in terms of anxiety to explore an aversive bright compartment. The Dark-Light Box consisted of two plastic chambers, connected by a small tunnel. The dark chamber measured 20x15 cm² and was covered by a lid. The other chamber, measuring 30x15 cm², was white and illuminated from above with an intensity of 600 Lux. Mice were placed into the dark compartment and latency to first exit, number of exits, and total time in the light compartment were recorded for 300 s.

![Figure 11: Dark-Light Box Test](image)

Assessment of exploratory drive and anxiety-like behaviour during bright illumination based on an approach-avoidance conflict.
2.3.1.6 O-Maze

In the O-Maze mice are analysed regarding anxiety to enter the elevated, exposed sections of a round maze, inflicting an approach-avoidance conflict. The maze consisted of a grey plastic annular runway (width 6 cm, outer diameter 46 cm, 50 cm above ground level), covered with black cardboard paper to prevent mice from slipping off the maze. Two opposing 90° sectors were protected by inner and outer walls of grey polyvinyl (height 10 cm). Animals were placed in one of the protected sectors and observed for 300 s. The maze was illuminated with 25 Lux. The following parameters were analysed: latency to first exit, number of exits to, and total time spent in the open compartments.

![Figure 12: O-Maze Test](image)

Assessment of anxiety-like behaviour in mice confronted with an aversive situation on the unprotected and elevated sections of the maze

2.3.1.7 Marble-Burying (Defensive Burying)

In the Marble-Burying Test, an anxiolytic-evaluated test to assess anxiety-like behaviour in rodents (160), the mice were placed into a standard macrolon cage type III containing 5 cm of compactly compressed sawdust bedding covered with 20 conventional glass marbles (diameter: 1.5 cm) for 30 minutes in red illumination. Since mice show an aversion when exposed unknown reflecting objects and crave to hide them, the number of buried marbles was assessed as an indicator of defensive behaviour.

2.3.2 Learning

2.3.2.1 Fear Conditioning

Fear Conditioning was done as described previously (82) and represents a type of emotional learning that is hippocampus- as well as amygdala-dependent. For both, contextual and cued conditioning, mice were individually placed into the conditioning chamber (58x30x27 cm³, TSE, Bad Homburg,
Material and Methods

Germany) and allowed to habituate for 120 s, before the onset of a discrete conditioned stimulus (2800 Hz tone; 85 dB) that lasted 30 s. At the end of the tone, animals were subjected to the unconditioned stimulus (2 s of continuous footshock of 0.8 mA). 24 h after training, the hippocampus-dependent context conditioning was assessed by measuring freezing, defined as a complete lack of movements apart from respiration. Context learning was tested in the same plexiglass chamber that was used during the training. Freezing behaviour was scored at intervals of 10 s for 300 s. The amygdala-dependent cued conditioning was analysed in a visually and olfactorically novel context at 48 h after training by exposing the animals to the tone for 3 min, during which freezing was scored as described above.

![Figure 13: Fear Conditioning](image)

Assessment of Fear Conditioning in cued and context conditions after presentation of an aversive stimulus in the Fear Conditioning chamber

2.3.2.2 T-Maze

The T-Maze Test is a spatial short-term working memory paradigm, analysing the animals’ ability to recognize and differentiate between a new unknown and a familiar compartment (43, 44, 133). The T-shaped maze was made of black wood with two 20 cm long arms, which extended with a right-angle from a 40 cm long alley. The arms and the alley had a width of 10 cm and were surrounded by 25 cm high walls. The test consisted of two trials with an intertrial interval of one hour, during which the animals were put back to their home cage. During an 8 min acquisition trial, one of the short arms was closed. In a second 180 s retention trial, mice had access to all three arms. Number of visits and time spent in either of short arms were assessed. Light intensity was 20 Lux.
Material and Methods

2.3.3 Depression-linked Behaviour

2.3.3.1 Porsolt Forced Swim Test

Mice were placed into a glass cylinder (23 cm height, 13 cm diameter), which was filled with water (22°C) up to a height of 8 cm, as described earlier (255). A testing period of 6 min was used to determine the onset and the percentage of time spent immobile. Immobility was defined as motionless floating in the water, only allowing movements necessary for the animal to keep its head above the water. In contrast, swimming was defined as time spent with active escape or struggling movements. 24 h after the first testing, a re-test was performed under the same conditions as before.
2.3.3.2 Sucrose Consumption

“Anhedonia” represents one of the crucial signs of depression and a suitable behavioural indicator of a depressive-like state in rodents (209, 243). The Sucrose Consumption Test assesses whether mice demonstrate a loss of interest in consuming pleasurable sweet solutions when they have the possibility to choose between a sugar solution and plain tap water. Despite of being a valuable tool for the examination of depression, the Sucrose Consumption Test is not stable, reproducible experiment, and therefore remains restricted in its application. However, a modification of the test resulted in an improvement of the representability of the results. For 5 weeks, 5 days a week, 2 hours per day (10:00-12:00 am), each normal water bottle was replaced by two bottles containing a sucrose solution at different concentrations (0.0, 1.0, 2.5 or 5.0 %, w/v). The position of the bottles was randomised. This phase is considered as necessary training so that the animals get used to the restricted availability of sucrose in the drinking bottles. In the following 16 days, a two-bottle choice test adapted from that used by Martinetti (143) was used. Again 5 test sessions were conducted per week. Thus, all animals received a series of 16 choice conditions, 2 h per day. During these choice conditions (summarised in Table 1) two solutions were concurrently available and the consumption of each one was calculated by weighing the bottles after each session using an electronic scale accurate to 0.1 g.

![Figure 16: Sucrose Preference Test:](image)

Assessment of “anhedonia” by analysing the quantity of sugar solution consumed relative to the consumption of water.

2.3.4 Novel Object Test

In the Novel Object Test assesses potential neophobic features of animals, which may indicate a depressive-like state (209), towards an unknown object placed into an Openfield arena. The same arena and test conditions were employed as for the Openfield Test. At the end of the Openfield testing period, a new and unknown object (a water-filled 50 ml Falcon tube, placed top down) was introduced to the centre of the arena. Object exploration was assessed for 300 s, monitoring the mean distance to the object, the latency of first approach, as well as the total number of approaches.
2.3.4.1 Learned Helplessness

In the Learned Helplessness paradigm, as described by Reif et al. (180), the animals were exposed to a transparent plexiglas shock chamber (18 x 18 x 30 cm), equipped with a stainless steel grid floor (Coulborn precision regulated animal shocker, Coulborn Instruments, Düsseldorf, Germany), through which they received 360 footshocks (0.150 mA) on two consecutive days, respectively. The footshocks applied were scrambled across the rods and unpredictable, with varying shock- (1-3 s) and interval-episodes (1–15 s), amounting to a total session duration of approximately 52 min. 24 h after the second the shock procedure, Learned Helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf, Germany). The shuttle box consisted of two equal-sized compartments (18 x 18 x 30) that were separated by a small gate (6 cm wide and 7 cm high). The shuttle box also contained a grid floor, through which current could be applied, and a signalling light at the top of both compartments. Spontaneous initial shuttles from one compartment to the other were counted during the first two min by redlight beams at the bottom of each of the two divisions. Performance was analysed according to the behaviour during 30 shuttle escape trials. Each trial started with a light stimulus of 5 s, announcing a subsequent footshock of maximum 10 s duration. The intertrial interval was 30 s. The following behavioural reactions were defined (numbers of responses): avoidance as adequate reaction to the light stimulus by changing to the other compartment immediately, escapes as shuttling to the other section as reaction to the electric shock, and failures, when no attempt to escape was made. Escape latency was assessed computerized by the determination of the time (seconds) it took the animal to escape after onset of the footshock. For determination of the activity during the intervals, shuttles in between the trials were recorded. Total time of testing for Helplessness was about 20 min, the exact time period depending on the animal’s ability to learn the paradigm.
Material and Methods

**Figure 17: Learned Helplessness**
Assessment of helpless behaviour by analyzing avoidance, escapes, failures, escape latency, ITI activity considering failures and escape latency as the most relevant parameters for the definition of Helplessness.

Shock procedure:
360 shocks of 0.15 mA on 2 consecutive days 24 h prior to testing

Test procedure with 30 trials:
2 min. of free exploration (initial activity) 5 s light followed by 10 s shock (0.15 mA) 30 s intertrial interval (ITI activity)

To exclude pain sensitivity as a confounding factor, all mice were tested on the Hotplate (ATLab, Vendargues, France) at a temperature of 52°C for 45 seconds. Latency to first reaction, licking hind paws or jumping was assessed.

**Figure 18: Hotplate Test**
Assessment of pain sensitivity by analysing the latency to first reaction the Hotplate, *i.e.* licking hindpaws or jumping

### 2.4 Stress Endocrinology

#### 2.4.1 Corticosterone Determination

*Basal corticosterone levels:* To test the circadian secretion of corticosterone mice were sacrificed at respective timepoints of their active (dark) and inactive (light) phase.
Corticosterone levels after restraint stress: Mice were restrained for a period of 30 min during their light phase in plastic tunnel. 40 and 60 min after the termination of the immobilization stress plasma corticosterone levels were assessed.

![Restraint Stress](image)

The animal is restrained in a plastic tube equipped with holes to ensure oxygen delivery for 30 minutes.

2.4.2 Dexamethasone Test (Dex-Test) and Dex/CRH-Test

The mice received an intraperitoneal (*i.p.*) injection of dexamethasone (3 µg/100g body weight, Sigma-Aldrich, Schnelldorf, Germany) 6 hours prior decapitation and determination of corticosterone suppression.

6 hours after application of dexamethasone, the mice were injected i.p. with 5 µg of corticotropin releasing hormone (CRH Ferring, Kiel, Germany) and sacrificed 30 min later. All corticosterone levels were analysed by a commercial radioimmunoassay kit (ICN Biomedicals, Eschwege) as described elsewhere (255).
Material and Methods

**Figure 20: The dexamethasone suppression Test.** This test assesses a negative feedback function of the HPA axis typical for depressive individuals determining the dysfunction as a characteristic non-suppression following the challenge of the system by the synthetic glucocorticoid dexamethasone

2.4.3 ACTH Determination

ACTH serum levels were analysed using commercially available radioimmunoassay kits (ICN Biomedicals, Eschwege, Germany).

2.5 Neurotrophins and Monoamines

The determination of neurotrophins and monoamines in BDNF+/− mice, as well as the time course experiment, was done in cooperation with Prof. Dr. Rainer Hellweg and Prof. Dr. Heide Hörtnagl, Department of Psychiatry, Charité Berlin.
Material and Methods

2.5.1 Brain–Derived Neurotrophic Factor and Nerve Growth Factor

Each frozen brain tissue was homogenized by ultrasonication in 10-20 vol of lysis buffer containing 0.1 M Tris-HCl pH 7.0, 0.4 M NaCl, 0.1 % NaN3 and a variety of protease inhibitors (99) and was stored at –80°C until analysis. Endogenous NGF levels in the re-thawed homogenates were determined by a fluorometric two-site enzyme immunoassay (ELISA), which has been described in detail elsewhere (98, 99). The mean recovery of mouse NGF (125 pg/ml) added to the homogenate ranged from 60 to 90 %. NGF content was expressed as equivalents of mouse 2.5 S NGF. The detection limit of the assay was 0.25 pg/ml. Endogenous levels of BDNF were measured in the re-thawed homogenates using commercial ELISA kits in principle according to the manufacturer’s instructions (Promega Inc., Mannheim, Germany), but were adapted to the fluorometric technique also used for NGF determination as described in detail previously (33). The BDNF content was expressed as equivalents of recombinant human BDNF. The detection limit of the assay was 1 pg/ml. Each brain tissue was consecutively processed for quantification of each neurotrophin, i.e. NGF and BDNF. Determinations of recovery, specific, and unspecific neurotrophin binding (the latter against mouse IgG1 obtained from MOPC 21) involved quadruplicate fluorescence determinations for each tissue sample. The neurotrophin levels were expressed as pg/mg tissue (wet weight). In order to minimize the influence of possible variances between experiments, neurotrophin levels from treated animals were normalized as a percentage of control, and refer to those from untreated control tissues, always being included in the same experiment.

2.5.2 Time Course Experiment

To assess the time course of BDNF protein regulation after foot shock stress, the mice were sacrificed by decapitation at different time points within the period of a robust helpless state. Time points included 0, 3, 6, 12, 24 and 48 hours post shock as well as 7 and 14 days, when Helplessness is not apparent anymore.

2.5.3 Monoamines and Choline Acetyltransferase

Dissection of the brains and homogenisation procedures: Mice were sacrificed, the brains were rapidly removed, immediately frozen on dry ice, and stored at -80°C until use. Various brain areas, including hippocampus, striatum, frontal cortex, and hypothalamus, were dissected on a cold plate (-16°C) according to Franklin and Paxinos (74). The tissue samples were weighed and stored at -80°C until homogenisation. The hippocampus, striatum, and frontal cortex from the left hemisphere and the hypothalamus were used for the measurement of monoamine levels and activity of choline
acetyltransferase (ChAT). For the determination of neurotrophin levels the hippocampus, striatum, and frontal cortex of the right hemisphere were used.

Homogenisation procedure: Each frozen tissue sample of the various brain areas was homogenized by ultrasonication in 10-20 vol of deionized water at 4°C. Immediately after sonication an aliquot of the homogenate (200-300 µl) was added to an equal volume of 0.2 N perchloric acid and centrifuged at 25000 x g for 10 min at 4°C. The supernatant was used for the measurement of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), NE, DA, and homovanillic acid (HVA). The remaining aqueous homogenate was used for the measurement of the activity of ChAT.

Determination of monoamines and metabolites: 5-HT, 5-HIAA, and HVA were analysed as described previously (205) using high performance liquid chromatography (HPLC) with electrochemical detection. NE and DA were measured by high-performance liquid chromatography with electrochemical detection after extraction to alumina according to Felice et al. (70) with minor modifications (206).

Determination of ChAT activity: The activity of ChAT was determined according to Fonnum (72) with minor modifications described previously (98). ChAT activity was expressed as microunits (µU) per milligram wet weight, i.e., picomoles acetylcholine per minute per milligram wet weight.

2.6 **Interleukin-6 (IL-6)**

For the determination of shock exposure effects during the Learned Helplessness paradigm on IL-6 expression, shocked and unshocked animals were quickly sacrificed by decapitation and hippocampal tissue was dissected and frozen on dry ice. The analysis of IL-6 protein levels was done in cooperation with the group of PD Dr. Markus Schwaninger, Department of Neurology, University of Heidelberg.

2.7 **Pharmacological Experiments**

2.7.1 **Pharmacological Validation**

Especially with regard to animal models it is essential to validate that the output of what is modelled is due to the assumed basic principle. Since the examination of depression in animals is restricted by the fact that the emotional status cannot be assessed like in human conditions, one has to adduct molecular markers on one hand and well-characterized behavioural features on the other hand. Proper description of depressive-like behaviour always requires a validation with an antidepressive therapy that is effective in the human disease and capable to convert depressive symptoms.
Material and Methods

To evaluate the readout of the Learned Helplessness paradigm in mice, the animals were treated subchronically, i.e. for five days, with two different doses of imipramine (10 and 30 mg/kg bodyweight Sigma, Germany) starting one day after the shock and shuttle box procedure. Shocked animals and respective controls were retested in the shuttle box on day 6 after induction of Learned Helplessness to verify the effect of the treatment. To exclude the effect of handling and stress caused by the injection, an additional cohort of mice were included in the study receiving just saline injections. Animals that were selected for the study had to fulfil the criteria of having more than 6 failures and an escape latency higher than 4.75, which was applicable for a subgroup of approximately 30 %, a proportion that reflects fairly well the percentage of depressive patients referring to the total population. The analysis and definition of marginal values was conducted on the constantly adjusted data basis of helpless animals (for detailed description see appendix).

2.8 Statistical Evaluation

For statistical evaluation mainly parametric statistical measures were used, since distribution of values was normal in most of the cases. One and two-way ANOVAs were calculated with XLstat (Version 7.5, Addinsoft). In cases in which data were not normal distributed, non-parametric statistics were applied. A detailed description of the particular statistical analyses can be found in the Results section.
3 Results

3.1 Establishment of the Learned Helplessness Paradigm

The Learned Helplessness, originally developed in dogs, represents one of the most valuable animal models of depression with good predictive validity (242). One current approach to determine which genes are involved in vulnerability to Helplessness, is the examination of specific candidate genes of depression in mouse knock-out models (a technique, that is not applicable in rats), which makes it necessary to look for standardized reproducible protocols that incorporate not only changes caused by the disrupted gene (84), but also the consideration of background and genotype as well as consistency of housing and testing conditions. Since the mouse inbred strain C57BL/6 is one of the most frequently used strains, the establishment and characterization of the Learned Helplessness was accomplished in this particular strain. To construct a databank comprising suitable data from valid experimental procedures, the experimental design included different protocols published in literature, which were used and modified and subsequently analysed statistically. The process was conducted in male approximately 12 months old, single-housed C57BL/6N mice.

For a more precise understanding of the procedure it is essential to be aware of the relevant parameters of the paradigm, as well as the mechanisms (Table 1). Escape failures as well as escape latency are considered as the most relevant parameters for the characterization of a helpless state by defining the coping deficit most precisely.

Table 1: Learned Helplessness Parameters

<table>
<thead>
<tr>
<th>Shuttle Box Protocol:</th>
<th>Recorded parameters:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaptation: 2min</td>
<td>- initial activity (number of shuttles)</td>
</tr>
<tr>
<td>Light: 5s</td>
<td>- avoidance (shock prevention)</td>
</tr>
<tr>
<td>Shock: 10s (0.15 mA)</td>
<td>- failure (Helplessness)</td>
</tr>
<tr>
<td>ITI (intertrial interval): 30s</td>
<td>- escapes (shock reaction)</td>
</tr>
<tr>
<td></td>
<td>- escape latency (Helplessness)</td>
</tr>
<tr>
<td></td>
<td>- ITI (number shuttles between ITI)</td>
</tr>
</tbody>
</table>

3.1.1 Protocols

For the optimal output, different protocols were compared and level of Helplessness as well as the amount of animals to become helpless were considered as markers for quality concerning the practicability of the model in future experiments. The protocols compared are listed below (Table 2).
Table 2: Learned Helplessness Protocols

<table>
<thead>
<tr>
<th>Trial</th>
<th>Protocol</th>
<th>Shock</th>
<th>Shuttle Box</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Anisman, 1988</td>
<td>360×0.150mA 2sec</td>
<td>30 trials: 2 min adapt.</td>
<td>activity ↑</td>
</tr>
<tr>
<td></td>
<td>(permanent houselight)</td>
<td></td>
<td>5sec HL, 10sec shock</td>
<td>esc.lat. ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 min ITI (3 days)</td>
<td>esc. lat. ↓</td>
</tr>
<tr>
<td>B</td>
<td>Anisman, 1988</td>
<td>360×0.150mA 2sec</td>
<td>30 trials: see above (1day)</td>
<td>activity (con)↑</td>
</tr>
<tr>
<td></td>
<td>Caldaronc, 2000</td>
<td>vs scrambled (1-3 sec)</td>
<td></td>
<td>Ø other difference</td>
</tr>
<tr>
<td>C</td>
<td>Caldaronc, 2000</td>
<td>360×0.150mA scrambled (2days)</td>
<td>30trials: see above (1day)</td>
<td>scrambled shock</td>
</tr>
<tr>
<td></td>
<td>Newton, 2000</td>
<td>vs steady shock in shuttle box</td>
<td></td>
<td>most effective</td>
</tr>
<tr>
<td></td>
<td>Ukai, 2001</td>
<td>vs steady shock 0.300mA (1day)</td>
<td></td>
<td>esc.lat./failures↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(permanent darkness)</td>
<td></td>
</tr>
</tbody>
</table>

*= modified protocol  esc.lat.= escape latency  con= control  HL.= houselight  vs= versus

When evaluated in different conditions, the readout, i.e. escape deficits, of the Learned Helplessness paradigm turned out to be most effective when preceded with two days of 360 scrambled shocks/day at an intensity of 0.150mA in constant darkness (Fig. 21).

![Bar chart A: Learned Helplessness - Escape failures](chart.png)

![Bar chart B: Learned Helplessness - Escape latency](chart.png)

**Figure 21: Evaluation of three potential protocols for the establishment of the Learned Helplessness.**

Scrambled “double shock” (ds) on two consecutive days results in the most prominent escape deficits regarding the number of failures and escape latency when compared to a protocol in which the shocks are applied within the shuttle box itself (shuttle) or the application of strong shocks (strong) with equal shock duration and intervals. Bars represent mean + SEM.

The duration of Helplessness was assessed by re-testing the shuttle-box procedure at different time points after the shock procedure (Fig. 22). Due to the learning progression in the re-test the level of Helplessness decreased, but compared to control animals escape failures and latency Helplessness was still constantly detectable after 10 days post shock exposition, a period – which, related to the lifetime of a rodent - resembles the situation in humans. After 12-14 days, Helplessness starts fading in most of the cohorts examined.
Results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>shocks</th>
<th>LH 100%</th>
<th>spontaneous course 83%</th>
<th>LH 67.5%</th>
<th>LH 33.3%</th>
<th>LH 22.2%</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Time:</th>
<th>day 0, day 1, day 2, day 3, day 4, day 5, day 6, day 7, day 8, day 9, day 10, day 11, day 12, day 13, day 14</th>
</tr>
</thead>
</table>

Figure 22: Time course of coping deficits: Percentage of helpless animals. 360 footshock with an intensity of 0.150mA on two consecutive days result in significant coping deficits, i.e. increase of failures and escape latency in the shuttle box Learned Helplessness paradigm 24 hours later (p < 0.05), lasting at least 10 days. The time-period between day 10 and 14 represents a critical labile condition in which a significant improvement becomes detectable as indicated by a sign-test (p = 0.0172) with principally merely tendencies of helpless behavioural standards.

According to the acquired practice concerning the described protocols one final version was selected for further experiments in male animals and for creation of the databank.

3.1.2 Final Protocol for the Learned Helplessness:

In the Learned Helplessness paradigm the animals were exposed to a transparent plexiglas shock chamber (18 X 18 X 30 cm), equipped with a stainless steel grid floor (Coulborn precision regulated animal shocker, Coulborn Instruments, Düsseldorf, Germany), through which they received 360 footshocks (0.150 mA) on two consecutive days. The footshocks applied were scrambled and unpredictable with varying shock- (1- 3 s) and interval-episodes (1 – 15 s), amounting to a total duration of approximately 52 minutes. 24 hours after the second day of the shock procedure, Learned Helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf, Germany). The shuttle box consists of two equal-sized compartments (18 X 18 X 30) that were separated by a small gate (6 cm wide and 7 cm high), also containing a grid floor, through which the current was applied, and a light at the top of both sections. Spontaneous initial shuttles from one compartment to the other were counted during the first two minutes by redlight beams at the bottom of each of the two divisions. Performance was analysed according to the behaviour during 30 shuttle escape trials (light stimulus: 5 s, footshock: 10 s, intertrial interval: 30 s), defining avoidance as adequate reaction to the light stimulus by changing to the other compartment immediately, escapes as shuttling to the other section as reaction to the electric shock and failures, when no attempt to escape was made. Escape latency was assessed computerized by the determination.
of the time (seconds) it took the animal to escape after onset of the footshock. For determination of the activity during the intervals, shuttles in between the trials were recorded. Total time of Helplessness training was about 20 minutes, depending on the animal’s ability to learn the paradigm.

3.1.3 Validation

For the pharmacological validation of the Learned Helplessness paradigm, helpless mice were treated subchronically with two different doses of imipramine (10 and 30 mg/kg body weight) and saline injections respectively and re-tested in the shuttle box. Re-tests were performed within 7 days after induction of Helplessness, when characteristic response features according to our time course experiment, are still detectable. As expected, control animals treated with saline injections did not differ in their coping performance (apart from the regularly observed subtle learning effects, when exposed to the shuttle box for the second time not revealing statistically significant differences). Since broad effects of imipramine on pain sensitivity or activity were excluded, these findings suggest a beneficial effect of imipramine (p = 0.0095) in a dose of 10 mg/kg body weight in this paradigm, while imipramine at a dose of 30 mg/kg body approached statistical significance (p = 0.083) (Fig. 23) (For detailed procedure see appendix).

![Graphs showing treatment effects in the Learned Helplessness paradigm.](image)

**Figure 23**: Treatment effects in the Learned Helplessness. While NaCl treatment does not result in a statistically significant reduction of Helplessness, imipramine in a dose of 10 mg/kg body weight significantly decreases the number of escape failures after 5 days of treatment (1 = before pharmacological treatment; 2 = after pharmacological treatment).

3.1.4 Neurotrophin Timecourse

Since BDNF levels are decreased after different types of stressors including restraint stress (229), we analysed the regulation of BDNF as well as NGF in a time course experiment after the regular shock exposition, which is capable of inducing a Helplessness behavioural phenotype. Neither BDNF nor
NGF revealed any specific induction pattern when assessed after 0, 3, 6, 12, 24, 48 hours or 7 and 14 days, respectively.

### 3.1.5 Associated Parameters

Since the Learned Helplessness paradigm is employed in the examination of depressive-like features it is supposed to be associated to depressive-like characteristics in corresponding behavioural tests. For a primary establishment of the protocol, this hypothesis was tested in male C57BL/6N mice, revealing a significant correlation of all relevant parameters in the Porsolt Forced Swim Test (Fig 24), a pharmacologically evaluated behavioural test for assessment of despair behaviour (175). General parameters assessed by the tests, which are employed in our test battery, did not interact with Helplessness.

**Figure 24: Associated Parameters.** Correlation between depressive-like parameters in the Learned Helplessness and the Total floating time in the Porsolt Forced Swim Test (Escape Failures; \( p = 0.027, R^2 = 0.485 \) and Escape Latency; \( p = 0.036, R^2 = 0.440 \))

### 3.1.6 Chronic Stress and Housing Conditions

As housing represents a fundamental aspect, which is controversially debated to affect the animals’ emotionality, this experiment aimed at investigating the implication of social and environmental stimulation for the development of a depressive-like syndrome.

#### 3.1.6.1 Group Housing Affects Exploratory Behaviour but not Locomotion

In the Openfield Test, the housing conditions did not affect basal locomotor activity of the animals, *i.e.* the total distance moved (Fig 25A) or the locomotor velocity (Table 3). However, a single exposure to
the Openfield Test also confronts the animals with an approach-avoidance conflict, in which the exploration of the animals, measured by the time spent in the centre of the Openfield arena [10], was significantly influenced by social housing conditions \( F/ (1,62)=10.962; p < 0.002 \). Posthoc tests demonstrated that both group housed cohorts (impoverished and enriched) spent more time in the centre than the respective single housed groups (Fig 25B). P-values of Fisher’s (LSD) posthoc tests are presented in Table 3. There was no statistically significant effect for the interaction between the factors structural and social housing (34).

3.1.6.2 Group Housing and Enrichment Evoke Reduced Anxiety-like Behaviour

Both housing factors, social \( F/ (1,62)=7.797; p < 0.007 \) and structural conditions \( F/ (1,62)=4.86; p < 0.031 \), significantly affected the latency to enter the aversive light compartment, while the interaction between factors did not show a significant effect (Fig. 25C). Posthoc tests (Table 3) demonstrated significantly reduced anxiety-like behaviour, i.e. latency to enter the lit compartment, in group enriched animals compared to single enriched (\( p < 0.008 \)) or compared to group impoverished mice (\( p < 0.031 \)). Single impoverished mice showed a statistical trend towards increased anxiety-like behaviour, when compared with group impoverished animals (\( p = 0.06 \), Fig. 25C). Moreover, ANOVA showed that structural rearing (impoverished vs. enriched) affected the number of exits into the aversive compartment \( F/ (1,62) = 6.005; p < 0.017 \). Posthoc tests also revealed a significant difference between single impoverished and group impoverished rearing (\( p < 0.016 \), Fig. 25D). The time spent in the aversive light compartment was additionally dependent on social conditions \( F/ (1,62)=8.025; p < 0.006 \), with group housing resulting in decreased in anxiety levels, while structural housing conditions only caused a trend for reduced anxiety under enriched conditions \( F/ (1,62)=3.613; p < 0.062 \). The interaction between the factors social and structural housing did not reach statistical significance for any of the three parameters investigated (i.e. latency, number of exits, time in lit compartment). All p-values of Fisher’s (LSD) posthoc test are presented in Table 3 (34).
Figure 25: Basal Behaviour. While no changes in locomotor activity were observed, housing significantly affected the exploratory drive in the Openfield arena as indicated by the time spent in the centre. In the Dark-Light Box, housing conditions exerted effects on anxiety in terms of the latency to enter the bright compartment, as well as concerning the time they spent therein. Bars represent mean + SEM.

Table 3: Significant inter-group differences: Fisher’s (LSD) posthoc test p-values. Group comparisons: p-values indicate inter-group differences in all parameters assessed, according to a Fisher’s (LSD) post hoc comparison. n.s. = not significant; enr. = enriched; imp. = impoverished.

<table>
<thead>
<tr>
<th></th>
<th>Single impoverished</th>
<th>Single enriched</th>
<th>Group impoverished</th>
<th>Group enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Openfield locomotion</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Openfield velocity</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Openfield centre time</td>
<td>vs group imp: p = 0.042</td>
<td>vs group enr: p = 0.002</td>
<td>vs single imp: p = 0.042</td>
<td>vs single enr: p = 0.002</td>
</tr>
<tr>
<td>Dark-Light Box latency</td>
<td>n.s.</td>
<td>vs group enr: p = 0.008</td>
<td>vs group imp: p = 0.001</td>
<td>vs group imp: p = 0.001</td>
</tr>
<tr>
<td>Dark-Light Box exits</td>
<td>n.s.</td>
<td>n.s.</td>
<td>vs group imp: p = 0.008</td>
<td>vs group imp: p = 0.008</td>
</tr>
<tr>
<td>Dark-Light Box time in lit compartment</td>
<td>vs group imp: p = 0.044</td>
<td>n.s.</td>
<td>vs single imp: p = 0.044</td>
<td>n.s.</td>
</tr>
<tr>
<td>Learned Helplessness failures</td>
<td>vs group imp: p = 0.009</td>
<td>n.s.</td>
<td>vs single imp: p = 0.008</td>
<td>vs single enr: p = 0.005</td>
</tr>
<tr>
<td>Learned Helplessness escape latency</td>
<td>vs group imp: p = 0.054</td>
<td>n.s.</td>
<td>vs single imp: p = 0.007</td>
<td>vs single enr: p = 0.007</td>
</tr>
<tr>
<td>Hotplate latency to 1st reaction</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

↑ = increased helpless performance
↓ = decreased helpless performance
3.1.6.3 Housing Conditions Affect the Coping Behaviour in the Learned Helplessness Paradigm

Before analysing potential effects of housing on learned Helplessness, we wanted to know, whether housing affects the two-way avoidance task, which is the “behavioural read-out” for Helplessness in this paradigm. Therefore, groups of non-shocked mice were compared by ANOVA for possible effects of the different housing conditions on the parameters escape failures and escape latencies, respectively. However, no differences were observed (Fig. 26). These results indicated that housing conditions do not produce an effect on avoidance learning per se.

Non-shocked and shocked groups subjected to the same specific housing conditions significantly differed in their shuttle-box performance (failures: single impoverished \( p < 0.034 \); single enriched \( p < 0.038 \); group impoverished \( p < 0.021 \); group enriched \( p < 0.067 \), escape latency: single impoverished \( p < 0.035 \); single enriched \( p < 0.45 \times 10^{-7} \); group impoverished \( p < 0.15 \times 10^{-5} \); group enriched \( p < 0.26 \times 10^{-9} \), Fig. 2). These results confirmed for all 4 housing conditions, that exposure to the uncontrollable shock protocol produced increased Helplessness.

In the learned Helplessness paradigm, ANOVA of the shocked groups subjected to the different housing conditions did not show a main effect for enrichment nor for group housing, but a significant interaction of both factors (Fig. 26). This finding was true with for both major parameters of interest, escape failures \( [F/(1,64)=8.46; \; p < 0.005, \; \text{Fig. 26A}] \) and escape latencies \( [F/(1,64)=5.223; \; p < 0.026, \; \text{Fig. 26B}] \). This effect was confirmed by posthoc analyses, which revealed that group housed impoverished mice show significantly increased Helplessness with more failures and prolonged latencies, compared to single impoverished as well as to group enriched mice (Fig. 26, Table 1). Thus, when reared in an impoverished environment, group housed animals showed the poorest coping abilities. However, group housing per se as well as impoverished environment per se did not affect the extent of helpless behaviour (p-values of Fisher’s (LSD) posthoc tests are presented in Table 1).
Results

Figure 26: Learned Helplessness after different housing. In the Learned Helplessness no difference occurs between the different rearing conditions in unshocked animals, while significant interactions occur for both, escape failures and escape latency, when animals were exposed to the stressful experience of the Learned Helplessness paradigm. Bars depict mean + SEM.

3.1.6.4 Hotplate: Housing Conditions do not Affect Pain Sensitivity

As analysed by a two-way ANOVA, pain sensitivity was not changed in the Hotplate Test conducted with animals from each housing condition.

3.2 Genetic Approach

The contribution of mutations in BDNF, CREB, and GR was examined regarding behavioural and stress-relevant parameters. Furthermore, mutations of IL-6 as well as eNOS, two additional genes of interest for depressive-like features, were implicated in behavioural testing and the Learned Helplessness paradigm. Mutations of the BDNF and CREB gene, respectively, did not reveal any effect referring to earlier results (223) in any of the parameters assessed while IL-6, eNOS, and especially the over- and underexpression of the glucocorticoid receptor resulted in alterations that are illustrated below.

3.2.1 BDNF+/− Animals do not Represent an Animal Model of Depression

According to the Neurotrophin Hypothesis of Depression, the reduction of BDNF would predict depressive-like behavioural alterations. In our hands such changes did not become evident, confirming the results of a study of MacQueen et al., in which the positive result in the Learned Helplessness paradigm was an artefact caused by altered pain sensitivity in these mice (137).
3.2.1.1 Basal Behaviour

BDNF+/− mice with approximately 50% reduced levels of BDNF mRNA and protein are behaviourally indistinguishable from their control littermates when analysed in a battery of tests for locomotor, exploratory, and anxiety-related behaviours (Fig. 27) (33, 137).

General and Anxiety-related Behaviour were unchanged in BDNF Heterozygous Mice

The Openfield Test evaluates the general locomotor and exploratory behaviour of mice exposed to a large open arena under dimmed light conditions. In this test, the total distance moved and the velocity of moving were similar in BDNF+/− mice and controls (Fig. 27A,B). However, BDNF-heterozygous animals spent significantly more time in the centre of the Openfield (Fig. 27C) (p = 0.036). In the Novel Cage Test, a paradigm investigating exploratory behaviour by measuring vertical activity, BDNF+/− mice showed regular numbers of rearings (Fig. 27D). The elevated O-Maze, as well as the Dark-Light Box Test, analyses anxiety-related behaviours by inflicting an approach-avoidance conflict on the animals, in which the level of anxiety correlates with the avoidance of the aversive compartments of the arena. In the Dark-Light Box Test, BDNF+/− mice demonstrate a regular latency to enter the open arms, a normal number of exits to these aversive compartments, and they spent similar time therein (Fig. 27 E,F). In elevated O-Maze, BDNF+/− mice and wildtypes also did not differ with regard to latency, total exits, and time spent in the aversive compartment. BDNF-heterozygous animals showed regular motor abilities when tested on a rotarod (data not shown). Furthermore, both genotypes learned to improve their motoric abilities when repetitively tested on consecutive days. At the age of 3 months, BDNF+/− mice exhibited a significantly higher body weight when compared to their wildtype littermates (24.35 ± g vs. 21.5 ± g; p = 0.034) as recently described by Lyons et al. (136).
3.2.1.2 Depressive-Linked Behaviour

In contrast to the predictions of the Neurotrophin Hypothesis of Depression, BDNF+/- mice do not differ from wildtype controls in their despair behaviour as observed in the Forced Swim Test or regarding their hedonic capacity as measured by sucrose consumption (33, 137). In the Learned Helplessness paradigm, a delayed escape latency has been observed (32). However, the authors attributed this deficit to a reduced sensitivity to the electric foot shocks. Their conclusion is in agreement with ours: BDNF-heterozygous mice are not more likely to display anxiety-related or depressive-like behaviours than wildtypes and, consequently, do not represent a genetic depression model.

**Fear Conditioning was not Altered in BDNF Heterozygous Mice**

Fear Conditioning is an associative (Pavlovian) learning paradigm that can be tested in two different forms. The context version is dependent on both hippocampus and amygdala, while the cue version is reported to be exclusively amygdala dependent (183). In both versions, the amount of freezing (immobility) represents a correlate of associative memory. BDNF+/- mice and their littermates
Results

demonstrated similar freezing scores in both versions of the test. In agreement with the locomotor data obtained in the Openfield, both genotypes revealed no difference in horizontal and vertical locomotor activity in the pre-training phase of the test (data not shown).

**BDNF Heterozygous Mice Behaved Inconspicuously in the Porsolt Forced Swim Test**
The Forced Swim Test is an established paradigm to measure the tendency "to give up" on attempts to escape from an unpleasant environment, with fewer attempts to escape interpreted as evidence of behavioural despair. This test possesses high predictive validity and good face validity as a screen for depression-like behaviour (45, 174). BDNF<sup>+/−</sup> mice and wildtype animals did not exhibit differences in total duration of floating. In the test on day 1, BDNF<sup>+/−</sup> mice displayed a longer latency to start floating (p = 0.027), but exhibited the same total floating time. On day 2, the two groups of mice revealed no differences in any of the parameters studied (Fig. 28).

![Porsolt Forced Swim Test](image)

**Figure 28: Porsolt Test in BDNF<sup>+/−</sup> mice.** Mice of both genotypes exhibit a significant reduction of the latency to start floating when compared with day 1, but there is no intergroup difference. Again both genotypes also reveal a similar total floating time. Columns represent means + SEM.

### 3.2.1.3 Stress Endocrinology

The HPA axis is not affected by the reduction of BNDF as shown by analyses of serum corticosterone and ACTH levels. BDNF<sup>+/−</sup> mice had normal values under baseline conditions and after 30 min of restraint stress (Fig. 29) (33).
Results

Figure 29: Corticosterone and ACTH levels in BDNF<sup>+/−</sup> mice. Mutant mice show normal corticosterone (A) and ACTH levels (B) under baseline conditions at nadir as well as after 30 min of restraint stress. Columns represent means + SEM.

3.2.1.4 Neurotrophins and Monoamines

BDNF<sup>+/−</sup> mice demonstrated a reduction of BDNF protein expression to about 60 % in all brain areas as analysed by ELISA, i.e. hippocampus, frontal cortex, striatum, and hypothalamus (Fig. 30A). In contrast, BDNF<sup>+/−</sup> mice showed normal NGF levels in these brain regions (Fig. 30B).

Figure 30: Neurotrophins in BDNF<sup>+/−</sup> mice. BDNF protein levels as measured by ELISA, are significantly reduced in the hippocampus (HC 64.0 %; p < 0.01), frontal cortex (FC 64.8 %; p < 0.001), striatum (ST 57.5 %; p < 0.001), and hypothalamus (HT 56.6 %; p < 0.001) of BDNF<sup>+/−</sup> mice as compared to their wildtype littermates (A). In contrast, NGF protein levels do not differ in any of the four brain regions investigated (B). Columns represent means + SEM.

Analyses of monoaminergic neurotransmitter systems (5-HT, NE, and DA) revealed regular tissue levels of all three biogenic amines and their degradation products 5-HIAA and HVA (Table 4). However, the activity of ChAT, the key enzyme for acetylcholine synthesis, was significantly reduced in the hippocampus of BDNF<sup>+/−</sup> mice to 81 % of wildtype levels (Table 2).
### Table 1: Tissue contents of monoamines and cholin-acetyltransferase in BDNF+/− mice

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>5-HT</th>
<th>5-HIAA</th>
<th>5-HIAA/5-HT</th>
<th>NA</th>
<th>DA</th>
<th>ChAT</th>
<th>BDNF+/− (n = 9)</th>
<th>wildtype (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>683.0 ± 15.5</td>
<td>644.8 ± 42.4</td>
<td>0.896 ± 0.050</td>
<td>404.8 ± 23.1</td>
<td>74.6 ± 17.2</td>
<td>136.5 ± 5.56</td>
<td>735.8 ± 50.1</td>
<td>710.1 ± 27.5</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>440.1 ± 22.4</td>
<td>14.6 ± 1.3</td>
<td>167.37 ± 10.07</td>
<td>n.d.</td>
<td>160.78 ± 5.83</td>
</tr>
<tr>
<td>Striatum</td>
<td>880.3 ± 72.8</td>
<td>712.8 ± 49.4</td>
<td>0.757 ± 0.040</td>
<td>129.2 ± 21.0</td>
<td>14708.1 ± 770.0</td>
<td>629.04 ± 30.26</td>
<td>889.7 ± 42.8</td>
<td>729.5 ± 34.2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>573.2 ± 38.3</td>
<td>701.6 ± 81.7</td>
<td>1.054 ± 0.080</td>
<td>1459.3 ± 50.3</td>
<td>299.6 ± 50.3</td>
<td>127.72 ± 11.57</td>
<td>647.9 ± 56.2</td>
<td>835.3 ± 72.5</td>
</tr>
</tbody>
</table>

Table 2: Tissue content of monoamines, their degradation products, and choline acetyltransferase in different brain regions of BDNF+/− mice (5-HT: serotonin; 5-HIAA: 5-hydroxyindolacetic acid; NA: norepinephrine; DA: dopamine; HVA: homovanillic acid, ChAT: choline acetyltransferase). The values of monoamines and metabolites are expressed as pg/mg wet weight. ChAT (µU/mg wet weight) was significantly reduced in the hippocampus of BDNF+/− mice to 81.6 % of wildtype controls (n = 8 in both groups; p = 0.02). n.d.=not done

#### 3.2.2 CREB<sup>S142A</sup> Animals do not represent an Animal Model of Depression

Despite the characteristical phase shift in locomotor activity as described by Gau et al. (83), CREB<sup>S142A</sup> mutant mice failed to demonstrate further indications of a depressive behaviour or changes in corticosterone release.
3.2.2.1 Basal Behaviour

CREB$^{S142A}$ mice demonstrated normal horizontal and vertical locomotor activity in both, the Openfield and the Novel Cage Test. To assess their activity at different circadian time points, locomotion was analysed in activity chambers during subjective day and night, respectively. At both time points, CREB$^{S142A}$ mice moved equivalent distances as their control littermates.

CREB$^{S142A}$ mice did not exhibit alterations of anxiety-related behaviours, as monitored in the elevated O-Maze by the latency to visit the aversive open compartment of the maze and the time spent therein. Similar findings were obtained for the anxiety-related parameters of the Dark-Light Box Test. Again, CREB$^{S142A}$ mice showed similar latencies to enter the aversive compartment as the littermate controls, and also spent comparable time periods therein. In addition, CREB$^{S142A}$ mice demonstrated unaltered behaviour in the Openfield with respect to the parameters, which correlate with anxiety, namely mean distance to the walls and time spent in the centre of the arena (data not shown).

3.2.2.2 Depressive-Linked Behaviour

When given a free choice between water and a 1 % sucrose solution, CREB$^{S142A}$ mice did not display any difference in sensitivity to the rewarding properties of the sweet solution, as seen by a normal amount of total liquid intake (Fig. 31A) and a regular sucrose preference (Fig. 31B). Thus, CREB$^{S142A}$ mice did not display anhedonic behaviour compared to littermate controls. In the Forced Swim Test, CREB$^{S142A}$ mice did not differ from wildtype mice in despair behaviour, as monitored by the latencies to start floating, as well as by the immobility times in different phases of the test (Fig. 31C). Both genotypes showed decreased latencies to start floating and an increased total floating time, when the test was repeated 24 h after the first trial (Fig. 31D), an indication for unaltered coping strategies of CREB$^{S142A}$ mice. Furthermore, CREB$^{S142A}$ mice revealed regular emotional learning both in contextual and in cued Fear Conditioning as indicated by unaltered freezing (Fig. 31E,F).
Results

Figure 31: Depressive-like behaviour in CREB<sup>S142A</sup> mice. A,B) In the Sucrose Preference Test CREB<sup>S142A</sup> mice show similar total liquid intake and equivalent preference for sucrose as the wildtype controls. C,D) In the Porsolt Test, CREB<sup>S142A</sup> mice reveal unaltered latencies to start floating and similar total floating on day 1 and day 2. E,F) CREB<sup>S142A</sup> mice exhibit regular emotional learning in context and cued Fear Conditioning. During the training period, mutant mice do not show differences in locomotor behaviour. Columns represent means ± SEM.

3.2.2.3 Stress Endocrinology

CREB<sup>S142A</sup> mice showed similar baseline corticosterone levels as control littermates (Fig. 31A). After 30 min of immobilization stress, a significant increase of the corticosterone level was observed in both genotypes, but again no difference was found between the CREB<sup>S142A</sup> and their littermates (Fig. 31B).

Figure 32: HPA system analyses in CREB<sup>S142A</sup> mice. (A) CREB<sup>S142A</sup> mice have similar corticosterone serum levels as their control littermates, both under baseline conditions at the beginning of their inactive phase (nadir), and (B) after 30 minutes of restraint stress. Columns represent means ± SEM.
3.2.3 GR+/- Animals Display a Depressive-Like Phenotype with Characteristic Stressphysiology in the DEX/CRH Test and Reduction of Neurotrophins

Heterozygous mice with a 50% reduction of GR expression can be used to elucidate the role of this receptor in mood disorders. While their HPA system regulation is not affected under baseline conditions, stress causes higher corticosterone plasma peak levels and a prolonged elevation. At the behavioural level, they do not differ from wildtype controls in a battery of tests assessing anxiety- and despair-related behaviours. Again, when subjected to stress in the Learned Helplessness model, GR heterozygous mice show increased helpless behaviours: fewer escapes, longer escape latencies, and more escape failures than wildtypes. Furthermore, GR heterozygous mice show a reduction of BDNF protein in the hippocampus. Thus, these mice may represent a line with a genetic predisposition to depression-like behaviours (Fig. 33).

![Figure 33: Role of the glucocorticoid receptor.](image)

A heterozygous reduction of GR induces depressive-like changes in the Learned Helplessness paradigm. Reduction of the receptor causes an increase of glucocorticoids after stressful challenge and leads to a decrease of BDNF protein. Antidepressive treatment, on the other hand, can antagonize these effects.
3.2.3.1 Basal Behaviour

**GR**<sup>+/-</sup> mice display normal behaviour under basal conditions

To analyse, whether the reduced GR expression also influences the animals' behaviour, a series of behavioural tests was performed. **GR**<sup>+/-</sup> mice exhibited normal behaviour under basal conditions and were indistinguishable from their wildtype littermates in the Openfield Test (Fig. 34A) and the Novel Cage Test (Fig. 34B). These two tests evaluate various aspects of locomotor and explorative behaviour. **GR**<sup>+/-</sup> mice also showed unaltered anxiety-related behaviour in two tests based on an approach-avoidance conflict, *i.e.* the elevated O-Maze (Fig. 34C) and the Dark-Light Box paradigm (data not shown). In both tests, **GR** heterozygous and wildtype mice exhibited similar scores in visiting the aversive, anxiety-related compartments of the maze, respectively. In addition, no differences between the two genotypes were observed in Fear Conditioning experiments that tested both context- and cue-dependent fear-associated learning (data not shown). **GR**<sup>+/-</sup> mice also demonstrated normal scores in the Porsolt Forced Swim Test. In this assay, the latency to start floating and the total time spent immobile are regarded as correlate of “despair-like” behaviour (Fig. 34D).

![Figure 34: Basal behaviour in **GR**<sup>+/-</sup> mice. **GR**<sup>+/-</sup> mice show normal basal behaviour in terms of locomotion, exploration, anxiety, and despair (A) **GR**<sup>+/-</sup> mice exhibit regular locomotor activity in the Openfield as demonstrated by similar scores in the total distance moved and velocity measurements. (B) In the Novel Cage Test, **GR**<sup>+/-</sup> mice reveal a similar vertical activity as their control littermates, as measured by the number of rearings. (C) **GR**<sup>+/-</sup> mice display normal anxiety-related behaviour in the elevated O-Maze, with regular latencies of first exits to the aversive open arms of the maze and similar time periods they spend thereon. (D) **GR**<sup>+/-</sup> mice demonstrate unaltered behaviour in the Forced Swim Test. Both, latency to start floating and total floating time are similar in both genotypes.](image-url)
3.2.3.2 Depressive-Linked Behaviour

The Learned Helplessness paradigm evaluates the coping capabilities of mice in an aversive test situation after two days of intense stress evoked by exposure to a series of unpredictable and uncontrollable foot shocks. When tested for helpless behaviour, GR<sup>+</sup>- mice displayed significantly increased escape latencies (p < 0.05; Fig. 35A) and a higher number of escape failures (p < 0.05; Fig. 34B). When mice were scored individually (Fig. 35C), the most helpless individuals were exclusively GR<sup>+</sup> (GR<sup>+</sup> r = 0.984, wildtype r = 0.929, α = 0.05). These results reflect true coping deficits as they were not caused by altered pain sensitivity in a Hotplate Test (data not shown) or general changes in activity (Fig. 35).

![Learned Helplessness](image)

Figure 35: Learned Helplessness in GR<sup>+</sup> mice. GR<sup>+</sup> mice display increased Helplessness in a Shuttle Box Test after exposure to inescapable footshocks on the two days preceding the test. (A) GR<sup>+</sup> mice exhibit significantly increased escape latencies compared to their wildtype littermates (p < 0.05). (B) GR<sup>+</sup> mice also show a higher number of escape failures (p < 0.05). (C) Latencies and numbers of failures are highly correlated when plotted for individual mutant and wildtype mice, demonstrating that the subjects with the worst coping (in the right upper area of the graph) are all GR<sup>+</sup> mice.

3.2.3.3 Stress Endocrinology

GR<sup>+</sup> mice have normal basal but increased stress-induced corticosterone levels

To investigate the effects of reduced GR expression on the regulation of the HPA system, corticosterone levels of GR<sup>+</sup> mice and wildtype littermate controls were analysed by a
radioimmunoassay. Under basal, unstressed conditions, no differences were observed between GR<sup>+/−</sup> and control mice, neither during the dark phase nor during the light phase of the circadian rhythm (Fig. 36a). Both genotypes showed about three times higher levels of corticosterone in the dark than in the light phase (Fig. 36A). Since the HPA system is more active under stressful than under basal conditions, we also analysed corticosterone levels following 30 min of restraint stress. Immobilization caused a strong increase of corticosterone levels in both genotypes, but levels were higher in GR<sup>+/−</sup> mice when measured at 40 and 60 min (p < 0.05) after restraint stress (Fig. 36B). These data indicate a disturbed feedback control of the HPA system in GR<sup>+/−</sup> mice under stress.

![Figure 36: Corticosterone levels in GR<sup>+/−</sup> mice.](image)

Under basal and stressful conditions, (A) Plasma corticosterone levels are unchanged in GR<sup>+/−</sup> mice as compared to wildtype mice, both during the animals’ active (dark) and inactive (light) phase. (B) After stress exposure, however, corticosterone levels are higher in GR<sup>+/−</sup> mice at 40 and 60 min (p < 0.05) following immobilization.

### 3.2.3.3.1 Dex-Test and Dex/CRH Test

**GR<sup>+/−</sup> mice exhibit a depression-like Dex/CRH Test**

Since coping deficits in the Learned Helplessness paradigm have been postulated to reflect depression-like behaviour, we subjected the animals to two clinically established neuroendocrinological tests for a depressive state, the dexamethasone suppression Test and the combined Dex/CRH Test. 6 h after injection of dexamethasone, GR<sup>+/−</sup> mice exhibited significantly higher corticosterone levels than wildtype controls (p < 0.01; Fig 37A). In the combined Dex/CRH Test, GR<sup>+/−</sup> mice showed also significantly higher corticosterone levels in response to the CRH challenge than the control littermates (p < 0.001; Fig. 37B). Similar changes are typical in patients with severe depressive episodes.
Results

Figure 37: Dex/CRH Test in GR<sup>+/−</sup> mice. GR<sup>+/−</sup> mice are non-suppressors in the DEX/CRH Test. (B) After administration of dexamethasone, wildtype mice show decreased corticosterone levels, while GR<sup>+/−</sup> mice exhibit non-suppression (p < 0.01). (B) In the combined Dex/CRH Test, corticosterone levels are significantly elevated in GR<sup>+/−</sup> mice compared to the levels of wildtype mice (p < 0.001).

3.2.3.4 Neurotrophins

GR<sup>+/−</sup> and YGR mice show altered levels of hippocampal BDNF expression

Since downregulation of BDNF has been postulated to play a critical role in the pathogenesis of depressive disorders, we analysed the levels of BDNF protein in the hippocampus by ELISA. GR<sup>+/−</sup> mice exhibited a significant downregulation of BDNF to 56.9 % of controls.

(See Fig. 41 for comparison of neurotrophins in GR heterozygous and YGR mice)
3.2.4 YGR Mutant Animals Demonstrate an Anti-Depressive Phenotype

In order to investigate whether an overexpression of the glucocorticoid receptor would exert opposite effects at both, behavioural and stressendocrinological level, the experiments that were done in GR heterozygous animals were transferred to YGR mice, revealing exactly what was suggested by the hypothesis.

![Diagram of glucocorticoid receptor role](image)

**Figure 38: Role of the glucocorticoid receptor.** Overexpression of GR induces depression-resistant changes in the Learned Helplessness paradigm. Reduction of the receptor causes a reduced increase and faster decrease of glucocorticoids after stressful challenge and leads to an elevation of BDNF protein.

3.2.4.1 Basal Behaviour

As observed in the GR heterozygous animals, basal behaviour in the GR overexpressing animals was unchanged.

3.2.4.2 Depressive-Linked Behaviour

Finally we intended to investigate, whether increased levels of GR would also lead to altered behaviour. Similar to GR$^{+/}$ mice, YGR mice did not show a conspicuous phenotype in the test battery for baseline behaviour (Openfield, Novel Cage, O-Maze, Dark-Light Box, Fear Conditioning, Porsolt
Results

Test; data not shown). Again, significant differences were restricted to the Learned Helplessness paradigm. In this test, YGR mice displayed significantly lower escape latencies (p < 0.05; Fig. 39A) and - correlating to that - lower numbers of escape failures (+/YGR r = 0.898, wildtype r = 0.997, α = 0.05) compared to their littermate controls (p < 0.05; Fig. 39B). Again, altered pain sensitivity could be excluded to account for these differences, since both groups showed similar results in the Hotplate Test (data not shown). Thus, while GR+/- mice were more prone to develop depression-like coping deficits, YGR mice were more resistant to develop Helplessness.

![Learned Helplessness Graph](image)

**Figure 39: Learned Helplessness in YGR mice.** YGR mice are more resistant in the Learned Helplessness paradigm. (A) YGR mice exhibit significantly decreased escape latencies compared to their wildtype littermates (p < 0.05). (B) YGR mice also show a reduced number of escape failures (p < 0.05). (C) Latencies and numbers of failures are highly correlated when plotted for individual mutant and wildtype mice, demonstrating worse coping (in the right upper area of the graph) in wildtype mice, whereas YGR mice cluster at the left lower area of the graph.

### 3.2.4.3 Stress Endocrinology

After the observation that the loss of one copy of the GR leads to a “depression-like syndrome” we tried to confirm the specificity of these findings by analysing mice that overexpress GR. We therefore asked whether these mice have accordingly a lower sensitivity to stress-induced behavioural and endocrinological alterations. Similar to GR+/- mice, YGR mice had unaltered corticosterone levels under basal conditions (Fig. 40A). Following 30 min of restraint stress, YGR mice exhibited significantly decreased peak levels of corticosterone at 40 min after stress exposure (p < 0.0048), while at 60 min no difference was observed anymore (Fig. 40A).
3.2.4.3.1 Dex-Test and Dex/CRH Test

In the dexamethasone suppression Test, corticosterone levels in YGR mice were significantly more suppressed than in wildtype controls (p < 0.01; Fig. 40B). In the combined Dex/CRH Test, corticosterone levels were lower in YGR mice than in control littermates, but this difference did not reach statistical significance (p = 0.07; Fig. 40C). In summary, YGR mice demonstrated exactly the opposite changes in HPA-system (dys)regulation than GR\textsuperscript{+/-} mice.

![Graph showing Corticosterone levels](image)

Figure 40: HPA system in YGR mice. YGR mice have a stress-resistant HPA-system and are over-suppressors in the DEX Test. (A) After stress exposure, corticosterone levels are significantly lower in YGR mice at 40 min following immobilization than in wildtype controls (p < 0.05). (B) After administration of dexamethasone, YGR mice show a significant over-suppression of corticosterone levels compared to wildtype mice (p < 0.01). (C) In the combined Dex/CRH Test, corticosterone levels are lower in YGR mice compared to the levels of wildtype mice (p = 0.07).

3.2.4.4 Neurotrophins

YGR mice revealed a significant upregulation of 51.7 % of BDNF (Fig. 41). In contrast, the expression levels of NGF protein, another key member of the neurotrophin family, were unaltered in both GR mutated strains, indicating the specificity of the results obtained for BDNF (Fig. 41).
Figure 41: Neurotrophin levels in GR+/- and YGR mice. BDNF protein levels in the hippocampus are significantly diminished in GR+/- mice (p < 0.05), and significantly increased in YGR mice (p < 0.05). In contrast, the expression levels of NGF are unaltered in both mutant strains (wt = wildtype).

3.2.5 IL-6 Deficient Animals Demonstrate a Stress Resistance

In order to investigate the effects of a decreased expression of Interleukin-6, which, according to current hypotheses, is postulated to be involved in the pathophysiology of depression (6), IL-6 knock-out animals were investigated in terms of basal and depressive-linked behavioural performance, demonstrating a resistance to stressful challenge. The finding, that IL-6 is involved in the development of a helpless phenotype was furthermore substantiated by an experiment, in which the content of IL-6 protein in the hippocampus of helpless wildtype animals was analysed, revealing an induction of IL-6 in these animals.
Figure 42: Role of Interleukin-6. The knock-out of IL-6 causes a decrease in depressive-like behaviour in the Learned Helplessness paradigm. Furthermore, when mice are subjected to the Learned Helplessness procedure, an induction of IL-6 was assessed.

3.2.5.1 Basal Behaviour

When investigated in our behavioural test battery, including the examination of locomotion, anxiety, exploration, and learning, IL-6 deficient mice did not show a conspicuous phenotype (data not shown).

3.2.5.2 Depressive-Linked Behaviour

IL-6 mutant mice displayed regular “despair” behaviour, when they were examined in the Forced Swim Test, but significantly differed in terms of depression-like behaviour when subjected to a matched law procedure of sucrose consumption, as well as in the Learned Helplessness paradigm, where they exhibited a resistance to develop a depressive-like phenotype, demonstrating less coping deficits in terms of failures (p = 0.0048) and escape latency (p = 0.049) (Fig. 43).
Results

**Figure 43: Role of Interleukin-6.** IL-6 mice are more resistant in the Learned Helplessness paradigm. (a) IL-6 mice exhibit significantly decreased escape latencies compared to their wildtype littermates (p < 0.05). (b) IL-6 mice also show a reduced number of escape failures (p < 0.05). (c) Latencies and numbers of failures are highly correlated when plotted for individual mutant and wildtype mice, demonstrating worse coping (in the right upper area of the graph) in wildtype mice, whereas mutant mice cluster at the left lower area of the graph.

### 3.2.6 NOS III (−/−) Mutant Animals

NOS III knock-out animals were investigated for differences in depressive-like behavioural characteristics, since it was postulated that altered neurogenesis in these mice may be associated to a depressive-like phenotype.

#### 3.2.6.1 Basal Behaviour

NOS III mutant mice, when examined in our behavioural test battery, did not exhibit an altered phenotype regarding locomotion, exploration anxiety or present any obvious abnormalities (Data not shown).

#### 3.2.6.2 Depressive-Linked Behaviour

Despite the hypothesis, that NOS III mice would demonstrate a phenotype with regard to depressive-like behaviour when subjected to the Learned Helplessness paradigm, NOS III deficient mice demonstrate a resistance to develop Helplessness regarding escape latency (p = 0.01) in the shuttle box procedure. This is not due to an altered pain sensitivity, which was ensured by examination of pain...
Results

threshold on the Hotplate, not revealing any differences. A fewer number of escape failures as well as an improved avoidance performance (p = 0.0015) were observed, which cannot be explained by general differences in locomotoric or activity features as observed in the Openfield Test. The Porsolt Forced Swim Test however, no differences in either direction occurred between the mutants and their wildtype littermates.

![Learned Helplessness graph](image)

**Figure 44: Learned Helplessness in NOS III⁻/⁻ mice:** NOS III knock-out animals demonstrated an improved avoidance and less escape deficits in the Learned Helplessness paradigm when compared to their wildtype littermates.
4 Discussion

4.1 General Discussion

This thesis had the set goal of shedding light on the phenomenon of Learned Helplessness by examining the relevant parameters in mice, (i.e. coping deficits in a shuttle box paradigm) and elucidating environmental (i.e. housing and stress) as well as genetic factors. To assess the impact of genetic factors, this thesis implies the investigation of transgenic mice, which have been hypothesized to represent animal models for depression according to characteristic alterations in their HPA system, neurotrophic factors or potentially associated aspects.

At this time animal models represent the unique option to expand the information about the mechanisms involved in the pathogenesis of defined diseases. Depression constitutes a major challenge because its characterization is difficult due to the multifactorial derivation of disease-specific traits. The consideration of the associations of diverse factors therefore represents a key concern of behavioural animal models of depression. Modelling a depressive-like state, such as the Learned Helplessness, consequently requires exact knowledge regarding relevant modulators to ensure representative results. This thesis aimed at investigating the role of target genes, suggested to be involved in the pathogenesis of depression, which makes it very important to elucidate potential distorting factors, such as the housing of the experimental animals, which is controversially debated to affect the animals' emotionality. The exclusion of such environmental factors, which evaluates the specificity of the behavioural findings in mutant animals, represented one of the assignments in this thesis. To guarantee a specific readout, which permits concrete statements regarding the quality of our model, we compared the effects of different social as well as structural factors with regard to general and Helplessness-specific effects on behaviour. Furthermore, the model was validated by a pharmacological regimen. Especially in studies of depression and emotionality it is essential to establish standardized protocols, involving the animal’s environment, to be able to precisely assess potential sources of stress and exclude artefacts. The high-quality design and modification of animal models, such as Learned Helplessness, subsequently bears the bonus not only to detect genetic aspects by investigating mice with mutations of particular target genes, but also to exploit elementary basics of depressive-like phenotypes.
4.2 The Learned Helplessness as a Model of Depression

Originally the Learned Helplessness model of depression was described by Seligman et al. in dogs (139), which was afterwards extended to many other species, including mice, rats, and even humans. It is characterized by performance deficits in subsequent learning tasks after exposure to acute inescapable stress, which are absent in individuals, who experienced the same stress but were able to control it (76, 110, 149, 192). Following a series of inescapable and unpredictable shocks, rats and mice show a variety of behavioural deficits, including a profound disruption of escape performance (194, 195) as well as neurochemical signs of depression-like changes in norepinephrine (NE), dopamine (DA), and serotonin (5-HT), neurotransmitters, which have been implicated in the human disease. Characteristic features, which arise in the animal as a consequence of the procedure, reflect the symptoms of a depressive state in men rather well (139). Furthermore these symptoms can be reverted by chronic administration of monoamine oxidase (MAO) inhibitors, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors, and electroconvulsive therapy (ECS), but not by chronic treatment with neuroleptics, stimulants sedatives, and anxiolytics (196). Therefore the Learned Helplessness is suggested to be a suitable model to mimic depressive-like symptoms in animals. The severity of depressive disorders and its high prevalence in the modern society increases the need of valid reliable animal models for depression, such as the Learned Helplessness paradigm, particularly in mice, which reach importance due to the possibilities opened by genetic engineering for the investigation of pathological mechanism. Thus, the reproducibility across laboratories remains to be one of the major problems, which makes it difficult to simply establish a model according to the description in the literature. Moreover, the understanding as well as interpretation of the effects observed in this paradigm requires further examination, ideally approaching from various disciplines. Only a fraction of rodents exposed to inescapable shock develop the Learned Helplessness syndrome, estimated to reach 10-80 % (58). Even though some of this variability can be explained by the stress parameters used to induce Helplessness, the difficulty and type of escape response, or the criterion used to define Learned Helplessness, a proportion of the variability is likely due to genetic variation. For instance, some inbred mouse strains exhibit severe escape deficits while other strains are not affected (194). Besides that, environmental factors have to be considered. Additionally, rat lines have been selectively bred for susceptibility to develop Learned Helplessness resulting in helpless lines with more than 95 % of helpless animals, with some rats even demonstrating this behaviour without being trained, and very resistant non-helpless lines (228, 229), in which only about 5 % become helpless (126).

One goal of this thesis included the characterization of the Learned Helplessness in mice (35), which required a modification of current protocols in literature (27, 194, 221). Besides the establishment of a new protocol for the assessment of depressive-like phenotypes in mutant mouse strains, it was
investigated in how far obligatory external factors, like housing conditions, affect this model to exclude the possibility of artefacts according to peripheral issues. Our data show that group housing has a profound impact in the development of Helplessness after uncontrollable exposure to electrical shocks, but that the exact direction of these changes depends on the level of structural enrichment of the environment in which the mice are reared. These results are in line with the few studies available at present (94, 114), and suggest that the manipulation of housing conditions may be a suitable model to study the impact of chronic social stress on helpless behaviour. In addition, these observations may be of interest for establishing standard housing conditions that ensure the animals welfare.

Thus, in the Learned Helplessness paradigm, group housed mice reared in impoverished cages displayed a higher number of coping deficits, *i.e.* Helplessness, indicated by an increased number of escape failures and larger latencies to escape than mice in other housing conditions. However, an enriched environment seems to have a protective effect on the development of helpless behaviour. No differences occurred between the individually housed groups indicating that enrichment improves the coping in a stressful situation only if it is combined with a respective social environment, where positive and negative interactions between the individuals may require a more pronounced behavioural repertoire than in the solitary animals.

Differences in Helplessness could arise from an effect of housing conditions in different components of the testing procedure. First, an increase on the number of failures and/or increased latencies to escape could result from motor deficits produced by specific housing configurations. However, as assessed in the Openfield (total distance) and Dark-Light Box (number of exits), the locomotion was not different among groups (34). Second, housing conditions could affect pain thresholds, subsequently changing the real impact of the uncontrollable shock exposure preceding Helplessness testing. This explanation does not seem to account for the Helplessness results either, since the pain thresholds, as measured in the Hotplate, were also unaffected by housing conditions. Third, anxiety-and mood disorders like depression show partial overlapping in both humans and rodents (239). Therefore, it could well be that housing conditions could alter Helplessness by changing anxiety-related parameters. However, although some differences among groups were found in anxiety-related measures, the pattern of these results was not coincident with those of the shuttle performance, and in fact no significant correlation between both sets of data was found. Finally, differences in shuttle performance were only observable after uncontrolled shock exposure, as non-shocked animals displayed similar avoidance regardless of their housing conditions. This observation suggests that these housing conditions did not affect two-way avoidance *per se; i.e.* affecting learning and memory processes (34).

Therefore, the results of the present study reveal a specific effect of housing conditions on the development of helpless behaviour. Thus, group housing can be a source of stress in territorial animals.
Discussion

such as mice (80) that may result in enhanced behavioural despair (114). Consequently, in our study, group housed animals reared in an impoverished environment showed the highest scores in two indices of Helplessness, the number of failures and the latency to escape in a two-way avoidance task. *Vice versa*, enrichment can have a protective effect in the development of these features, as reflected by the improved performance in the group housed animals reared in an enriched context. This behavioural observation is unprecedented but seems to be in accordance with previous studies that have shown that an enriched environment improves some physiological parameters, which accompany depression-like states in both rodents and humans (49, 69, 77, 219). Moreover, supporting this protective effect of environmental enrichment, it should be noted that the performance of mice individually housed was not sensitive to environmental enrichment, which again is in line with previous results showing an enhanced efficacy of imipramine treatment only in group housed mice (114).

In summary, the results of the present study reveal that the manipulation of housing conditions can be a suitable procedure to study behavioural despair in mice. We believe that this procedure presents some advantages over other available alternatives. First, it allows a bi-directional manipulation of this behavioural phenomenon, which is manipulated by social (*i.e.* group housing) as well as structural (*i.e.* enrichment) factors. Moreover, these manipulations are more naturalistic than those currently used in this field (80) and have been partially pharmacologically validated (114). In addition, as observed in our non-shocked animals, the manipulation of housing conditions does not produce helpless behaviour *per se*, but rather results in a predisposition that is only expressed after a precipitant factor (*i.e.* uncontrollable shock exposure), then mimicking the diathesis-stress model currently accepted for affective disorders such as major depression (90). On the other hand, our findings also have implications when considering animal welfare and the attempts to standardize the rearing of rodents. Thus, individual housing, which is usually criticized to be stressful (12), may be an adequate type of housing depending on the species and strains as well as on the experimental needs.

The awareness of such fundamental effects of social as well as structural environment represents a crucial factor considering the quality of the results in the species one is working with. The standardization of our protocols by including external factors therefore appears to fulfil the requirement of a valid model of depression, with regard to the investigation of additional aspects, such as genetic effects.
4.3 **Role of Neurotrophic Circuits**

Neurotrophins are postulated to be involved in pathogenetic as well as therapeutic pathways by a variety of authors (61, 158, 215, 224), describing the antidepressive properties of BDNF as well as stress-induced decreases of BDNF in rodent models. This appears to be in line with the decline of BDNF levels found in *postmortem* studies of depressive patients without antidepressive treatment. According to these findings two mutations of genes involved in the “Neurotrophin Hypothesis of Depression”, BDNF, and CREB, were analysed related to the results published in the literature and discussed in the following paragraphs.

4.3.1 **Brain-Derived Neurotrophic Factor**

The presented experiment assessed potential neurochemical, neuroendocrinological, and behavioural changes in adult mice with a heterozygous mutation of the BDNF gene. We showed that these mice have a circa 60% reduction of BDNF protein levels in several forebrain regions. Our aim was to test one of the predictions of the "Neurotrophin Hypothesis of Depression", *i.e.* that the reduction of BDNF levels would induce depression-like features in these mice. However, BDNF$^{+/−}$ animals exhibited regular levels of monoamines and their metabolites, a normal regulation of the HPA-system and - apart from minor differences - unchanged emotional behaviours.

Our behavioural data are in agreement with the results of a study by MacQueen and colleagues on BDNF-heterozygous mice (137). In this study, similar behavioural analyses to ours did not reveal any abnormalities apart from impaired pain sensitivity (137). Both, the latter study and ours, unambiguously show unaltered locomotor, anxiety-related, and depression-related behaviours. Similar as described by Lyons and colleagues (136), a significant difference occurred in the Openfield Test, where BDNF$^{+/−}$ mice spent significantly more time in the centre than control animals. In view of the normal anxiety-related behaviour of BDNF$^{+/−}$ mice in the elevated O-Maze and in the dark-light-box paradigm (Fig. 3E-F), this finding most likely reflects an increased exploration of BDNF deficient mice (255). Increased exploration, however, reflects "non-depressive" or "depression-resistant" behaviour, because depressed animals have a tendency to exhibit neophobic behaviours (239). In the Forced Swim Test, BDNF-heterozygous mice had a shorter latency to start floating on the first day of testing. However, BDNF$^{+/−}$ mice revealed an unchanged latency and total floating time on day 2 of the test, when depression-like behaviour, according to the reported reverse of this behaviour by antidepressant treatment is most evident (45, 46). MacQueen and colleagues did not detect any changes in the Forced Swim Test of their BDNF-heterozygous mice, also strongly arguing against a depression-like behavioural phenotype in this test (137). The fact that the most measures of emotional behaviour are unchanged in BDNF$^{+/−}$ mice is perplexing, considering the proposed role of BDNF in
Discussion

the pathophysiology of depression and other stress-related disorders (3, 61, 199). A possible explanation may be that a 60 % reduction of BDNF is not sufficient to induce strong behavioural effects. However, BDNF-heterozygous mice have been shown to display other behavioural abnormalities, i.e. increased aggressiveness, learning deficits, and hyperphagia, the latter being in accordance with the significantly increased body weights of BDNF+/− mice also observed in our study (60, 120, 131). Mice carrying a mutation since early embryogenesis may develop compensatory mechanisms to overcome the effects of gene targeting during development. However, NGF expression was normal in all brain areas with reduced BDNF levels. Nevertheless, mice with a complete forebrain-specific conditional BDNF knock-out displayed increased anxiety-related behaviours. Other behavioural tests more related to depression-like behaviours have not been performed as yet with this strain.

We also analysed the tissue levels of 5-HT, NE, and DA in several brain regions of BDNF+/− mice, because the monoaminergic neurotransmitter systems play an important role in the pathogenesis and therapy of depression. BDNF+/− mice exhibit normal amounts of all monoamines and degradation products investigated, which may explain their unaltered emotional behavioural phenotype. In line with these findings, BDNF+/− mice exhibit a normal regulation of the HPA-system, both under baseline conditions and after restraint stress. Our neurochemical analyses revealed a significant reduction of choline acetyl transferase (ChAT) activity to about 80 % selectively in the hippocampus of BDNF+/− mice. This loss of activity agrees with the previous finding that in the medial septum of BDNF+/− mice the number of cholinergic neurons is reduced at postnatal days 6 and 15 as compared to BDNF+/+ and wildtype littermates (234). Although the number of cholinergic septohippocampal cells is not reduced in BDNF+/− mice in the early postnatal period, the decrease in enzyme activity in the adult animal may reflect a disturbance in the maintenance of function and survival of these neurons in response to the reduced BDNF levels. It has been suggested that synaptic plasticity in the hippocampus is influenced by neurotrophins via reciprocal regulation of acetylcholine, BDNF, and NGF, representing a "molecular framework" (122). This may be explained at least in part by the fact that BDNF regulates postnatal maturation and neurite outgrowth of cholinergic forebrain neurons and their projections to the hippocampus (234). Furthermore, a protective effect of BDNF was demonstrated for ChAT mRNA levels following peripheral injury of motor neurons, indicating the existence of regulatory pathways for cholinergic gene expression responsive to neurotrophic factors (233). Basal forebrain cholinergic neurons projecting their axons throughout the hippocampal formation and the neocortex are important for learning and memory (68, 234). Thus, deficiencies in cholinergic projections to the hippocampus may be in part responsible for the learning deficits observed in mice with impaired BDNF-TrkB signalling. BDNF deficient mice, as well as mice with a conditional TrkB knock-out in the forebrain exhibited severe impairments in hippocampus-dependent spatial learning (131, 150). In contrast to
those deficits in explicit or declarative learning and memory, BDNF-TrkB compromised animals revealed normal results in hippocampus-dependent associative learning paradigms, such as Fear Conditioning in the present study and passive avoidance in the study of Minichiello (150). Similar as BDNF-heterozygous mice, TrkB conditional knock-out mice also demonstrate regular emotional behaviours (255).

The fact that mice with mutations impairing the BDNF-TrkB pathway do not show any features of depression challenges the hypothesis that this signalling pathway may play a major role in the pathogenesis of depression. However, despite the conflicting results on the role of this pathway in the pathogenesis of depression, this signalling cascade clearly seems to be involved in the mechanisms of antidepressive therapy. Thus, chronic antidepressive treatment evokes an increase in BDNF expression (158, 248). Mice with reduced BDNF expression as well as mice with impaired TrkB function do not show the normal reduction of behavioural despair seen in wildtype mice following antidepressant treatment, indicating that BDNF-mediated TrkB activation is necessary for at least some behavioural effects of antidepressants (187). These findings in transgenic mice, together with the direct antidepressive effects of BDNF in mouse models of depression (197, 199), as well as the increased BDNF levels in patients under antidepressive medication (32), suggest a cumulative evidence for a central role of this neurotrophin and its receptor in the molecular mechanisms of antidepressive therapy. There are several possible biological mechanisms that could mediate the antidepressant effects of BDNF. On a molecular level, the MAP kinase cascade is activated by BDNF-TrkB signalling and induces the plasticity-related transcription factor c-fos. On a cellular level, BDNF is involved in synaptic remodelling and functioning (141, 145, 173). Such cellular adaptations may also improve monoaminergic signalling in the forebrain, which is a major participant in the therapy of depression according to the monoaminergic hypothesis. While our results, as well as those of others, suggest that mice which are compromised in BDNF-TrkB signalling do not provide a genetic mouse model for depression, they may represent a valuable instrument to study molecular and cellular effects of antidepressive therapy.

4.3.2 CREB

Mice with a mutation of the CREB Ser142 phosphorylation residue were regarded as a candidate strain for depression-like behavioural or neuroendocrinological symptoms for several reasons. First, they have a desynchronization of their circadian rhythm (83), a key feature of subgroups of patients suffering from depression, e.g. Seasonal Affective Disorder (SAD) patients (24, 155, 208). Second, CREB is downregulated and/or underactivated in patients with depression (57, 249). Third, CREB induction and activation is induced by antidepressive therapy in mice and men (for review see (155)).
However, in the present study, CREB^{S142A} mice did not show behavioural or neuroendocrinological features of depression when investigated in a test battery for locomotion, anxiety, despair, anhedonia, and emotional learning or stress-induced HPA-system abnormalities. The lack of a depression-like phenotype of CREB^{S142A} mice is in line with the behavioural data in other strains of mice with a genetically modified expression of CREB, i.e. mice overexpressing dominant-negative mutant CREB in striatal regions, which blocks CREB function, show decreased immobilization in the Forced Swim Test, while an opposite effect is observed in mice virally overexpressing CREB in that region (172). In accordance with this, Eisch et al. found brain-region specific effects of BDNF and its receptor TrkB exerting depressive-like behavioural effects when injected in the ventral tegmental area – nucleus accumbens (67). CREB^{αΔ} mutant mice, which lack the α and Δ CREB isoforms, leading to a more than 90% reduction of CRE-binding activity in the brain, demonstrate less despair behaviour in the Forced Swim Test and in the tail suspension Test than wildtype controls (41). Mice with decreased CREB activity in the forebrain due to expression of a dominant-negative CREB-mutant polypeptide show reduced depression-like behaviours in the learned-Helplessness paradigm (156). However, despite their lack of depression-like behaviours, CREB^{αΔ} mutant mice and also mice with a brain-specific lack of all CREB isoforms exhibit increased levels of anxiety when tested in the elevated T-Maze and the Dark-Light Box Test (89, 225). In summary, despite the obvious role of CREB in the molecular mechanisms of antidepressant therapy, CREB expression and/or function does not seem to be involved in the pathogenesis of depression-like symptoms in mice.

Our hypothesis is supported by the fact that our study did not provide evidence for a role of CREB Ser142 in the pathogenesis of depressive syndromes, neither in mice nor in men. However, depression is a multifactorial and also a multigenetic disease. Therefore it can be assumed, that different genetic factors as well as various psychosocial and environmental variables contribute to this disorder. Thus, it is also conceivable that manipulation of one single genetic function, like CREB Ser14,2 may be necessary but not sufficient to cause a complex syndrome of behavioural alterations. Improved strategies for genetic modelling of depression-like syndromes in animals may therefore require a simultaneous targeted dysregulation of several genes involved in the pathogenesis of depression. This approach can be complemented in human models by the identification of behavioural traits, which are thought to be encoded by a limited set of genes. These so-called "endophenotypes" (46, 87), may reveal novel insights into the molecular mechanisms underlying circadian rhythmicity as well as specific subgroups of depression, such as SAD. In this respect we have identified a molecular switch in the CREB gene, CREB Ser142, which seems to be responsible for a specific symptom, i.e. the (dys)regulation of circadian rhythms, in mice and men.
4.4 **Role of the HPA System**

Changes in the hypothalamus-pituitary-adrenocortical (HPA) system are typical for depression. Since the effects of glucocorticoids are mediated by intracellular receptors including, most notably, the glucocorticoid receptor, various studies have investigated the number and/or function of GR in depressed patients. Because an overall examination of CNS-related mechanisms in humans is restricted due to the unavailability of human brain tissue, it is necessary to search for corresponding animal models, in which pathophysiological changes can be investigated.

4.4.1 **Glucocorticoid Receptor**

The present study analysed the effects of reduced and increased expression of GR on behaviour and the HPA system in mice. On both levels, alterations only became evident after a stressful challenge, while none of the strains showed a phenotype under basal conditions in a test battery for locomotor, exploratory, anxiety-related, and emotional behaviour. In this context it is of interest that a selective loss of GR function in hepatocytes did not affect gluconeogenesis under basal conditions, but caused a gluconeogenesis deficit under challenge conditions by fasting the animals (163). Similarly, GR<sup>+/−</sup> mice exhibited a depression-like phenotype, *i.e.* increased Helplessness after challenge by uncontrollable footshocks. GR overexpressing mice, conversely, revealed a resistance to develop helpless behaviour. While GR<sup>+/−</sup> mice demonstrated a disinhibited HPA axis after stress exposure and a depression-like Dex/CRH Test (as the consequence of an altered feedback regulation due to a reduced GR gene dosage at the level of the PVN and the anterior pituitary), YGR mice had a stress- and challenge-resistant HPA system.

The Learned Helplessness model represents a rodent depression model with good face- and construct-validity (46, 197, 230). It is based on the concept that depressed individuals show a loss of coping strategies in aversive environmental situations (198). This paradigm has been successfully used to demonstrate depressive behaviour in genetically altered mice (157). In the present study, GR<sup>+/−</sup> mice demonstrated significantly prolonged escape latencies and increased failures in the active avoidance task of the Learned Helplessness paradigm after two sessions of footshock exposure. The impaired coping behaviour strongly indicates the development of depression-like behaviour in GR<sup>+/−</sup> mice. This altered behavioural reactivity to stress is accompanied by depression-like alterations of the HPA system. Similar to the behavioural level, changes of the HPA axis are not present under basal conditions in GR<sup>+/−</sup> mice, but a significant disinhibition occurs after stress. Furthermore, GR<sup>+/−</sup> mice exhibit a pathological Dex/CRH Test, currently the most relevant biological marker in patients for both, florid depression and the risk to develop a depressive episode. This test combines the suppressive effect of dexamethasone with the stimulatory potency of CRH (102). In 70-80 % of severely depressive patients, the CRH-elicited ACTH and cortisol response is blunted, and
dexamethasone pretreatment does not elicit a suppressive effect and paradoxically induces enhanced cortisol levels following a CRH challenge (102). In healthy individuals, in contrast, the Dex/CRH Test creates the situation of a pharmacological (partial and transient) adrenalectomy, in which hypothalamic CRH expression is increased due to a decrease in plasma cortisol (155). Similar as depressed patients, GR<sup>+/−</sup> mice display a characteristic non-suppression following dexamethasone treatment and the paradoxical increase of corticosterone levels after combined Dex/CHR application. Very recently, a depression-like phenotype similar as for GR<sup>+/−</sup> mice was described in mice with a forebrain-specific GR knock-out (21). These animals also exhibit impaired negative feedback regulation of the HPA axis, as well as increased depression-like behaviour (21). These findings indicate that crucial brain regions responsible for the phenotype of GR<sup>+/−</sup> mice may be located in the forebrain. Mice with a forebrain-specific complete GR-knock-out, induced by the calcium-calmodulin-dependent protein kinase II (CamKII) promoter, exhibit a depression-like phenotype at 3 weeks of age already under basal (unstressed) conditions (21).

Our results show that GR under- and overexpression from early development on does not lead to an overt phenotype under baseline conditions with low levels of stress. This could be due to a mere gene dosage effect. On the other hand, the lack of a specific phenotype might also be caused by early ontogenetic compensatory or adaptational processes, possibly reflecting the condition of patients with a high genetic (familiar) risk for depression, who are initially inconspicuous but develop depressive episodes following stressful life events. The lack of a phenotype under baseline conditions in the GR mutant strains examined here may also be due to the distinct genetic background of the animals. In accordance with the recommendations of the Banbury conference (38) all mice used in our experiments were F1 hybrids (from C57BL/6 and FVB/N parent mice). This results in a so-called hybrid vigour, which can reduce the impact of genetic alterations compared to a mixed or inbred genetic background. On the other hand, the detection of phenotypic alterations or pathologies in mice on a hybrid background (245) - in our GR mutant mice altered stress sensitivity - implies that the consequences of the underlying mutations are robust and reliable. These considerations regarding the behavioural findings can also be employed to physiological systems like the HPA axis. Despite their altered GR expression, GR mutant mice show normal baseline corticosterone levels during circadian rhythm. Following immobilization stress, however, significant changes in the response of the HPA system are present in GR<sup>+/−</sup> as well as in YGR mice. Since depression is a multigenetic disease, the dysregulation of a single system may not be sufficient to induce depression-like alterations in mice under basal conditions, in particular not in a robust F1 hybrid background. Therefore, provoking or destabilizing factors, such as stress or early childhood environmental factors may be necessary in addition to the existing genetic predisposition to effectively elicit a behavioural and also neuroendocrinological phenotype (36). This concept may explain why the depression-like phenotype
of GR\(^{+/−}\) mice manifests only after a stress challenge. The latter fact, however, can be regarded as strength of this model, because also depression-vulnerable humans often develop a depressive episode due to external or internal stress factors. An indirect genetic approach to prove the specificity of the depression-like alterations of GR\(^{+/−}\) mice seemed to be the behavioural and neuroendocrinological examination of mice overexpressing GR. In these mice one would expect the opposite phenotype of GR\(^{+/−}\) mice. Indeed, YGR mice carrying four copies of the GR gene demonstrate a stress-resistance on both the behavioural and the hormonal level. These animals exhibit less Helplessness, lower corticosterone levels after immobilization, and an over-suppression of corticosterone following dexamethasone treatment. This is in agreement with the predictions of our hypothesis. Recently it was reported that mice overexpressing GR specifically in the forebrain display increased emotional lability in the Porsolt Forced Swim Test indicated by increased baseline immobility (235). Furthermore, these animals exhibited increased anxiety-like behaviour in the Elevated Plus-Maze and in the Dark-Light Box (235). This was not observed in our YGR mice that express GR under control of its own regulatory sequences. These conflicting results could be explained by the late onset and forebrain restricted mutation induced by the calcium-calmodulin-dependent protein kinase II promotor (CamKII) used in the former study (235). As already discussed above, in our mice developmental adaptive processes as well as a robust F1 hybrid background may account for the lack of a phenotype under basal conditions. Given that depression is a multifactorial disease (36), we assume that our GR mutant mice represent models for combined effects of both genetic and environmental manipulations. The results in GR\(^{+/−}\) mice resemble the human situation in depressive disorders, in which individuals at risk are predisposed to develop depressive episodes after stress. In this respect, GR\(^{+/−}\) mice differ from mice with a forebrain-specific complete GR knock-out (21), which already demonstrate depression-like behavioural and neurochemical alterations under baseline conditions. However, the findings in both strains indicate that compromised GR function constitutes a crucial molecular risk factor in the pathophysiology of depression. Therefore we suggest that the mouse lines with altered GR expression are suitable tools for further physiological, biochemical, and pharmacological investigations of GR function with regard to depressive disorders. These analyses are also of clinical relevance, since in preliminary studies GR antagonists have been of value in the treatment of severe depressive episodes (15).

Recently, the so-called Neurotrophin Hypothesis of Depression postulated that a downregulation of BDNF is essential for the pathogenesis of depression in humans and rodents, and can be reversed by antidepressant therapy (46, 61, 127, 155, 247). In concordance with this hypothesis, GR\(^{+/−}\) mice – which have a predisposition to stress-induced depression-like behaviour – show a significant downregulation of BDNF in the hippocampus, while YGR mice exhibit a significant BDNF upregulation. Indeed, this is the first experimental evidence that reduced GR function concurrently
evokes a BDNF dysregulation and a predisposition to depressive behaviour. In future experiments with these strains we will investigate further mechanisms of neural plasticity thought to be involved in the molecular and cellular pathogenesis of depression, e.g. monoaminergic systems, arginine vasopressin and neurogenesis. In addition to the potential involvement of neurotrophins, respective experiments could also reveal a link between these systems, GR function, and depression-like behaviour.

4.5 Role of Interleukin-6
Depression is accompanied by an immune response. It is not clear whether activation of the immune system and in particular an increased production of IL-6 is a cause or consequences of depressive disorders. In addition to its well-known role in the immune system (e.g. induction of fever), IL-6 is constitutively expressed in various areas of the brain where it can exert neuromodulatory effects. IL-6 deficiency has been shown to increase emotional reactivity to a novel environment. In this study, we subjected IL-6-/- mice to a battery of tests for anxiety and depression-related behaviours. Our findings confirm that they are more anxious than wildtype controls and display a less depressive-like behaviour in the Learned Helplessness paradigm. Additionally, IL-6-/- mice are more sensitive to the rewarding properties of sucrose. Moreover, in normal C57BL/6 mice, IL-6 levels are elevated in the hippocampus after the Learned Helplessness procedure. Thus, IL-6-deficient mice are more anxious but less depressive than control mice and our results suggest that IL-6 activation might be more a cause than a consequence of depression.

In summary, the investigation of IL-6 knock-out animals revealed a decrease in Helplessness in the same paradigm, suggesting depression-resistance, which was supported indirectly by the detection of IL-6 induction in helpless wildtype animals. This is in line with the finding in eNOS knock-out animals, which demonstrated better coping performance in the Learned Helplessness paradigm which may contribute to the judgment of IL-6 action due to a recent hypothesis of Rönnbeck et al. (184), who state a contribution of IL-6 as well as NO in the modulation of glutamatergic neurotransmission by impairing astroglial glutamate transport. As glutamate signalling is crucial for information intake and processing within the brain, and due to the pivotal role for glutamate in brain metabolism, dynamic alterations in glutamate transmission could be of pathophysiological importance for mental fatigue, which may be of utmost importance for breaking the vicious cycle, which comes with the risk for secondary anxiety and depression (184). Although mental fatigue is not exactly the same as depression there are overlaps (e.g. the enhanced production of proinflammatory cytokines (4) and “sickness behaviour” (107, 116)) and both disorders present behavioural manifestations such as a reduction in motivation that would appear similar in animal models, where the affective state is either
irrelevant or difficult to assess. Even “sickness behaviour” contains a component of fatigue (116). Mental fatigue appears as a decreased ability to absorb and process information over time, and becomes pronounced when cognitive tasks have to be performed for longer time periods without breaks (cognitive loading). Often the symptoms are absent or mild in a relaxed stress-free environment (184), which refers to our findings in which depressive-like symptoms occur after stress exposition but not under basal conditions.

In states of anxiety and stress, increased levels of glucocorticoids have been demonstrated. Interestingly, long term increases of glucocorticoids have been shown to result in the production of proinflammatory cytokines such as TNF-α and IL-1β (56), which, from a hypothetical point of view, seems to be in line with the behavioural results in glucocorticoid-deficient and overexpressing mice. Combining these findings with what is described in literature reveals an appropriate idea of how factors that may be investigated by means of the Learned Helplessness or other suitable animal models might be correlated and help to explain interrelations.

**4.6 Role of NOS III**

The role of adult neurogenesis in the pathogenesis of depression represents a core question for the understanding of pathological mechanisms of this disease (119). As suggested by Santarelli et al., an increase in adult neurogenesis might be a phenomenon of antidepressive treatment or contribute to the mechanism of antidepressive therapies (188). Since, according to these findings, the robust reduction in adult neurogenesis in NOS III deficient mice was hypothesized to lead to a depressive-like phenotype, we subjected these animals to the Learned Helplessness paradigm. Surprisingly, the opposite phenotype with a good performance in the task, was found, while no behavioural changes occurred in the Forced Swim Test. In line with this is the finding that NOS III-/- demonstrate good learning capabilities in the Morris Watermaze, indicating improved spatial learning (75). Anxiety-like behaviour was not affected apart from subtle alterations on the Elevated Plus-Maze (75), in our experiments, neither in those of others (51, 52). According to these observations, in which artefacts concerning the Learned Helplessness were excluded, since general effects of this mutation on pain sensitivity and locomotoric features were not evident, it may be concluded that adult neurogenesis does not represent a decisive factor for the genesis of a depressive-like phenotype. This idea was previously supported by Vollmayr et al. (231), however, despite current improvements in the conductibility of animal models one has to keep in mind the problem of transferability of results of animal models to the human situation (46). Possibly the reduction of adult neurogenesis, as it is found in NOS III knock-outs, was not sufficient or compensatory mechanisms were involved. Since cell
survival was unchanged, maybe this represents the decisive mechanism with regard to a depressive state (180). The improvement in the Learned Helplessness, however, continues to be difficult to understand. Since neither neurogenesis nor depression are likely to be explainable by simple rules, but are regulated by fine-tuning of several regulatory systems, which remain to be illuminated, a continuous exchange between disciplines is required to clarify the behavioural impact of neurogenesis.

### 4.7 General Conclusions

For evaluation of the Learned Helplessness paradigm in mice different aspects were investigated, considering the induction of a helpless state by stress as the primary criterion, which can be reverted by antidepressive therapy. While no alteration of BDNF levels were detectable after footshock stress, and a heterozygous mutation of BDNF did not reveal true depressive-like characteristics, thus challenging the Neurotrophin Hypothesis, when exposed to Learned Helplessness stress, GR over- and underexpression affected BDNF levels in opposite directions, thereby confirming the postulation of the Stress Hypothesis of Depression. This indicates, that BDNF itself has to be questioned regarding its role in the pathogenesis of depression, but potentially represents a cofactor in the stress-induced course of this disease, with great relevance in the treatment of a depressive state.

GR<sup>wt</sup> and YGR mice demonstrated converse coping in the Learned Helplessness, which refers to the respective up- and downregulation of BDNF protein as suggested by the Neurotrophin Hypothesis. Once more this emphasizes the assumption, that BDNF is significant as a secondary coaspect under particular conditions, *i.e.* increased vulnerability by a disturbed HPA system. When exposed to...
Discussion

(restraint) stress, GR<sup>−/−</sup> and YGR mice differed again, suggesting a depression-like phenotype in GR heterozygotes and a depression resistance in GR overexpressing mice. These results were furthermore underlined by characteristic findings in the Dex/CRH Test, which, in summary, substantiates the validity of these two mutant lines as models for depression and depression-resistance, respectively.

The investigation of IL-6 knock-out animals revealed a decrease in Helplessness in the same paradigm, suggesting, similar as in YGR mice, depression-resistance, which was supported indirectly by the finding of an IL-6 induction in helpless wildtype animals, which may be related to the opposite findings in GR mutant mice.

Furthermore, it was found, that NOS III knock-out animals, with a robust reduction in adult neurogenesis demonstrated better coping performance in the Learned Helplessness paradigm. Probably the reduction of adult neurogenesis, as it is found in NOS III deficient mice, was not sufficient or compensatory mechanisms were involved. However, in view of the fact that neither neurogenesis nor depression are likely to be explained by simple mechanisms, but are modulated by an interplay of several regulatory systems, which remain to be illuminated, a permanent exchange between disciplines is required to elucidate the behavioural impact of neurogenesis.

In summary, the individual behavioural, pharmacological, and genetic contribution to the results of this thesis validate the Learned Helplessness model as a valuable instrument for the research on depression. The adjustment and modification of this paradigm, which is currently mainly used in rats, to mice bears the advantage of simultaneous examination of environmental as well as genetic factors by investigating the effects of manipulated target genes in transgenic mice. General problems of such procedures may therefore be solved, since an approach from different perspectives becomes possible when working with mice. Current ideas might be investigated and verified by the reflection of findings, which are suggested to overlap with the identified phenomena, thus completing hypotheses, which are examined by animal models of depression.

The fact that the Learned Helplessness model, as it was established in this thesis, is plastic to environmental influences, i.e. housing stress, which is in line with what is discussed in the literature, and thereby supports its position in reflecting diseases, which are also dependent on environmental conditions. Furthermore, this model proved to be representative not only by being reactive to antidepressive treatment, but also when it was employed for the detection of depressive-like effects at behavioural level in genetic animal models of depression, like GR deficient mice. The suitability of this model for the investigation of the pathogenesis and pathophysiology of depression is moreover supported by the finding that only a proportion of animals develop a helpless state which mimics the human situation, representing a realistic illustration of the disease, which should be implied in future scientific issues.
4.8 Perspectives
The high prevalence of depression in humans and only partially effective treatment possibilities suggests that valid animal models could play a central role in further understanding of this disorder. Thus, the Learned Helplessness in mice represents a promising tool, which can be expanded. Among the issues which should be expanded it remains how far the induction of a helpless state may modulate the mechanisms of the HPA-system. If this is the case these findings do correspond to the observed vulnerabilities caused by environmental conditions. Moreover, the pharmacological validation of the GR+ and YGR mice needs to be carried out to evaluate these models suitability for studying neuroplasticity in depression. Another interesting aspect would be the identification of particular brain regions involved by expression studies and chip analyses as well as the examination of neurogenesis-related processes.

Another essential question would be, arising from own studies is if IL-6 is changed in GR-heterozygous and overexpressing animals. Since the association to the other findings presented in this thesis is still unclear, additional aspects have to be included in future hypotheses. One factor to focus on may be the heat shock factor 1 (HSF1), which to some extend appears to cross-talk with the current candidate systems examined in this thesis by regulating the transcription capacity of the glucocorticoid receptor as well as Interleukin-6. Another subject, related to HSF 1, which must be considered in upcoming physiological and behavioural research on consequences of genetic and environmental causes for depression, is the examination of female mice, which are frequently excluded due to their hormonal cycles. Thus it seems a crucial issue to establish reliable methods for the assessment and/or synchronization of the animals’ oestrus cycles. This would allow defined experiments, incorporating natural occurring gender variations as possible causative factors, too. Since women in the human population are more affected by this disease, it appears as an imperative object to look for differences that may cause this predisposition at genetic and/or environmental level.
5 Own Publications

Original Research Articles:


Reviews (Peer-Reviewed):


Appendix

6 Literature


Appendix


responses to novelty, learning, and memory, and the circadian rhythm in cortisol in growing pigs. *Physiol Behav* **68**: 571-578.


Appendix


Appendix


Appendix


Appendix


Appendix


Appendix


Appendix


Appendix


Appendix


Appendix


7 Appendix

Abbreviations

a.m. ante meridiem
ACTH “adrenocorticotropic hormone”
ANOVA “analysis of variance”
BDNF “brain-derived neurotrophic factor”
BDNF+/− heterozygous BDNF knock-out mice
cAMP “cyclic adenosine monophosphate”
CET Central European Time
ChAt “choline acetyltransferase”
cm centimetre
CNS central nervous system
con control
CRE “cAMP response element”
CREB “cAMP response element binding protein”
CRH “corticotrophin releasing hormone”
Dex Dexamethasone
DNS “desoxyribonucleic acid”
ds double shock
ECT electroconvulsive therapy
enr. enriched
e.g. exempli gratia
esc. lat. Escape latency
et al. et alteri
Fig. Figure
g gram
h hour
GR glucocorticoid receptor
GR+/− heterozygous glucocorticoid receptor knock-out mice
HPA “hypothalamus-pituitary-adrenal”
i.e. id est
IL interleukin
IMI imipramine
imp. impoverished
kg kilogram
K.O. “knock-out”
LH Learned Helplessness
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTP</td>
<td>“long-term potentiation”</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoaminoxidase</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>“messenger ribonucleic acid”</td>
</tr>
<tr>
<td>n</td>
<td>number of experimental subjects</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NaCl</td>
<td>natriumchloride</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>p</td>
<td>probability</td>
</tr>
<tr>
<td>PBS</td>
<td>“phosphate-buffered saline”</td>
</tr>
<tr>
<td>PCR</td>
<td>“polymerase chain reaction”</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>potential hydrogenii</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>p.m.</td>
<td>post meridiem</td>
</tr>
<tr>
<td>RIA</td>
<td>“radioimmunoassay”</td>
</tr>
<tr>
<td>RNA</td>
<td>“ribonucleic acid”</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>“standard error of the mean”</td>
</tr>
<tr>
<td>Ser142</td>
<td>“serine 142”</td>
</tr>
<tr>
<td>Tab.</td>
<td>Table</td>
</tr>
<tr>
<td>TrkB</td>
<td>“tyrosine kinase receptor B”</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>wt</td>
<td>wildtype</td>
</tr>
<tr>
<td>YGR</td>
<td>glucocorticoid receptor overexpressing mice</td>
</tr>
</tbody>
</table>
Appendix

Chronological Table

10000 a.d.: Ritual culture of the scull
           Trepanations in living persons, i.e. opening of the scull, are reported,
           but proof of medical indication is missing

3000 a.d.: No psychiatric proofs, but indications that sickness in general, was
           caused by obsession (Mesopotamia)

500 a.d.:  Inauguration of psychiatric curing history primary occurs in the
           greek antique and mental cases are described in the literature.
           Reversal of credence in obsession by supernatural beings to the
           assumption that psychiatric diseases represent organic illnesses

460-370 a.d.: Hippokrates creates the theory of the “humuralpathology”, in which
             an imbalance between the body juices (blood, bile, and slime) is
             illustrated

80-130 a.d.: Description of melancholy and mania by Aretaios from Kapadokia

9th century: Foundation of a psychiatric compartment in Bagdad

1493-1541:  Paracelsus distinguishes psychiatric illnesses as natural diseases

1621:        “Anatomy o the melancholy” by Robert Burton is published in
            Oxford

1790-1830:  “Romantic Psychiatry”: “psychic” vs. “somatic” theories draw an
            opposite pathogenesis concerning cause and consequence of
            psychiatric disorders

1803/1808:  The term “psychiatry” is manifested by Christian Reil
            (“Rhapsodias”) and the common treatment of patients is criticized

1845:       Foundation of the “German Society for Psychiatry and Juristic
            Psychology”

1859:       Discussion of the “Social Darwinism”

1878:       First psychiatric University Hospital in Heidelberg is founded

1895:       Sigmund Freud and Josef Breuer create the psychoanalysis

1903:       Barbitural (Barbiturat) is synthesized

1913:       Karl Jaspers publishes one of the most important books in
            psychiatry: “General Psychiatry”

1929:       Hans Berger publishes about the EEG
### Appendix

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1933</td>
<td>The National Socialists enact the “law of avoidance of inherited diseases in the next generation”</td>
</tr>
<tr>
<td>1938</td>
<td>Electroconvulsive therapy is applied first by Celetti and Bini</td>
</tr>
<tr>
<td>1939-1941</td>
<td>Systematic elimination of psychiatric patients in Germany (“T4-action”)</td>
</tr>
<tr>
<td>1946</td>
<td>Meprobomat (Tranquilizer)</td>
</tr>
<tr>
<td>1949</td>
<td>Lithium (Antimanic)</td>
</tr>
<tr>
<td>1950</td>
<td>“Clinic Psychopathology” is published by Kurt Schneider</td>
</tr>
<tr>
<td>1952</td>
<td>Chlorpromazin (Neuroleptic); Reserpin (Antipsychotic)</td>
</tr>
<tr>
<td></td>
<td>DSM (US-american “Diagnostic and Statistical Manual of Mental Disorders”)</td>
</tr>
<tr>
<td>1957/1959</td>
<td>Imipramine (Tricyclic Antidepressant) is discovered by Kuhn</td>
</tr>
<tr>
<td>1958</td>
<td>Haloperidol (Neuroleptic) discovered by Janssen</td>
</tr>
<tr>
<td>1959</td>
<td>“Antipsychiatry”</td>
</tr>
<tr>
<td></td>
<td>“The divided Self” by Laing describes schizophrenia as consequence of a negative social environment, not as disease</td>
</tr>
<tr>
<td>1960</td>
<td>Chlordiazepoxide (Benzodiazepine)</td>
</tr>
<tr>
<td>1963</td>
<td>Diazepam (Benzodiazepine)</td>
</tr>
<tr>
<td>1967</td>
<td>Lithium as Moodstabilizer</td>
</tr>
<tr>
<td>1968</td>
<td>DSM-II</td>
</tr>
<tr>
<td>1972</td>
<td>Clozapine (atypical Neuroleptic)</td>
</tr>
<tr>
<td>1980</td>
<td>DSM-III</td>
</tr>
<tr>
<td>1991</td>
<td>ICD-10</td>
</tr>
<tr>
<td>1994</td>
<td>DSM-IV</td>
</tr>
</tbody>
</table>
Appendix

Learned Helplessness Protocol:

Validity and Reliability of Depressive-Like States in Mice

The Learned Helplessness paradigm is a depression model in which animals are exposed to unpredictable and uncontrollable stress, *e.g.* electroshocks, and subsequently develop coping deficits for aversive but escapable situations (9). It represents a model with good face, construct, and predictive validity in rats. Despite an increased need to investigate emotional, in particular depression-like behaviors in transgenic mice, so far only a few studies have been published using the Learned Helplessness paradigm. One reason may be the fact that – in contrast to rats (18) - there is not a generally accepted Learned Helplessness protocol available for mice. This prompted us to develop a reliable helplessness procedure in C57BL/6N mice, to exclude possible artifacts, and to establish a protocol, which yields a consistent fraction of helpless mice following the shock exposure. Furthermore, we validated this protocol pharmacologically using the tricyclic antidepressant imipramine. Here we present a mouse model with good face and predictive validity that can be used for transgenic, behavioral, and pharmacological studies.
Appendix

1. Type of research

(1) The protocol described here allows murine studies of molecular, neurochemical, stress-physiological, and behavioral consequences of the Learned Helplessness paradigm, a behavioral model of depression.

(2) A detailed characterization of this model, defining a time course of coping deficits (helplessness) and employing clearly structured definitions of helpless behavior, also evaluated by pharmacological treatment, supplies good validity and reliability of this procedure.

(3) As a result, this protocol can be used for transgenic and pharmacological approaches to study depression in mice.

2. Time required

The course of the Learned Helplessness paradigm starts with an inescapable shock procedure on two consecutive days, each of these shock sessions lasting approximately 52 minutes. Learned Helplessness is assessed 24 hours after the second shock procedure and lasts up to 24 minutes, depending on the animals’ performance in the shuttle box. More then 80 % of helpless animals remain helpless for at least 1 week (Fig. 1).

3. Materials

3.1. Special equipment

The shock procedure was applied in a transparent plexiglas shock chamber (18x18x30 cm³), equipped with a stainless steel grid floor (Coulborn precision regulated animal shocker, Coulborn Instruments, Düsseldorf, Germany).

The two-way avoidance test was conducted in a two-compartment shuttle box (Coulborn Instruments, Düsseldorf, Germany), equipped with red-light beams at the bottom of each of the two compartments to monitor spontaneous shuttling as well as behavioral responses to a light (conditioned) or an aversive footshock (unconditioned) stimulus, respectively. The shuttle box consisted of equal-sized compartments (18x18x30 cm³) that were separated by a small gate (6 cm wide and 7 cm high). Both compartments of the shuttle box contained a grid floor, through which the current was applied, and a signaling light at the top of each compartment. Protocol charts for both, the shock procedure and shuttle box testing, respectively, were designed using Graphic State software (Coulborn Instruments, Düsseldorf, Germany).
Appendix

3.2. *Hotplate*
To exclude altered pain sensitivity as a confounding factor, all mice were tested on the hotplate (ATLab, Vendargues, France).

3.3. *Pharmacological treatment*
The model was pharmacologically validated with the tricyclic antidepressant imipramine in two different doses (10 and 30 mg/kg body weight). We assessed the capacity of imipramine to revert helpless behavior in the shuttle box. NaCl served as vehicle control.

4. **Detailed Procedure**

4.1. *Animals*
10 weeks old male C57BL/6N mice were purchased from Charles River, Sulzfeld, Germany, and acclimatized to single housing in macrolon cages (type II) at constant conditions with a 12 h dark-light cycle and an average room temperature of 22°C for two weeks prior to the experiments, with food and water *ad libitum*. All experiments were approved by German animal welfare authorities (Regierungspräsidium Karlsruhe).

4.2. *Inescapable shock procedure*
(1) Mice were exposed to inescapable shocks during their active (dark) phase. Animals had to be transported in their homecages to the experimental room, and were then placed into the shock chamber.
(2) The shock procedure comprised 360 scrambled footshocks (0.150 mA) on two consecutive days. The footshocks were unpredictable with varying duration (1-3 s) and interval-episodes (1–15 s), amounting to a total session duration of approximately 52 min. During the shock exposure lights were turned off.
(3) Control animals underwent the same handling and contextual procedures without receiving the footshocks. By thorough cleaning with 70 % ethanol, we took care that control animals, which did not receive electroshocks, were exposed to the shock chambers without being distressed by the smell of shocked mice.

4.3. *Assessment of Learned Helplessness*
24 h after the second the shock procedure, Learned Helplessness was assessed by testing shuttle box performance (Graphic State Notation, Coulborn Instruments, Düsseldorf,
Appendix

Germany). Each trial started with a light stimulus of 5 s, announcing a subsequent footshock of maximum 10 s duration (intensity: 0.150 mA). The intertrial interval was 30 s. The following behavioral reactions were defined: *avoidance* as adequate reaction to the light stimulus by changing to the other compartment immediately, *escape* as shuttling to the other compartment as reaction to the electric shock, and *failure* when no attempt to escape was made. Furthermore, the parameter *escape latency* was recorded as the time needed to shuttle into the other compartment after onset of the footshock. For determination of the general activity, the shuttles before the first footshock (*initial activity*), as well as the activity in-between the trials (*intertrial interval activity* or *ITI*) were recorded. Total time of testing for helplessness lasted about 20-24 min, the exact time period depending on the animal’s ability to learn the paradigm and to respond properly. Before each trial the apparatus was thoroughly cleaned with 70 % ethanol.

4.4. Pain sensitivity
To exclude potential artifacts by altered pain sensitivities, which could influence the effect of the electroshocks, a subgroup of mice was tested on the hotplate prior to the learned helpless procedure at a temperature of 52°C. The latency to first reaction (jumping or licking the hind paws) was monitored.

4.5. Defining helplessness
Following the evaluation of the behavioral parameters, the shocked animals were classified as “helpless” or “resistant”, depending on their performance in the shuttle box test. Failures and escape latencies were taken as indicators for helplessness, and a k-means (k = 2) clustering algorithm was applied to a data pool including 212 mice subjected to the described protocol (see 5.3). The number of failures and the escape latencies were used as performance scores of the individual animals, because these are the most commonly reported indices of helplessness (2, 11, 12, 15, 16, 18). These behavioral indices were normalized (i.e. transformed to Z scores) to prevent differences in the range of each variable, which could produce a bias, and then inadvertently be used to implement a clustering process. This classification was further refined by means of a two-step discriminant-canonical analysis, which also provided classification equations for identification of helpless/non-helpless mice following this protocol.

4.6. Pharmacological validation
94 additional C57BL/6N mice were trained and tested in our protocol. Prior to any pharmacological treatment, these mice were classified as “helpless” or “non-helpless” using
the classification equations previously obtained (see sections 4.5 and 5.1) which takes into account the number of failures and the latency to escape.

The duration of helplessness for approximately 10 days dictated a subchronical antidepressive treatment interval of 5-6 days (Fig. 1). Thus, the animals underwent 5 days of vehicle (NaCl), 10 or 30 mg/kg b.w. imipramine regimen. On day 6, animals were re-tested in the escape task. The classification equations were used again to classify each subject, but now the values of the re-test session were used in for the calculation. The changes in this categorical classification (i.e. mice moving from “helpless” to “non-helpless” group) after imipramine treatment were considered as an index of sensitivity of the provided operational definition of helplessness. This analysis was complemented by the assessment of variations in the squared mahalanobis distance (for detailed explanation see 5.3) to the centroid of the non-helpless group before/after the pharmacological treatment to have a continuous rather than categorical index of imipramine effects.

5. Results

5.1. Definition of helplessness

Cluster analysis (Fig. 2) is a group of exploratory data analysis tools, which aim at sorting different objects into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise. Thus, cluster analysis can be used to discover structures in data without providing an explanation or interpretation.
Among the different clustering algorithms available we used the $k$-means procedure, because it allows the *a priori* specification of the number of clusters suspected to be in the sample. In general, the $k$-means method will produce exactly $k$ different groups of greatest possible distinction. In our case this number was restricted to two clusters, aimed at separating helpless and non-helpless mice. In our sample, 65 subjects (approximately 30% of the sample) were categorized as our “helpless” cluster, and 149 subjects were in the “non-helpless” cluster, respectively. The Euclidean distance between both clusters was 2.007933 (squared Euclidean distance $= 4.031235$) and two independent ANOVAs revealed that the obtained clusters significantly differed in the number of failures [$F_{(1, 212)} = 668.716, p < 0.000001$] and latencies to escape [$F_{(1, 212)} = 643.243, p < 0.000001$] (for descriptive statistics see Table 1A).

To further confirm that these empirically defined clusters were mainly reflecting differences in the variables of interest (failures and escape latencies) and to obtain a classification equation for new cases, we performed a two-step discriminant-canonical analysis (Fig. 2). Discriminant function analysis is used to determine which variables discriminate between two or more naturally occurring groups, and when applied to two groups its computation and interpretation is almost identical to a multiple linear regression. We used our clusters as naturally occurring groups and different dependent variables of our escape procedure (failures, escape latency, intertrial interval activity, and initial activity) as possible discriminant factors. The number of escapes and avoidances were not included in this procedure, because these indices are not mutually independent and they are redundant towards others already included (*i.e.* the number of escapes is an algebraic combination of failures and avoidances). Results indicated that only the number of failures and, to a lesser extent, the escape latencies defined both groups ($[F_{(2,214)} = 7.634, p < 0.001] \text{ and } [F_{(2,214)} = 4.728, p < 0.01]$, respectively). The factors “intertrial activity” and “initial activity” failed to enter in the model. Therefore, this model is exclusively based on the number of failures and the latency to escape, because these indices accounted for 100% of the variance (Eigenvalue: 3.200). Thus, the activity parameters can be ruled out as a confounding factor in this paradigm. Although it differed according to the group, this model presented an average concordance of 97% (see Table 1B for detailed results).
### Table 1: A) k-means clustering revealed two distinct clusters among a total number of 214 animals, which reveals a distribution of 30.37 % and 69.26 %, respectively. Means ± SEM of the 2 parameters of relevance, failures, and escape latency.

<table>
<thead>
<tr>
<th>Number of subject</th>
<th>Cluster 1 &quot;Helpless&quot;</th>
<th>Cluster 2 &quot;Non-Helpless&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(% of total sample)</td>
<td>65 (30.37 %)</td>
<td>149 (69.26 %)</td>
</tr>
<tr>
<td>Failures</td>
<td>19.80 ± 0.79</td>
<td>2.55 ± 0.24</td>
</tr>
<tr>
<td>Latency to escape(s)</td>
<td>7.51 ± 0.21</td>
<td>2.49 ± 0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>PREDICTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helpless (p = 0.30374)</td>
<td>Non-helpless (p = 0.69626)</td>
</tr>
<tr>
<td>O</td>
<td>Non-Helpless</td>
</tr>
<tr>
<td>B</td>
<td>Helpless</td>
</tr>
<tr>
<td>S</td>
<td>Total</td>
</tr>
</tbody>
</table>
B) **2-step discriminant-canonical analysis** confirmed the categorization according to $k$-means clustering with a probability of 90.77% in helpless animals, and was in a 100% accordance with the classification of non-helpless animals, respectively.

**Figure 2: Statistical characterization of helplessness.**

Statistical analysis was applied to a datapool comprising relevant parameters of the paradigm, *e.g.* escape latency or escape failures. $k$-means clustering indicates a classification of helpless and non-helpless individuals. For confirmation of appropriateness of the classification a canonical analysis was performed, including intertrial interval activity in the parameters evaluated, in order to determine its relevance for the categorization of helplessness. Canonical analysis verified a proper classification and subsequent defined criteria for helplessness with a probability of 97%.

In a second step, canonical coefficients (data not shown) and classification equations (adjusted to the sample size) were obtained. While these results are presented in Table 2, we would like to highlight that these equations can be used to calculate the classification of any subject tested in the avoidance task described in section 4.3 the probability of being classified as “helpless” or “non-helpless”. To estimate the possible incidence of “spontaneous helplessness” as a
confounding factor, 93 control (non-shocked) mice were tested in this procedure. As expected, all these subjects were classified as “non-helpless” according to the given equations. Figures 3A and 3B show the frequency histograms of the distribution of number of failures and escape latencies for these three groups.

Table 2: Classification equations obtained after a two-step discriminant-canonical analysis. Cells display the constant and the weights corresponding to the two parameters relevant for each subject’s classification as “helpless” or “non-helpless”, respectively. These values are combined in a linear equation: NLH = -8.19995 + (-2.26963 * failures) + (8.60186 * escape latency) or LH = -20.5300 + (-1.6956 * failures) + (9.7177 * escape latency). These equations can be used to perform a priori classifications of any subject tested with the protocol. The case would be classified as belonging to the group for which it has the highest classification score, which reflects a smaller squared Mahalanobis distance towards the centroid of the corresponding group. Also and because we assume a multivariate normal distribution around each centroid, these distances (i.e. the scores of these equations) can be used to calculate the posterior probabilities (i.e. probability of belonging to the group to which each subject has been ascribed).
Figure 3: Distribution of critical parameters for helplessness
A) Escape latencies and B) number of failures observed in our Learned Helplessness paradigm. Mice (n = 307) were tested in a shuttle box for escape deficits as described in section 4.3 after uncontrollable shock exposure (n = 214) or sham-treatment without footshocks (n = 93). The distribution curves demonstrate that shock-exposed mice, which are classified as “non-helpless” by k-means clustering (n = 155, see text for details), and “control” (i.e. non-shocked) animals display the lowest values in both variables. The overlapping distribution of these values in both groups also suggests that all these mice fit into a unique population. Conversely, mice classified as “helpless” (n = 59) show a different (i.e. non-overlapping) distribution and display high values in both parameters, confirming the interpretation that these mice belong to a different population with different behavioral features.

5.1. Time course of helplessness
The average duration of robust Learned Helplessness was 10 days after the second footshock exposure (Fig. 1). This period was followed by a “critical phase”, in which significant improvements of the coping deficits were observed. The given time window of 10 days corresponds closely to the time course of Learned Helplessness in rats (17). This time course restricted the pharmacological validation of this model and will also limit future pharmacological experiments to a subchronic treatment interval of 5-6 days.
5.3. Pharmacological validation of the model

The duration of helplessness for approximately 10 days dictated a subchronical antidepressive treatment interval of 5-6 days. Thus, the animals underwent 5 days of vehicle (NaCl), 10 or 30 mg/kg b.w. imipramine regimen. 94 male C57BL/6N mice were trained and tested according to the methods described in sections 4.2 and 4.3. These mice were classified as “helpless” or “non-helpless” according to the classification equations presented in Table 2A. The next day, vehicle or imipramine treatments were administered as described in 4.5 followed by re-testing in the avoidance task on day 6. The change (after - before treatment difference) in the squared mahalanobis distances to the centroid of the “non-helpless” group was used as main indicator of effects of imipramine treatment (Fig. 4). This is a measure of the distance between two points in the multidimensional space defined by the predictor factors similar to the Euclidean distance, which does not presuppose that the predictors are orthogonal. By taking the centroid of a group as one of these points, and each individual score as the other, the probability of belonging to that group can be estimated, and considering the difference after - before, the impact of the imipramine treatment in the two criteria involved in the helpless definition can be quantified simultaneously. Thus, a two-way ANOVA revealed a significant effect of both factors, group (helpless vs. non-helpless) and treatment (vehicle (NaCl), 10 or 30 mg/kg b.w. imipramine)) [F(1,88) = 35.49, p < 0.0001] and [F(2,88) = 5.25, p < 0.01], respectively. As the interaction between both factors failed to reach statistical significance, appropriate Bonferroni corrections were performed in the subsequent mean comparisons. These comparisons revealed a significant (p < 0.05) dose-dependent reduction of the squared mahalanobis distances to the centroid of the “non-helpless” group in helpless animals treated with imipramine. Interestingly, the same pharmacological treatment did not show any significant effect in the same variable in non-helpless mice, thus discarding an indiscriminate improving effect of imipramine in learning/memory processes. Therefore, as it is selectively reverted by antidepressant treatment, these results seem to confirm that poor avoidance performance after uncontrollable shock exposure is indicative of helplessness. We also compared the individual’s classification provided by the equations presented in Table 2 before and after pharmacological treatment. Pharmacological treatment only affected the classification of “helpless” mice, thereby confirming the results of Fig. 4. Thus, in the cohort of “helpless” mice treated pharmacologically, imipramine produced not only a significant average improvement of latencies and failures, but also in more than half of the animals a reversal from “helpless” to “non-helpless”. As expected, these changes were also related to the imipramine dose, a higher dose being more effective (percentage of subjects becoming “non-
Appendix

helpless”: 57.1, 50.0 and 0.0 % of subjects after 30, 10 and 0 mg/kg of imipramine, respectively, see also Table 3).

### Table 3: Effects of pharmacological treatment with imipramine (10 or 30 mg/kg b.w. vs. saline) on the classification as helpless or non-helpless. Cells display mean ± SEM of failures (F) and latency to escape (L) before and after treatment. Numbers in brackets contain the number of helpless animals before/after treatment in each group.

<table>
<thead>
<tr>
<th>PHARMACOLOGICAL TREATMENT</th>
<th>Saline</th>
<th>Imipramine 10</th>
<th>Imipramine 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEFORE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failures</td>
<td>24.4 ± 0.82</td>
<td>24.0 ± 1.58</td>
<td>23.6 ± 2.8</td>
</tr>
<tr>
<td>Latency</td>
<td>8.74 ± 0.45</td>
<td>8.63 ± 0.41</td>
<td>9.03 ± 0.42</td>
</tr>
<tr>
<td>(8)</td>
<td></td>
<td>(8)</td>
<td>(7)</td>
</tr>
<tr>
<td>AFTER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failures</td>
<td>21.7 ± 3.54</td>
<td>13.6 ± 4.48</td>
<td>16.4 ± 5.47</td>
</tr>
<tr>
<td>Latency</td>
<td>7.68 ± 1.03</td>
<td>5.4 ± 1.2</td>
<td>5.97 ± 1.70</td>
</tr>
<tr>
<td>(8)</td>
<td></td>
<td>(4)</td>
<td>(3)</td>
</tr>
</tbody>
</table>

Table 3: Effects of pharmacological treatment with imipramine (10 or 30 mg/kg b.w. vs. saline) on the classification as helpless or non-helpless. Cells display mean ± SEM of failures (F) and latency to escape (L) before and after treatment. Numbers in brackets contain the number of helpless animals before/after treatment in each group.

**Figure 4**

Figure 4: Pharmacological validation

Effects of imipramine on murine helplessness: bars depict mean ± SEM of the difference before-after pharmacological treatment in the squared Mahalanobis distances from the “non-helpless” cluster.
Appendix

centroid (see methods section for details). Mean comparisons revealed that imipramine does not modify this distance in “non-helpless” animals but it significantly (p < 0.05) reduces it in “helpless” mice. Repeated injection of vehicle solution (0.9 % saline) does not modify this parameter in any group. Taken together, these results suggest that imipramine produces a selective and specific reduction of helplessness.

Alternatively, it has to be considered that the pharmacological treatment produced a variable improvement of depression-like symptomatology within each group. Thus, as an example of another possible application of the herein proposed operational definition of helplessness, the classification equations in Table 2 can be used to provide a qualitative index of the response to antidepressant (i.e. “responsive” vs. “resistant”) or vehicle (i.e. “spontaneous remission”) treatment.

5.4. Assessment of potential artifacts
Statistical evaluation of the activity in-between the shuttle-escape trials showed that helplessness represents a true shock effect, which is not related to general changes in activity that could falsify the results by assessing spontaneous shuttling. Furthermore, alterations in pain sensitivity were excluded to correlate with the level of helplessness (p = 0.998 and $R^2 = 0.0000000108$), which could be responsible for escape deficits because of a lack of sensitivity to the unconditioned aversive stimulus.

6. Discussion
6.1. Setup of the protocol
In order to establish a valid and reliable model of depression as it has been described in rats (18), we designed a Learned Helplessness procedure, in which two series of unpredictable and uncontrollable footshocks applied over 2 days under dark conditions evoke helpless performance in a shuttle box. This is in line with most rodent depression models that use stress as a tool to induce depression-like symptoms.

6.2. Time course
Supporting the face validity of our paradigm, time course experiments revealed a helpless period of about 10 days, which seems comparable to the duration of a depressive episode in humans if set in relation to the lifespan of a mouse. This time window should also be considered, when the Learned Helplessness paradigm is embedded in a behavioral test battery, which includes stressful handling or testing, because this may prolong the period of helplessness.
6.3. Categorization: Definition of Learned Helplessness

According to our criteria for the definition of Learned Helplessness, approximately 30% of the stressed mice become helpless after shock exposure, which is high in comparison to the general prevalence of depression in humans. On the other hand, this rather large fraction allows the investigation of several relevant biological factors, because the procedure yields enough animals for several cohorts, *e.g.* with different antidepressant treatment. Furthermore, a robust fraction of 30% is not prone to floor or ceiling effects.

6.4. Pharmacological validation of the model

The pharmacological validation of our model confirms its specificity by demonstrating a statistically significant improvement of helplessness following antidepressive treatment, while NaCl was not effective. Consequently, helplessness, as defined by our experiments, seems to represent a specific depression-like syndrome, since it can be antagonized by pharmacological treatment (57.1% after 30 mg/kg imipramine) with a similar efficiency as in humans, which have an approximate remission rate of 60% (1).

6.5. Potential artifacts and troubleshooting

To confirm a depressive-like phenotype, several artifacts due to general changes in behavior have to be considered, *i.e.* general changes in activity, which may alter the performance in the shuttle box, or pain sensitivity, which may alter the impact of footshock stress. In our set-up, using C57BL/6N mice, artifacts due to alterations in locomotion (*i.e.* ITI activity) or pain (*i.e.* hotplate) could be excluded, again supporting the validity of this model. Similarly, anxiety-like effects, which could also modify the performance, could be excluded. It is crucial that these potential confounding aspects are investigated and always included in the experimental design, especially when phenotyping mutants, in which general changes in baseline behavior could account for “false positive effects” in the Learned Helplessness paradigm and other behavioral depression models.

6.5.1. The animals are hyperactive

If a large proportion of animals shows hyperlocomotion, which could minimize the read-out of the Learned Helplessness, it is suggested to decrease stress levels by i) reducing noise pollution in the housing facilities, ii) supplying nesting material [17,18], and iii) separating the genders, in order to reduce olfactoric stimulation. It goes without saying that adequate and standardized handling (*i.e.* with gloves) is fundamental. Prior to the Learned Helplessness...
procedure, an openfield can be performed to identify and differentiate between hyperactive and normal animals. Since hyperactivity may be a confounding factor in models of anxiety and depression, it is suggested to solve this problem by a covariance analysis, thereby excluding the effects attributed by the hyperlocomotion. Nonetheless, severe hyperactivity will cause a floor effect, which cannot be compensated, and will therefore limit the suitability of the Learned Helplessness paradigm, and any other model that implicates locomotion.

6.5.2. Too few or too many animals are helpless

If, after inescapable shock treatment, too few or too many animals turn out to be helpless, this may indicate that the paradigm is not sufficiently stressful or includes too much stress, respectively. It is essential that the protocol allows a broad spectrum of behavioral responses, therefore floor and ceiling effects have to be avoided, possibly by changing current intensity (see (8)) or the length of the unconditioned stimulus. Additionally, a faecal bolus can lead to short-circuiting of the shock generator, resulting in only minimal current delivery or even an absence of the electric stimulus to the mouse. Permanent monitoring is therefore crucial to exclude those animals from the experiment. In general, housing conditions in terms of predispositions for stress-susceptibility (i.e. group or single, enriched or impoverished), should to be considered for a successful experimental design.

6.6. Conclusions and preview

To investigate depressive-like behaviors in mice, we designed a Learned Helplessness protocol with good face, construct, and predictive validity. A reliable and valid protocol in mice is particularly important with respect to the investigation of transgenic animals, representing potential genetic models of affective disorders. Apart from the analysis of mutant mice this model can be generally used as a test for emotional behavior, to investigate neurobiological mechanisms in mice that exhibit helplessness or good coping performance in the shuttle box, respectively. Last but not least, this paradigm may turn out to represent a valuable tool for the assessment of new antidepressant strategies.

7. Quick procedure

(a) Expose the mice to 360 scrambled footshocks (intensity: 0.150mA) on two consecutive days.

(b) 24 hours later perform a two-way avoidance paradigm (30 shuttle escape trials) in a shuttle box.
Appendix

(c) Analyze the data by considering “failures” and “escape latency” as the most relevant parameters for the identification of helpless animals.

8. Alternative protocols

Training procedures reported previously, based on the paradigm by Shanks and Anisman (1988) and Caldarone (2000) may represent alternative protocols (2, 14). In these protocols, Learned Helplessness is induced by administering 120 inescapable 4 s footshocks (0.30 mA) with a random interval (range 3-50 s) over a 1 h session. Training is given in two sessions that are spaced approximately 24 hours apart. Mice are placed on either side of the shock chamber, so that the shock is administered either to one or two mice simultaneously. Mice of the same gender are always shocked together. A control group does not receive shock but is exposed to the apparatus for an equal period of time. Shuttle escape testing is conducted approximately 24 h after the second LH training session. The side of the chamber, on which each mouse is placed at the start of the test session, is alternated. Mice are given 30 shuttle escape trials with 30 s intervals between the start of each trial. A gate between the two chambers opens when the shock turned on and the trial terminated when the mouse crosses through the gate into the adjacent compartment. Shock termination is delayed by 1 second after crossing. If an escape response is not made, the trial is terminated 24 s after shock onset.

MacQueen et al. (2001) make use of another modification, in which the animals receive 360 shocks, at an intensity of 0.150 mA, lasting 2 seconds with inter-shock intervals of 9 s. Testing in a 30 shuttle escape trial paradigm is conducted immediately after application of the footshocks (6). The chambers, in which testing takes place, are equipped with a hurdle that opens 4 s after onset of the shock. Shock is terminated as soon as the mouse crossed to the other chamber. The gate is closed immediately and reopens until 4 s after initiation of the next escape trial.

Alternatively to our proposed canonical analysis, the subjects could be classified by using the 95% confidence intervals. This classifies animals, which have scores higher than the mean ± 2 standard deviations of the non-helpless population, as defined by k-means clustering, as helpless. In our case, animals with an escape latency ≥ 4.75s and failures ≥ 6 qualified as helpless. This method, though, has the caveat of splitting the classification into two independent variables. Thus, an animal, which qualifies as helpless by having a high escape latency may still display a low number of failures, and consequently has to be excluded from the study.
Appendix

9. Essential literature references

(2-5, 7, 10, 13, 15, 18)

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft to P.G. (GA427/4-2, SFB636/B3). S.C. had a scholarship from the GK 791, University of Heidelberg.
Appendix

Literature Learned Helplessness Protocol


Curriculum Vitae

Personal Data

Family Name: Chourbaji
Maiden Name: Rein
First Name: Sabine
Date of Birth: December 3rd, 1974
Place of Birth: Heidelberg
Nationality: German
Marital State: married to Said Chourbaji
Parents: Dr. Hans Rein, Dr. Claudia Rein

School Career

1981-1985 Dalberg Elementary School, Ladenburg
1985-1994 Carl-Benz Gymnasium, Ladenburg
June 1994 Abitur (School-Leaving Examination)

Education/University

1994/95 Studies in Spanish /English, English Institute, Language School, Heidelberg
1995-1997 Basic Studies Agriculture Biology, University of Stuttgart-Hohenheim
1997-2001 Advanced Study Period Biology, University of Münster
Studies in Ethnology, History and Romanistics, University of Münster
2001 Diploma Thesis: The Effects of Social-, and Housing Conditions on the Acute Experimental Pancreatitis in C57BL Mice (Main subject: Zoology) at the Institute of Neuro- and Behavioural Biology, University of Münster
2002-2005 Doctoral Thesis: Coping with Stress: Impact of the Hypothalamus Pituitary Adrenal (HPA) System and Neurotrophic Circuits in the Learned Helplessness Model of Depression at the Central Institute of Mental Health, Mannheim, University of Heidelberg (supported by the GK791, University of Heidelberg)
9 Acknowledgements

At this point I want to sincerely thank my doctoral advisor PD Dr. Peter Gass for excellent scientific supervision and education throughout this thesis. I always appreciated his systematic as well as amicable support concerning all issues of discussing, planning and executing the experiments. He was always a patient and helpful supervisor with regard to the successful preparation of this doctoral thesis as well as scientific manuscripts.

Also I would like to thank Prof. Dr. Dr. Fritz A.Henn, for splendid supportive scientific supervision and for generating a really unique and stimulating research environment.

In addition I have to express thanks to my colleagues from the Department of Behavioural Biology at the Central Institute of Mental Health for excellent overall support and “happy hours”.

Especially I am grateful to my friend and colleague, Christiane Zacher, for the outstanding cooperation, indispensable encouragement and friendship during the preparation of this thesis. Moreover it was always a pleasure to elaborate theoretical, technical as well as statistical designs with the help and knowledge of Dr. Carles Sanchis-Segura, who was anytime up to help.

I want to thank all colleagues, who participated in this work, especially Steffi Ridder for exceptional teamwork in a main part of the topic of this thesis.

I am grateful to my husband Said Chourbaji for patience and appreciation throughout the period of this thesis, as well as my parents Hans and Claudia Rein and my friends Thurid Wagner and Oliver Adrian, who always supported me during my studies and encouraged me to work on this particular subject.