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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines
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Für Thomas

und meine Eltern

Contents

Abbreviations

ACTH	Adrenocorticotropic hormone
AD	Alzheimer´s disease
AUC	Area under the curve
AVP	Vasopressin
BNST	Bed nucleus of the stria terminalis
CNS	Central nervous system
CORT	Corticosterone
Con	Control
CRH	Corticotropin releasing hormone
CRH-R1	CRH receptor type 1
CRH-R2	CRH receptor type 2
DA	Dopamine
EMPReSS	European Mouse Phenotyping Resource for Standardized Screens
ENU	N-ethyl-N-nitrosourea
ES cells	Embryonic stem cells
EPM	Elevated Plus Maze
EUCOMM	European Conditional Mouse Mutagenesis
EUMODIC	European Mouse Disease Clinic
EUMORPHIA	European Union Mouse Research for Public Health and Industrial Applications
FELASA	Federation of European Laboratory Animal Science Association
FST	Forced Swim test
GC	Glucocorticoid
GMC	German Mouse Clinic
GR	Glucocorticoid receptor
GWAS	Genome wide association study
HMGU	Helmholtz Zentrum München, German Research Centre for Environmental Health
HPA axis	Hypothalamic-pituitary-adrenal axis

i.c.v.	Intracerebroventricular
IKMC	International Knockout Mouse Consortium
IMPC	International Mouse Phenotyping Consortium
i.p.	Intraperitoneal
IVC	Individually ventilated cages
LC	Locus coeruleus
LDB	Light-Dark-Box
MPI	Max-Planck-Institute
MR	Mineralocorticoid receptor
Mut	Mutant
NA	Noradrenalin
NMDA	*N*-methyl-D-aspartate
OA	Open arm
OF	Open field
PD	Parkinson´s disease
PFC	Prefrontal cortex
PVN	Paraventricular nucleus
ROS	Radical oxigen species
Shirpa	SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, Phenotype assessment
SN	Substantia nigra
SNS	Sympathetic nervous system
SPF	Specic pathogen free
SOP	Standard operating procedure
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
WT	Wild-type

Abstract

We are confronted every day with stressful situations: giving a talk in front of a full auditorium; meeting deadlines at work or just catching a bus or train home in the last second before it leaves. What happens when you're stressed? Two systems are activated: one is the sympathetic nervous system, which floods the body with noradrenalin and adrenalin, and the second system is the hypothalamic-pituitary-adrenal axis (HPA axis), with its end products, the corticosteroids. Both systems prepare the body to cope with the stressful situation. Your attention increases; energy is directed to muscle and brain; other systems, like the digestive tract, are inhibited, to mention but a few changes in response to a stressor. As soon as the last question from the audience has been answered, all the work has been done, or you sit in the bus or train homeward bound, your stress system is shut down and you relax. This is true for most of us. But disturbances in this highly regulated system can have devastating effects. An altered stress system has been implicated in many complex diseases, such as psychiatric disorders, like anxiety and depression, as well as neurodegenerative diseases, like Alzheimer´s and Parkinson´s disease as well as in diabetes.

To study the etiology of human diseases, mutant mouse lines are being generated. In various large-scale projects, like the IKMC (International Knockout Mouse Consortium) and the IMPC (International Mouse Phenotyping Consortium), genetically modified mouse lines for each gene are being generated and phenotyped. Amongst other places, phenotyping occurs at the German Mouse Clinic (GMC) in a comprehensive and standardized manner. Here these lines are checked in a variety of disease areas and organ systems. Thereby each mouse runs through the so-called Primary Screen in a standardized workflow under basal conditions. In this systemic screening, pleiotropic effects of the mutated gene are analyzed and interpreted, creating hypotheses for further in-depth analysis. The Primary Screen is the first step in elucidating gene function and impact, but it does not take into account the environmental challenges a human is facing in life. For this the GMC II has been established. Here five major environmental challenges to human health are simulated in mouse models. These are: physical activity, nutrition, infection, air pollution and stress.

The aim of my thesis was to establish an acute and a chronic stress test for the stress-platform. For both the acute and chronic stress tests, restraint was the method of choice. Here I present the successful establishment of the acute stress test, which reproducibly and reliably detects alterations in stress reactivity in a non-invasive mode, through a behavioural read-out. Animals of the C57Bl6 strain reproducibly show a stress-induced increase in vertical and horizontal activity in the first five minutes of the Open Field Test. Using two mutant mouse lines with known opposing stress-reactivity, a CRH overexpressing line (Cor26Nes) and a *CRH-R1*-KO mouse line I validated the stress test. I could show that mutants of the higher stress-reactive

line, the Cor26Nes line, already increase activity after 15 minutes stress, whereas mutants of the *CRH-R1*-KO line show no stress-induced alterations in these parameters even after a two hour stress exposure. I could further demonstrate that the secretion of corticosterone (CORT) is necessary for the effect of stress on behaviour, which makes the test a highly valuable tool to non-invasively detect differences in CORT response. Also a wide number of other possible factors impacting on the behavioural read-out, e.g. different behavioural tests, time points of testing and methods of testing behaviour, were evaluated. Furthermore, I demonstrated that repeated testing is feasible, revealing that the homotypic stressing reliably induces CORT secretion. This provided the possibility of successive investigation, for example of blood CORT levels and pharmacological manipulations. Although this acute stress test has been established with C57Bl6 males it is applicable to other mouse strains, females as well as old-aged animals. With the established acute stress test we have already successfully analyzed several mutant mouse lines, which are now being analyzed in more detail, based on the observed differences in stress responsivity.

The aim of establishing a chronic restraint stress test was to be able to subject mutant mouse lines to stress throughout a defined time and to analyze different end points, in order to determine the effects of stress on disease onset and progression. The goal of my thesis was to establish a chronic stress protocol and identify reliable and easily measurable parameters as a read-out of stress efficacy. To this end I subjected both wild-type C57Bl6 and mutant mouse lines, with different stress-reactivity, to diverse protocols. I could show that unpredictable chronic stress over a two week period induced anxiety-related behaviour in C57Bl6 males. Further testing revealed that behavioural tests as a read-out can easily be confounded by as yet undefined factors. Nonetheless, changes in body weight emerged as a reliable and reproducible read-out, which was validated by the two mouse lines, which showed opposing effects in the acute restraint stress; the Cor26Nes and the *CRH-R1*-KO mouse line. I could also show in the Cor26Nes mouse line that two weeks of stress had an influence on the morphology of hippocampal neurons, which was time-dependent.

In conclusion I have established both a robust acute and chronic stress test, with which mutant mouse lines can be tested to determine gene x environment (in my case stress) interactions. The acute stress test is reproducible, non-invasive and reveales alterations in corticosterone response. This can be used as an indicator for further in-depth analysis of the functionality of the HPA-axis. Also it can be used as a first exploratory test for stress x genotype interactions, which can predispose for stress-related disorders. To elucidate such interactions, the chronic stress test can be applied and different biomarkers relevant for disease progression should be measured.

Zusammenfassung

Wir werden täglich mit belastenden Situationen konfrontiert: einen Vortrag vor einem vollem Auditorium halten, das Einhalten von Fristen bei der Arbeit oder den Bus oder Zug, der nach Hause geht, in der letzte Sekunde erwischen, bevor er wegfährt. Was passiert, wenn wir gestresst sind? Zwei Systeme werden aktiviert: Das eine ist das sympathische Nervensystem, welches den Körper mit Noradrenalin und Adrenalin überschwemmt, und das zweite System ist die Hypothalamus-Hypophysen-Nebennieren Achse, mit seinen Endprodukten, den Kortikosteroiden. Beide Systeme bereiten den Körper darauf vor, mit der stressvollen Situation umzugehen. Unsere Aufmerksamkeit erhöht sich, Energie wird an Muskeln und Gehirn geleitet, andere Organe, wie der Verdauungstrakt, werden inhibiert, um nur einige stressbedingte Änderungen zu nennen. Sobald die letzte Frage aus dem Publikum beantwortet wurde, die ganze Arbeit getan ist, oder wir im Bus oder Zug nach Hause sitzen, wird unser Stressbewältigungssystem heruntergefahren und wir entspannen uns. Dies gilt für die meisten von uns. Aber Störungen in diesem stark regulierten System können verheerende Folgen haben. Veränderungen in der Fähigkeit Stress zu bewältigen stehen im Zusammenhang mit vielen komplexen Krankheiten, wie zum Beispiel mit psychiatrischen Störungen, wie Angst und Depression, sowie mit neurodegenerativen Erkrankungen, wie Alzheimer und Parkinson, als auch mit Diabetes.

Genetisch veränderte Mauslinien werden in zunehmendem Umfang eingesetzt, um die Entstehung humaner Krankheiten zu studieren. In verschiedenen Großprojekten, wie dem IKMC (International Knockout Mouse Consortium) und dem IMPC (International Mouse Phenotyping Consortium), werden genetisch veränderte Mauslinien für jedes Gen erzeugt und phänotypisiert. Unter anderem, wird die Phänotypisierung an der „German Mouse Clinic" (GMC) in einer umfassenden und standardisierte Art und Weise durchgeführt. Diese Mauslinien werden hier in einer Vielzahl von Krankheitsfeldern und Organsystemen untersucht. Dabei durchläuft jede Maus den sogenannten Primärscreen in einem einheitlichen Ablauf unter basalen Bedingungen. In diesem systemischen Screening werden pleiotrope Effekte des mutierten Gens analysiert und interpretiert, wodurch Hypothesen für weitergehende Studien generiert werden. Der Primärscreen ist der erste Schritt in der Aufklärung der Genfunktion und dessen Einflusses, jedoch werden Umweltbelastungen, denen der Mensch in seinem Leben ausgesetzt ist, dabei nicht berücksichtigt. Dazu wurde die GMC II aufgebaut. Hier werden im Mausmodell fünf großen Herausforderungen der menschlichen Gesundheit simuliert. Diese sind: körperliche Aktivität, Ernährung, Infektion, Luftverschmutzung und Stress.

Das Ziel meiner Arbeit war es, einen akuten und einen chronischen Stresstest für die Stressplattform zu etablieren. Sowohl für den akuten, als auch für den chronischen Stresstest, war Immobilisierung die Methode der Wahl. Hier präsentiere ich die erfolgreiche Etablierung des akuten Stresstests, der reproduzierbar und zuverlässig Änderungen in der Stressreaktivität nicht-invasiv durch Verhaltensänderungen detektiert. Tiere des C57Bl6- Stamms zeigen reproduzierbar eine stressinduzierte Zunahme der vertikalen und horizontalen Aktivität in den ersten fünf Minuten des Open Field Test. Durch zwei genetisch veränderte Mauslinien mit bekannter, entgegengesetzter Stressreaktivität, d.h. eine CRH überexprimierende Linie (Cor26Nes) und eine *CRH-R1*-KO-Mauslinie, wurde der Stresstest validiert. Ich konnte zeigen, dass die Mutanten der stressreaktiveren Cor26Nes Linie bereits nach 15 Minuten Stress eine erhöhte Aktivität aufwiesen, während Mutanten der *CRH-R1*-KO Linie keine stressinduzierten Veränderungen in diesen Parametern zeigten, auch nicht nach einer zweistündigen Stressbelastung. Ferner konnte ich zeigen, dass die Sekretion von Kortikosteron (CORT) für die stressinduzierten Verhaltensänderungen notwendig ist, wodurch der Test ein sehr wertvolles Mittel ist, um nicht-invasiv Unterschiede in der CORT Antwort zu detektieren. Außerdem habe ich eine große Anzahl von anderen möglichen Einflussfaktoren auf das Verhalten untersucht und bewertet, zum Beispiel verschiedene Verhaltenstests, der Zeitpunkt des Tests und verschiedene Testmethoden. Darüber hinaus konnte ich zeigen, dass wiederholte Tests möglich sind, und somit das homotypische Stressen zuverlässig die CORT-Sekretion induziert. Damit ist die Möglichkeit gegeben, anschließend weitere Untersuchungen durchzuführen, wie beispielsweise die Bestimmung der CORT-Werte im Blut oder pharmakologische Manipulation. Obwohl dieser akute Stresstest mit C57Bl6 Männchen etabliert worden ist, ist er auch auf andere Mausstämme, Weibchen sowie alte Tiere anwendbar. Mit dem etablierten akuten Stresstest haben wir bereits erfolgreich mehrere genetisch veränderte Mauslinien getestet, die nun, basierend auf den beobachteten Unterschieden in ihrer Stressreaktivität, im Detail analysiert werden. Das Ziel der Etablierung des chronischen Stresstests war es, im Stande zu sein genetisch veränderte Mauslinien über einen definierten Zeitraum zu stressen und verschiedene Endpunkte zu analysieren, um die Auswirkungen von Stress auf den Ausbruch der Krankheit und deren Progression untersuchen zu können. Das Ziel meiner Arbeit war es, ein chronisches Stressprotokoll zu etablieren und zuverlässige und leicht messbare Parameter zu identifizieren, die die Stresswirksamkeit anzeigen. Zu diesem Zweck wurden sowohl wildtyp C57Bl6 als auch genetisch veränderte Mauslinien mit unterschiedlicher Stressreaktivität verschiedenen chronischen Stressprotokollen unterzogen. Ich konnte zeigen, dass ein unvorhersehbarer chronischer Stress über einen Zeitraum von zwei Wochen Angstverhalten in C57Bl6 Männchen induziert. Weitere Tests zeigten, dass diese Verhaltensänderung als read-out durch noch nicht definierte Faktoren beeinflusst werden kann und daher unzuverlässig ist. Dennoch stellte sich heraus, dass Veränderungen des Körpergewichts als zuverlässige und reproduzierbare read-outs für die Wirksamkeit des chronischen Stresses herangezogen werden können. Dies wurde auch durch die beiden Mauslinien, die entgegengesetzte Effekte im akuten Stresstest zeigten, die Cor26Nes und die *CRH-R1*-KO Mauslinie, vali-

diert. Auch konnte ich anhand der Cor26Nes Mauslinie zeigen, dass ein zweiwöchiger chronischer Stress einen zeitabhängigen Einfluss auf die Morphologie hippocampaler Neurone hat.

Zusammenfassend gesagt, habe ich einen robusten akuten und chronischen Stresstest etabliert, mit welchen genetisch veränderte Mauslinien getestet werden können, um die Interaktion von Genen und Umweltfaktoren (in meinem Fall Stress) zu bestimmen. Der akute Stresstest ist reproduzierbar, nicht-invasiv und zeigt Veränderungen der Kortikosteronantwort auf. Dies kann als Indikator für weitere, eingehende Analysen der Funktionalität der Hypothalamus-Hypophysen-Nebennieren Achse verwendet werden. Auch kann er als erster explorativer Test genutzt werden, um Stress-Genotyp Wechselwirkungen anzuzeigen, die prädisponierend für stressbedingte Erkrankungen sein können. Um solche Wechselwirkungen aufzuklären, kann der chronische Stresstest angewandt und verschiedene Biomarker, die für die Krankheitsprogression relevant sind, gemessen werden.

1 Introduction

1.1 The German Mouse Clinic

1.1.1 The German Mouse Clinic I

Standardized high-throughput phenotyping of mutant mouse lines

Genetically modified mouse mutants have revolutionized biomedical research. Mice are the geneticists´ favourite "pet". Not only because of the high homology their genome has to the human (we share approximately 95 % of homologous genes), but also because functional embryonic stem (ES) cells can only be generated from mice, which are needed for most of the genetic manipulations and establishment of mouse mutant lines in the genotype-driven[1] approaches (see Box 1). In contrast to rats, which have been used extensively in life science research until now, mice show a lower spontaneous mutation rate, which make them more suitable for genetic manipulations. The toolbox for inducing mutations is large. It ranges from N-ethyl-N-nitrosourea (ENU)- induced mutagenesis, constitutive and conditional knock-outs to knock-downs and knock-ins of human genes to recent developments like Zink-finger nucleases and TALENs and it is still growing [62, 91, 96, 124]. Box 1 illustrates various different technologies. Note that it is just a random selection of methods and not all-encompassing.

Mouse models mimicking human diseases are now extensively generated. Pleiotropic effects of gene mutations make it necessary to screen the whole animal "from head to toe" unravelling effects in different organs and at various time points in the developing animal. The challenge the mouse line creator is facing, is that he is an expert in his field but not in others. To overcome this problem the German Mouse Clinic (GMC)[2], headed by Martin Hrabě de Angelis, was set up as an open access platform.

[1] Genotype-driven approach means that a known gene is manipulated and the mutant mouse is screened for phenotypes. In the phenotype-driven approach, mice are screened for phenotypes after inducing a random mutation in their genome. Subsequently the mutation has to be identified.

[2] http://www.mouseclinic.de

Box 1:

Different technologies for the induction of mutations in mice

Phenotype driven:

Chemical mutagenesis:
ENU-induced mutagenesis: Male mice are injected with a synthetic alkylating agent, N-ethyl-N-nitrosourea (ENU), which leads mainly to point mutations in the spermatogonial stem cells. Offspring is then analysed for phenotypic dominant mutations and back-crosses are analysed for recessive ones. Thereafter identification of the mutated gene or genes starts.

Gene trapping:
Here no individually created gene vector has to be designed. A Gene-trap vector consists of the beta-galactosidase reporter gene (*lacZ*) and a neomycin-resistance gene. It inserts at a random site within the genome. Transcription of the gene with the inserted gene trap leads to a fusion protein, which can be easily detected, and disrupts functionality of that gene.

Genotype driven:

Transgenic mice:
An exogenous gene, a transgene, is microinjected into the pronucleus of a zygote and randomly integrates into the genome. It is frequently used for dominant mutations causing diseases, e.g. in Alzheimer´s disease, the overexpression of mutated human APP.

Virus Injection:
Viral vectors are used by injecting the shortened viral genome, which has no infectious and reproductive abilities any more, with the integrated transgene, into the region of interest. This enables to look at changes at a defined time point and a certain area. It is a relatively easy and fast way to analyse the effects of ectopic expression of a transgene.

Gene targeting / Knock-out mice:
Embryonic stem (ES) cells are transfected with a gene targeting vector, including generally a neomycin-resistance gene flanked by wild-type fragments to enable homologous recombination. After homologous recombination ES cells are injected into blastocysts, which in turn are transplanted into foster mothers. Resulting chimeras are then bred and back-crossed to create a mutant mouse line. These are called constitutive knock-out mice.

Gene targeting / conditional Knock-out mice:
Approximately 25% of the constitutive gene knock-outs are embryonical lethal. To circumvent this problem conditional knock-outs have been created. It enables the activation of the gene of interest in a certain cell-type. For this the essential exon is flanked by two *loxP* sites. After homologous recombination the chimeras can be bred to cre-deleter mice. The cre-recombinase cuts out the area between the *loxP* sites, resulting in a knock-out. The cre-recombinase can be under the control of a cell-specific promoter, thereby only being activated and leading to knock-out of the gene in a specific cell type.

Gene targeting / inducible Knockout mice:
To address the question of time dependent gene expression, the establishment of inducible mutants occurred. Here two systems have been used to influence cre-recombinase activity: transcriptionally by doxycyclin or posttranslational through tamoxifen

Anti-sense and RNA-interference:
Here genes are inhibited at the level of the mRNA. Anti-sense inhibition is achieved by adding anti-sense mRNA. These bind the complementary mRNA, which leads to inhibition of translation or degradation of the mRNA.

Zinkfinger nucleases:
Zinkfinger nucleases are sequence specific endonucleases that bind and cleave DNA. The double-strand break they introduce is repaired by homologous recombination or non-homologous end-joining through which mutations by deletions or insertions occur.

Talens:
TALENS are fusion proteins of a TAL (Transcription activator-like) and a nuclease. The TAL consists of seven repeats, which specifically bind one DNA base pair. TALENS can be easily generated for user-specific DNA sequences. Like for the Zinkfinger nucleases, the TALENS introduce a double-strand break, which is repaired by homologous recombination or non-homologous end-joining.

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The GMC was founded at the Helmholtz Zentrum München (HMGU, German Research Centre for Environmental Health) in 2001. It provides a comprehensive phenotyping of mutant mouse lines with standardized methods. The GMC combines the expertise from various fields of mouse genetics, physiology and pathology and collaborates with clinicians (see Figure 1.1). At the HMGU these experts work closely together, through which a comprehensive insight into the screened mouse line is achieved. These various fields are divided into 14 screens: Allergy, Behaviour, Bone and Cartilage, Cardiovascular, Clinical Chemistry, Energy Metabolism, Eye, Immunology, Lung Function, Molecular Phenotyping, Neurology, Nociception, Pathology and Steroid Metabolism (Order and time in which different tests occur see Figure 1.2). In these 14 screens mice are analysed routinely in a standardized workflow (see Figure 1.2) in the so-called Primary Screen, collecting in sum about 550 different parameters in multiple tests [63]. During their time in the GMC the mice

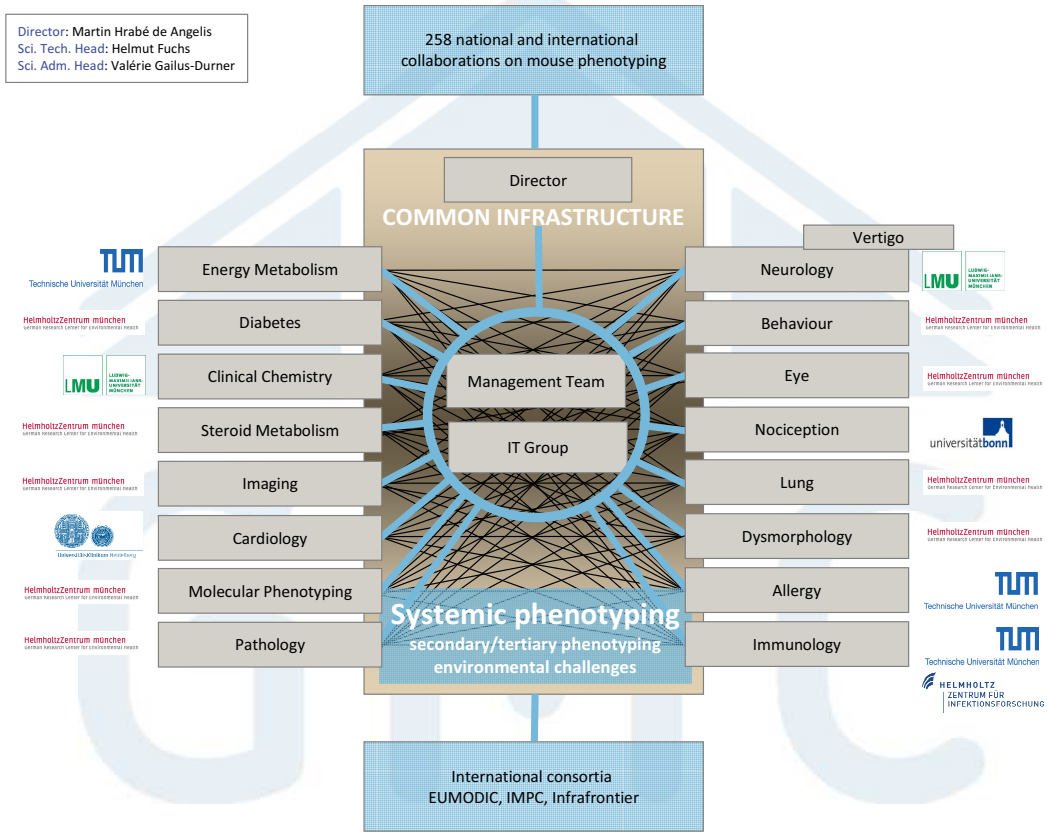

Figure 1.1: The GMC consortium

are housed in individually ventilated cages (IVC) to guarantee specific pathogen free (SPF) conditions, according to the FELASA (Federation of European Laboratory Animals Science Association) protocols. Each screen has its own lab and mouse

housing room next door. The test order is arranged based on their sensitivity and non-invasiveness and to the age at which the parameter measured is most robust. This is why the pipeline starts with the Behaviour Screen, where experimentally naive animals are tested, and for example the Dysmorphology Screen assesses parameters of bone and cartilage later in life of the mouse, when the skeleton has already matured. Data is collected from both males and females with a number of 10-15 animals per sex and genotype. Analysis of the data is done by uploading the raw data into a database, the so-called mouseDB [113], in which R-Scripts for statistical analysis are run. The results are then discussed and interpreted in a joint meeting with all screeners and the mouse provider present. If the Primary Screen reveals interesting changes in the phenotype and the screener and mouse provider find it worthwhile going into detail, then a new cohort of animals can be more closely analysed within the Secondary and Tertiary Screen, including here a tailor-made workflow for the specialized in-depth analysis.

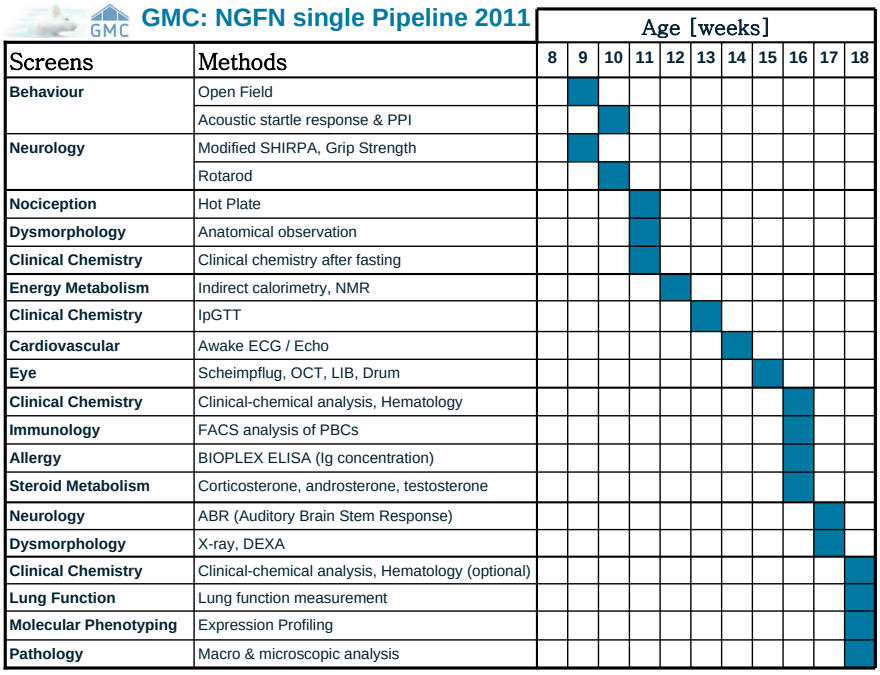

Figure 1.2: The GMC workflow
The GMC workflow consists of a single pipeline (adapted from [64])

The GMC was the first of its kind and soon other centres world wide adopted the idea of a comprehensive analysis. In October 2002 different centres from Europe (including the Medical Research Council Harwell (MRC, UK), the Institut Clinique de la Souris (ICS, France) and the Welcome Trust Sanger Institute (UK)) joined in the EUMORPHIA[3] (European Union Mouse Research for Public Health and Industrial Applications) program in which the standard operating procedures (SOP),

[3]http://www.eumorphia.org

named EMPReSS, were agreed on and a uniform workflow was established. This is the basis for a valid phenotyping of mutant mouse lines across different centres and the collection of the produced data in one huge database accessible for the scientific community. The database was also established within the EUMORPHIA program under the name of EuroPhenome[4]. The idea was to simplify the access to data of mouse mutant lines, which are of interest for different people within the scientific community. The EUMORPHIA project ended in march 2006 and phenotyping of 500 mutant mouse lines was continued in the EUMODIC[5] (European Mouse Disease Clinic) program. These mutant mouse lines mainly came from the EUCOMM[6] (European Conditional Mouse Mutagenesis Program) project, where mouse mutants are generated for each protein-coding gene on a C57BL/6N background. Further phenotyping, of the remaining mutants of the 20 000 and more genes, will now be conducted in a new project, the IMPC[7] (International Mouse Phenotyping Consortium), which began in 2011. This consortium combines the expertise of the pan-European project members with centres world wide including the US, Canada, Australia, China, Japan and Korea.

That the idea of the GMC is a very exciting and valuable tool in science, can be seen in the enormous popularity and the long waiting list mouse providers are willing to accept to let their mutant mouse line being screened at the GMC [1]. Also requests from different institutions from various countries asking for help in building up their own mouse clinic points to the fact that more of this large-scale standardized comprehensive phenotyping is needed.

1.1.2 The German Mouse Clinic II

Genome-environment interactions

Genetic diseases can be divided into two major groups: single gene disorders and multifactorial disorders. The single gene mutations are inherited in a dominant or recessive way, either on autosomal or sex-chromosomes, and can easily be detected in family trees. Multifactorial diseases, in contrast, are more complex. There is an extensive interrelationship between several genes and environmental factors, for instance age and lifestyle, including diet, activity and stress. These combined factors make up the phenotype of the disease, thereby causing a high variation between diseased individuals.

In Parkinson´s disease (PD) or Alzheimer´s disease (AD) it takes several decades for the disease to manifest. Several risk factors have been proposed and associated

[4]http://www.europhenome.org
[5]http://www.eumodic.org
[6]http://www.knockoutmouse.org/about/eucomm
[7]http://www.mousephenotype.org

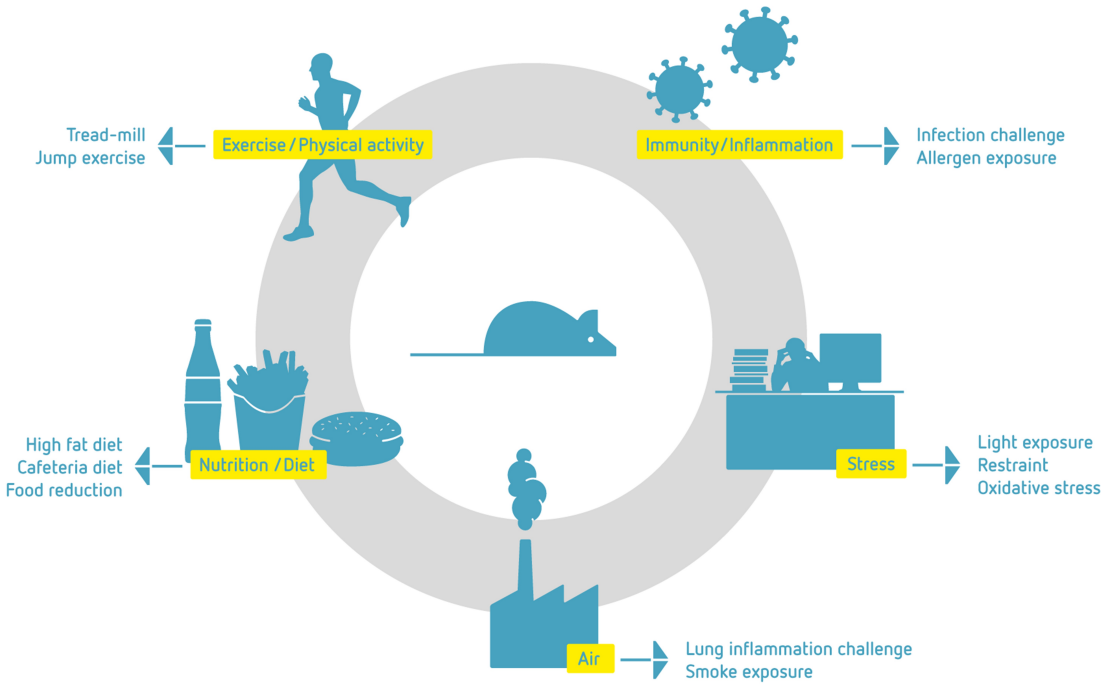

Figure 1.3: The GMC II Envirotype platforms

with different diseases. The major risk factors in neurodegenerative disorders are age, stress and lifestyle.

Checking phenotypes under basal, unchallenged levels in mouse models is surely the first step in elucidating endophenotypes[8] related to a particular gene or mutation, but does not give the whole picture. In order to evaluate environmental influences on genetics and to test these gene-environment interactions, the GMC II was started. The aim is to offer challenges mimicking major environmental threats to human health. The challenges incorporated in the envirotype platform are infection, pollution, diet and nutrition, physical activity, chemical as well as psychophysical stress (see Figure 1.3).

This platform is purely hypothesis driven and not, as the Primary Screen, explorative. Also a combination of different challenges is possible. The workflow here is highly flexible and needs to be discussed by the screeners involved and the mouse provider. Genetic mouse models, which do not show the full blown phenotype of a multifactorial disease under basal conditions, can be challenged in an attempt to tease out the medical condition.

After screening mouse models for phenotypes under basal, unchallenged levels and

[8]Endophenotypes are intermediate traits between the clinical symptoms and genetics

analysing the influence of environmental factors, the idea of screening for pharmacological compounds in these models is not far-fetched. Currently the GMC III is on its way with the goal of evaluating substances for their relevance in disease treatment and systemic effects.

1.2 Stress

Walter Canon, who never actually used the term "stress", studied the effects of environmental stimuli on the organism. In 1932 the physiologist at Harvard University was the first to use the word "homeostasis" to describe the maintenance of the animals internal equilibrium [24]. The well-known phrase "fight or flight" reaction was coined by him [23]. He described that a challenge to the animal results in an activation of the sympathetic nervous system (SNS) to fight or flight the stressful situation and regain homeostasis. According to Canon, disease is the result of a malfunctioning restoration of homeostasis.

Hans Selye, a physician, introduced "stress" into biomedical research to describe the effect of noxious agents on the organism [153]. The term "stress" originally comes from mechanics, where it is used as a measure of pressure on a deformable body. Hans Selye, who is often referred to as the father of modern stress research, developed his model of a General Adaptation Syndrome, which consists of three stages: the alarm reaction, the resistance and the exhaustion phase [154]. The alarm reaction includes the classical "fight or flight" reaction in which the autonomic nervous system is highly involved and body functions drop below optimum. In the second phase the organism tries to cope and overcome the stress or it reacts inappropriately leading to first pathological symptoms (e.g. increased adrenal size, thymic atrophy and gastric ulcers). In the third phase, the phase of exhaustion, the organism can no longer compensate and meet energy demands. Here, continuing stress is detrimental and even fatal. Selye thought of the stress response as being unspecific, meaning that all kinds of stressors lead to the same response.

Not all researchers agreed with his theory of unspecifity. One criticism was that even if the same stressor is applied, not all animals react the same way and that even the anticipation of stress can cause a physiological stress response [115]. John Mason, a psychiatrist, described three major psychological determinants that give rise to a stress response in most of the individuals. These were: novelty, unpredictability and uncontrollability [116]. Stressors can be divided into different groups: physiological stressors, such as heat or toxins (with which Selye worked most of the time), physiological stressors with a strong psychological component, like immobilization, and purely psychological stressors, such as exposure to a new environment and fear.

Bruce McEwen tried to overcome the terminological problems of "stress" and "homeostasis" by using the term "allostasis". With this term he describes the organisms ability to adapt to a new steady state as a response to an environmental challenge

[118]. In the short term this is beneficial and helps coping with stressors, but repeated challenges lead to allostatic (over)load. Increased frequency of exposure to stressors, increased intensity of stressors or mal-adaption of the response system lead to allostatic overload, which in turn causes permanent damage, not only in peripheral tissues but also in the central nervous system (CNS). For example hippocampal neurogenesis is disrupted, dendrites atrophy and even whole neurons are lost [119].

Challenging stimuli, of the internal or external environment, evoke a stress response which triggers physiological and behavioural responses in order to ensure the organisms survival. Two major systems are activated by this disruption of homeostasis, one of them is the SNS of the autonomic nervous system, with its major secretagogues noradrenalin (NA) and adrenalin (see next subsection) and the other one is the hypothalamic-pituitary-adrenal (HPA) axis (see subsection 1.2.2) with its final secretory products, the glucocorticoids (GC). Both systems contribute to the adaptive response, by physiological changes, for example by mobilizing energy (increasing gluconeogenesis, lipolysis and glycolysis), enhancing respiration and redistribution of blood flow, enhancing vigilance and focused attention and inhibiting vegetative systems such as the immune and reproductive system as well as digestive function. In response to a stressor the SNS is the first to react. NA and adrenalin are released within seconds. The HPA-axis takes longer to secrete GCs (within minutes), reaching a peak secretion approximately 15 to 30 minutes after the initial stress, and also needs more time to subside again. Depending on the stressor-type, the intensity and how it is perceived, different brain regions are activated leading in sum to different behavioural and neuroendocrine outputs [48, 85].

In the following subsections the two systems will be described in more detail.

1.2.1 The Sympathetic Nervous System

Stress triggers the release of catecholamines, namely NA and adrenalin, from different parts of the body, e.g. the adrenal medulla and catecholaminergic neurons in the brain. The release of NA and adrenalin is one of the quickest responses to a stressor. Both NA and adrenalin exert their functions on many different target tissues. One of the best known and well-studied actions are the effects on the cardiovascular system. Here they increase blood flow and pressure as well as cardiac output. In the brain, stress-induced NA release has been shown to be responsible for enhancing vigilance and alertness mediated by the prefrontal cortex (PFC) [16].

The locus coeruleus (LC; A6 region) is the primary source of NA in the brain. In the primate brain it accounts for approximately 70 % of NA. In the rat the LC contains about 3 000 neurons and in the human about 24 000 neurons [61]. They send their projections throughout the entire CNS, which will be explained in more detail in subsection 1.2.3.

Immobilization stress has been shown to increase LC neuronal activity by assessment of c-fos expression and tyrosine hydroxylase (TH) activity and its protein levels. TH is the rate limiting enzyme in catecholamine synthesis. Interestingly transcription factors and MAP kinase pathways are differentially modulated depending on single or repeated stress sessions [72].

In the periphery NA and adrenalin are synthesized in the adrenal medulla (approximately 20 % NA and 80 % adrenalin) and secreted by chromaffin cells acting as hormones on their target organs. NA, unlike adrenalin, plays a major role as a neurotransmitter in the SNS. Here NA is released from the post-ganglionic nerve terminals mediating the "fight or flight" response.

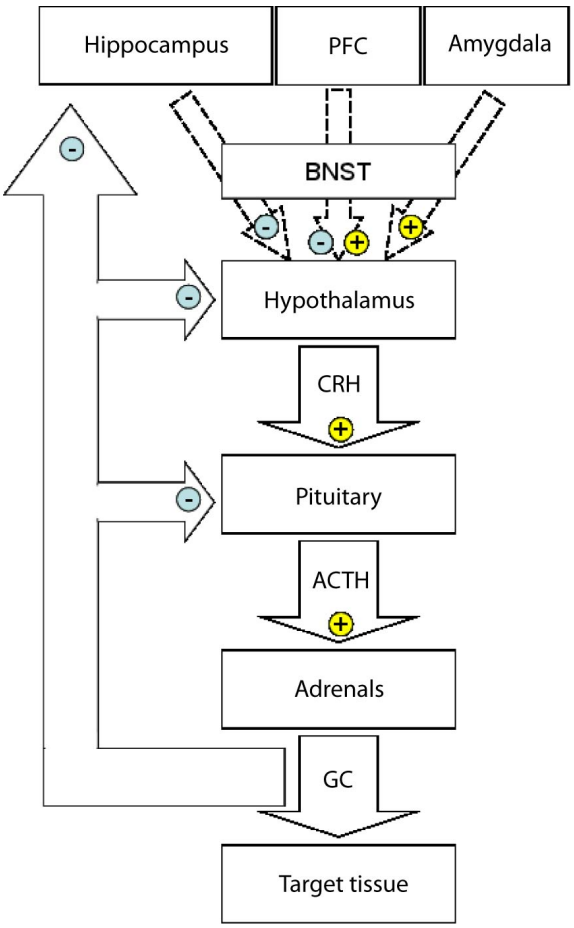

Figure 1.4: The HPA axis
The hippocampus, PFC and Amygdala have only an indirect influence (spotted arrows) via the BNST on the HPA axis. CRH is released from the hypothalamus and reaches its receptors in the anterior pituitary stimulating ACTH release. ACTH reaches the adrenal cortex via the circulation, where it stimulates glucocorticoid secretion. Glucocorticoids act on different target organs including the brain. The negative feedback loop reaches the pituitary, hypothalamus as well as the hippocampus. For abbreviations see text. Plus and minus indicate stimulation or inhibition respectively.

1.2.2 Hypothalamus-Pituitary-Adrenal Axis

Two major systems orchestrate the response to a challenging or stressful situation. In addition to the already mentioned SNS, which mediates the fast "fight or flight" response, the HPA axis (see Figure 1.4) is activated (for review see [47, 164]). In response to a stressor different brain regions are activated resulting in the secretion of corticotropin releasing hormone (CRH; aka CRF- corticotropin releasing factor) from the hypothalamus, specifically from the paraventricular nucleus (PVN), into the median eminence from where it reaches the anterior pituitary via the hypophyseal portal system. Acting on its receptors, here CRH receptor type 1 (CRH-R1), CRH leads to the release of a polypeptide hormone called adrenocorticotropic hormone (ACTH) into the blood stream. Most of the CRH containing neurons also express

vasopressin (AVP), which potentiates the effect of CRH at the level of the anterior pituitary. AVP itself can also stimulate the release of ACTH [152]. It is important to note that these AVP containing neurons originate in the parvocellular region of the PVN and project to the median eminence, while AVP containing neurons from the magnocellular division project to the posterior pituitary, where they are responsible for regulating salt-water homeostasis [55]. Via the circulation ACTH, released from the anterior pituitary, reaches the adrenal glands, atop of the kidneys, where it induces the synthesis and secretion of GCs (cortisol in humans and corticosterone (CORT) in rodents) from the zona fasciculata of the adrenal cortex. The GCs have different effects on various target organs, which culminate in the allocation of energy and resources to cope with the stressful situation [35, 85, 48]. About 30 minutes after the activation of the HPA axis, GC levels reach their maximal concentration and return to basal levels at about two hours after cessation of the stressor. Furthermore, the lipophilic GCs, which easily cross the blood-brain-barrier, act as negative-feedback regulators at different hierarchical stages in the brain, amongst others the hippocampus, the PVN and the pituitary, thereby terminating their own secretion. This ensures a rapid shut-down of the activated system, to return to homeostatic levels after the stressor has ceased.

GCs are not only released in response to stress but also in a pulsatile manner and a circadian rhythm, peaking before the onset of the active period and decreasing towards a trough at the start of the inactive period. Metabolic and behavioural processes have an influence on HPA activity and vice versa [7, 42].

GCs act on two different intracellular glucocorticoid receptors in the brain: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Both receptors have different functions, as indicated by different properties and distribution. The MR has a six to ten times higher affinity for GCs than the GR [142]. Therefore most of the MRs are occupied by GCs at basal levels (90 % of the MR and 10 % of the GR are occupied by GCs), whereas the GRs are activated only during circadian peak secretion (i.e. before waking) and in stressful situations when levels are high (MRs are then fully occupied and GRs are occupied between 67-74 %; [46]). The distribution patterns of MRs and GRs in the brain differ. MRs are exclusively expressed in the limbic system, whereas GRs are present more widespread in the subcortical (e.g. PVN and hippocampus) and cortical (e.g. PFC) areas as well as in the brain stem (e.g. LC) [142].

Upon GC binding, chaperones, like hsp90 and other heat shock proteins, dissociate from the receptor and the receptor-complex translocates to the nucleus. In the nucleus, the receptors bind as homodimers on the glucocorticoid response elements (GRE) by which transcription of the gene can be altered [89]. The receptor can also regulate transcription in an indirect way by interacting with other transcription factors, thereby preventing their translocation to the nucleus and binding to DNA. Besides this relatively slow mechanism of transcriptional change, there is also a fast mechanism by which GCs act, but the receptors involved have not been identified yet. It is probable that these receptors might be integrated in the cellular membrane

enhancing velocity of signal transmission [53, 133].

The strong expression of GRs in different brain areas indicates their importance in regulating and modulating the HPA axis. For example, both the hippocampus and the PFC have been shown to inhibit the HPA axis. The amygdala, a brain region highly involved in fear and emotion, exerts an activational effect on the stress axis [167]. Interestingly, none of the above mentioned structures show a direct input into the PVN. They project to the bed nucleus of the stria terminalis (BNST) where their signals converge and the integrated output is then directed to the PVN (see Figure 1.4). The BNST acts as a relay station and exerts both inhibitory and stimulating effects [73].

1.2.3 Orchestrating the Stress Response

CRH is the major mediator of the stress response initiation. The active form of CRH is a 41-amino acid long peptide. It was the first member of the CRF-related family, which also contain the urocortins [176]. Its structure is highly conserved across mammalian species and its receptors, CRH-R1 and CRH-R2, are widely expressed in the brain. CRH-R1 is highly expressed in the anterior pituitary, pointing to its central role in conducting CRH signals within the HPA-axis. It is also expressed in the BNST, the main integrative nucleus, as well as in the amygdala, the hippocampus and PVN, only to mention a few [49]. Here CRH exerts its function as a neurotransmitter modulating synaptic transmission. These regions, as well as the LC, have CRH-containing neurons. CRH has a much lower affinity for the CRH-R2 than for the CRH-R1. Both receptors exert different effects; CRH-R1 mediates most of the anxiety-related actions, whereas CRH-R2 mediates stress-effects more on vegetative functions. CRH receptors have seven transmembrane domains, are G-protein coupled and activate the adenylylcyclase. In the PVN the transcription of the *CRH* gene is inhibited by GCs [102].

The hypothalamus is a major integrative centre by mediating both the response to a stressor via the HPA-axis as well as the SNS. This was shown by injecting CRH i.c.v., which increased the basal firing rate of neurons from the LC [177]. NA, in turn, can stimulate the release of CRH from the PVN [139]. Different stressors (such as restraint or the forced swim test) activate NA neurons [41, 107]. These results suggest a close interaction of CRH and the NA-system. Different groups have shown that there is a reciprocal connection between CRH neurons in the PVN and NA neurons in the LC [178, 179, 180]. But not only the PVN innervates the LC. CRH-containing neurons from the central nuclei of the amygdala send their projections to the LC as well. NA neurons of the LC project to various brain regions, including the hippocampus [61, 178]. Other NA regions, such as A1 and A2, send projections to the BNST and the PVN respectively and receive input from their targets. This demonstrates the highly complex system involved in stress response.

1.2.4 Stress and Disease

All living organisms are exposed to external and/or internal stressors, which may be physical or emotional or even only perceived as such. These adverse effects challenge the internal equilibrium, called homeostasis. Adaptive responses, mainly driven by the HPA-axis and the SNS, come into play to regain homeostasis. The homeostatic system, including the stress system, exert their effects in an inverted U-shaped dose-response curve, where healthy homeostasis is in the middle, optimal range of the curve and to either side of the curve inappropriate adaptations would occur, harmful to the organism in either the long or short term [36]. The appropriate response of the stress system to the perceived stressor is essential for the well-being of the organism. As the stress system has pleiotropic effects on several target organs it is not astounding that an inappropriate activity of the stress system has a strong impact on other systems regulating growth, development and metabolism. This can lead to several different disorders, such as endocrine, metabolic, cardiovascular and autoimmune disorders. It has been shown, for instance, that hypersecretion of GCs leads to the metabolic syndrome, which can manifest in cardiovascular disease, diabetes, immunosuppression, gastric ulcers, only to name a few [118, 175]. The impact of a pathological dysregulation of the HPA-axis has also been shown to affect the CNS. Hence a link has been established between dysfunctional HPA activity and mood disorders [20, 76, 125] as well as cognitive impairments [109, 110] and neurodegenerative diseases [27, 161, 162].

One of the most prominent stress-related disorders is depression, or major depressive disorder as it is officially called. Depression is the most common disabling disorder with 121 million people affected world wide[9]. Depression is diagnosed according to the criteria of the Diagnostic Manual of Mental Disorders (DMS-IV), which delineates a major depressive episode by the following symptoms: depressed mood, anhedonia, sleep disturbances, fatigue, weight gain or loss, low self-esteem and thoughts of suicide, to name but a few. Although the disorder has been known since the ancient Greeks (Melancholia as it was called by Hippocrates around 400 B.C.) the pathomechanisms underlying are still unresolved [130]. Nowadays it has become clear that an altered stress system, especially the HPA-axis, plays a major role in the disease [76]. Yet it is still unclear, whether these alterations are the cause or a consequence of the depression. The common view is that an impaired HPA-axis seems to precede depressive episodes [48, 75, 76].

For understanding the underlying (patho-)mechanisms, animal models have been used, yet with varying success. It is not trivial mimicking a complex, multifactorial human disease such as depression in a mouse model. The different, sometimes even contradictory (weight loss vs weight gain) symptoms, especially low self-esteem and thoughts of suicide, are difficult, if not impossible to recapitulate in a mouse model. Still it is possible to establish valid models for mood disorders. But mimicking the full-blown human phenotype in a mouse model is more than difficult. It has become

[9] http://www.who.int/mental_health/management/depression/definition/en

accepted that a useful strategy is to mimic endophenotypes [40]. Endophenotypes are associated with the disease but have a closer link to genetics and therefore the variability between individuals is lower. In a mouse model for depression, endophenotypes, like anxiety-related behaviours, changes in body weight and neuroendocrine disturbances, can easily be measured. To achieve this, different methods have been applied. Exposure to stressful life events has been implicated in the development of depression. Thus many mouse models of depression are based on chronic stress, often applied in adulthood, for example chronic restraint stress, chronic mild stress or chronic social defeat. Also, exposure to stress during critical periods in life (i.e. pre- and postnatal development, infancy and adolescence) shapes the 'hard-wiring' of the stress-axis and leads to altered stress responses later in life [9, 105]. This is the basis for using early life stress models, like maternal separation and fragmentation of maternal care [143]. Still, reproducibility is not always given and not all the animals always show the expected changes in endophenotypes. This is also true for a different way of tackling the challenge of unravelling pathomechanisms in depression: the use of genetically engineered mouse models. Although epidemiological studies revealed a genetic risk for depression of about 40-50% [130] the search for specific genes remains cumbersome. The link between gene and disease is not as clear as in other diseases and reproducibility between Genome Wide Association Studies (GWAS) is not always achieved.

The difficulties encountered in modelling depression are also apparent in other complex disorders, such as AD and PD. Although the candidate genes are more obvious than in psychiatric diseases, not all models show the full-blown human etiopathology. By bringing the two major risk factors together, gene and environment, scientists hope to unravel the interactions leading to endophenotypes and/or the disease.

Studies of gene-environment interactions have already been successful. An example illustrating this is a study in non-human primates by Barr et al [14]. Rhesus monkeys, like humans, can carry a long or short variant of serotonin transporter gene-linked polymorphic region (5-HTTLPR), which results in differences in serotonin transporter expression. In humans these variants are associated with depression and anxiety amongst others, although not all studies could replicate these findings (for review see [26]). Barr et al [14] exposed young rhesus monkeys, carrying either the long/long or the short/long version, to different rearing conditions and measured ACTH release later in life after separation stress. It was shown that mother-reared animals did not differ in their response independent of genotype, but peer-reared animals did. Here animals with the short/long variant showed an increase in ACTH after stress, whereas the animals carrying the long/long variant did not. It illustrates the synergy of genetic predisposition and environmental factors and strengthens the theory that a genetic predisposition can alter vulnerability to environmental influences, which in the end can lead to the onset of a disease. Also gene-environment interactions are being investigated in the field of neurodegeneration. Mouse models of AD have shown an increase in A-beta levels after chronic restraint stress [33].

The gene-environment interactions are now being studied in the GMC II. One of the major environmental risk factors, as already mentioned above, is stress. Many studies have shown the influence of stress on disease. The link between cardiovascular illness, such as coronary heart disease, or immunological diseases and chronic stress is demonstrated by many medical studies [21, 118, 154]. But most of the information we acquired about stress and disease so far came from the research field of depression. Here, it has been shown that the onset of depression and relapse is associated with traumatic events, such as divorce or the death of a loved one. During the acute phases of depression, patients show elevated cortisol levels, blunted circadian fluctuations, decreased GC receptor function, exaggerated CRH/ACTH/cortisol response and enlarged adrenals [12]. Stress has been shown to have an effect on memory performance [108, 110, 119]. It is not astounding that the neuroanatomical region of learning and memory, the hippocampus, is affected. The hippocampus is reduced in size when excessive GC production is apparent, as in patients with Cushing´s syndrome or in patients chronically treated with GCs [20]. Due to high abundance of GRs and MRs, the hippocampus is one of the central players in HPA-axis regulation and is mainly responsible for the rapid shut-down of the axis after stress. Excessive exposure to GC have been shown to reduce hippocampal volume and retraction of dendritic branches possibly leading to a dis-inhibition of the HPA system resulting in increased GC production and starting a vicious cycle. Many of these effects have been recapitulated in animal models [8, 11, 28, 73, 79, 84, 117, 148, 163]. After repeated stressing rats showed learning and memory deficits, reduced hippocampal volume, reduction in dendritic branching and number of spines, as well as reduced neurogenesis [29, 108].

But the hippocampus is not the only region affected. Chronic stress has been shown to have dramatic effects on several other target organs, for example the thymus, which atrophies under the influence of excessive GCs, depicting the strong influence GCs have on the immune-system [18, 169]. In contrast, adrenals are enlarged by increased GC levels, pointing to their role as the main site of GC synthesis and release [174].

Also in neurodegenerative diseases, such as AD and PD, stress has a deteriorating effect [30, 159, 162]. Prominent features in AD are, amongst others, hippocampal degeneration and hypercortisolemia [70]. Patients treated with GCs or exposed to stress show worsening of their symptoms [111]. In animal models of AD chronic stress accelerates cognitive impairments, elevates senile plaque deposition, increases A-beta levels and provokes abnormal hyperphosphorylation of tau [99, 82]. Even neuronal loss occurs in the hippocampus. Also in Parkinson´s patients symptoms worsen when exposed to stress [30, 92, 158]. GRs are expressed in the basal ganglia and make them responsive to GCs [6]. It has been shown that immobilization stress in mice can selectively damage nigrostriatal neurons [94], probably through elevating dopamine (DA) and glutamate levels in the striatum and inhibiting their re-uptake. Both of these transmitters are neurotoxic and can form radical oxygen species (ROS), which in turn lead to oxidative stress [158, 159], which is also seen

in PD [81]. As neurodegenerative diseases have another major risk factor, ageing, it is interesting to note that hypersecretion of GCs has been implicated in ageing as a deleterious factor [99, 106, 150, 160].

Stress has become a clear risk-factor for many multifactorial diseases, but it is still difficult to test and evaluate its impact. In the scientific community different stressors have been proposed and used, by which stress can be applied to rodent models. But when searching the literature, not one of these protocols seems reproducible over time and/or applicable within the GMC II. For the GMC II reproducibility and reliability of the test are the most important factors. Also factors like time needed for conducting an experiment, costs and space requirements are of interest for selecting the appropriate stressor.

The goal of this thesis was to search for a test producing reproducible and reliable results. I established an acute stress test for evaluating stress-reactivity as an endophenotype in mouse models. Deciding on the right stressor for the acute stress was relatively straightforward. Restraint was chosen, as it is frequently used in different labs and consistently produces an increase in CORT levels (for review see [22, 65, 134]). Although there are many different methods in restraining an animal we used the method most widely applied: restraint was applied in well-ventilated 50 ml tubes (see section 5.2). This is a psychophysical stressor [44], with no physical harm to the animal.

For the chronic stress we conducted a more elaborate literature search. Here again stress procedures varied and even within the same research groups protocols changed over time. Also the results from behavioural tests at the end of the chronic stress period were inconsistent within one method. We stayed with restraint as the procedure of choice and found a publication by Kim and Han [93], which characterized the chronic restraint stress with behavioural read-outs and CORT analysis. They found that a chronic restraint stress for two hours per day for 14 days caused increased anxiety-related and depression-like behaviours in the stressed mice. The recapitulation of this method was the starting point of our chronic stress experiments.

2 Aim of the study

A dysregulated stress axis is a risk factor for developing serious diseases. An altered stress response is implicated in various severe disorders such as depression, AD and PD. It seems obvious to test mouse mutants modelling multifactorial diseases for differences within this system. The GMC, as a large-scale screening platform of mutant mouse lines, is predestined to implicate stress and stress-reactivity tests in its screening.

The aim of this thesis consists of two major parts:

1. Establishing an acute stress challenge and
2. Establishing a chronic stress challenge.

Restraint was the stressor of choice, a non-invasive psychophysical stress, which is often applied in rodents.

For the acute stress challenge, the objective was to establish a reliable and reproducible protocol for testing stress reactivity with a behavioural test as a read-out. The acute stress test shall supply the experimenter with information about the stress-reactivity, which is an indicator of stress perception and processing. The results aid in interpreting the behavioural acute stress response of a mutant mouse line and point out in which direction future or further research should go. The acute stress test should help to elucidate which mouse model might be most promising to test in the chronic restraint stress challenge.

The second part of the study consisted of the establishment of a chronic restraint stress protocol, which leads to reproducible changes in easily measurable parameters and hence ensures straightforward read-out of the efficacy of the stress applied. The idea of the chronic restraint stress is to reliably stress mutant mouse lines throughout a given time and check for disease related end-points, to see if stress has an influence on disease onset, progression and/or severity.

Incorporated into the GMC II, both stress challenges can be used as modules and therefore be combined with each other and other challenges or read-outs of different screens to investigate gene x environment interactions.

3 Results

3.1 Acute Stress Challenge

3.1.1 Establishment of the Acute Stress Challenge

The acute stress challenge was established with C57BL/6J male mice at the age of approximately two months. Animals of the stress group were restrained in well-ventilated tubes. After stress exposure, they were placed into a clean cage and left undisturbed for 20 minutes. Directly after this interval, animals were transferred into the Open Field (OF) arena and behaviour was automatically measured by the Actimot system. Unstressed animals were placed into the OF directly from their home cage.

Several different cohorts of male animals were tested with the same protocol (see Figure 3.1), only varying in the duration of stress, ranging from 15 minutes, over 50

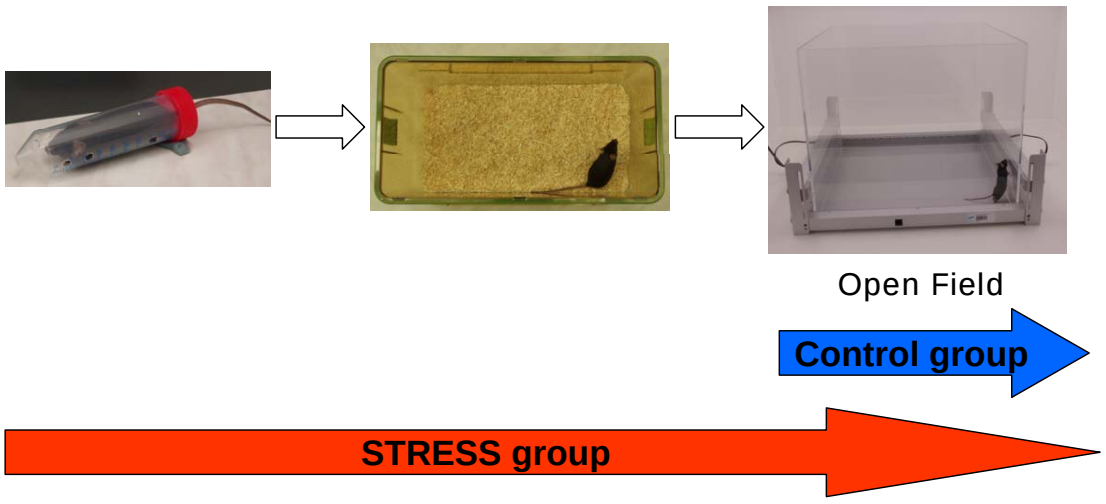

Figure 3.1: Scheme of the acute stress test
Animals of the stress group were restraint in well-ventilated 50 ml tubes. After the stress period these animals were placed into a clean animal housing cage for the interval period of 20 minutes. The OF is depicted as the behavioural read-out test. Animals from the control group were placed directly from their home cage into the OF arena.

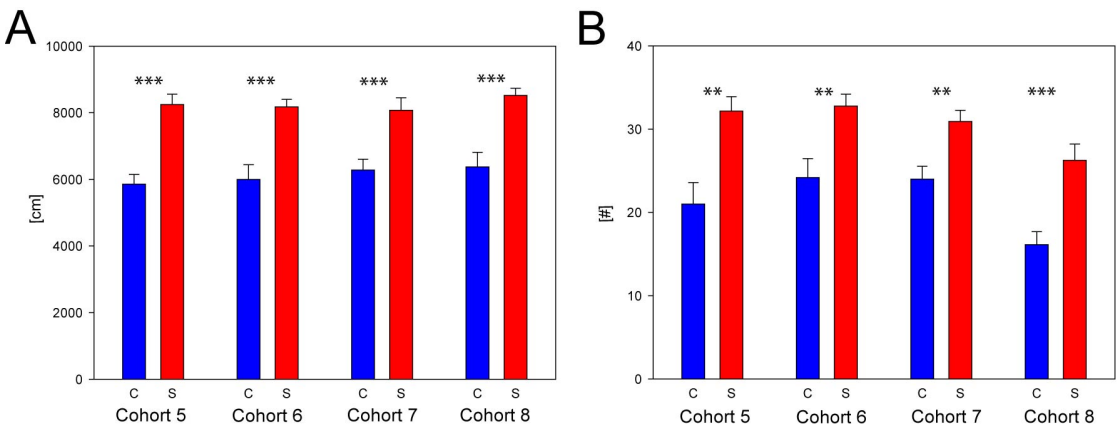

Figure 3.2: Two hours acute stress in C57BL/6 males

Wild-type C57BL/6 strains were tested with the two hour acute stress protocol. Depicted are the *distance travelled* (A) and the *number of rearings* (B) in the first five minutes of the OF. Control groups in blue and stress groups in red. Note that cohort 8 is of the C57BL/6N strain, whereas all the other cohorts are of the C57BL/6J strain. Error-bars shown as SEM; Significances: **- $p<0.01$; ***- $p<0.001$; n=11-13 per group

minutes up to two hours. The various parameters and the stress-induced changes, which were analysed for the OF are shown in Table 3.1 and Table 3.2. The only reproducible change throughout one test condition was found in the first five minutes of the OF in the parameters *"distance travelled"* and *"number of rearings"* with the two hour stress duration. Here the stressed group reliably shows an increase in both vertical and horizontal exploration (see Figure 3.2; *Distance travelled*: Cohort 5: t_{22}=-5.578, P≤0.001; Cohort 6: t_{22}=-4.606 P≤0.001; Cohort 7: t_{22}=-3.609, P=0.002; Cohort 8: U=19, P<0.001; *Number of rearings*: Cohort 5: t_{22}=-3.595, P=0.002; Cohort 6: t_{22}=-3.285, P=0.003; Cohort 7: t_{22}=-3.410, P=0.003; Cohort 8: t_{26}=-4.080, P≤0.001). None of the other stress durations did reliably show differences in these parameters. Note that not only the difference between the control and stressed group is robust, but also the absolute values have little variance between the different cohorts in *distance travelled* in the two hour stress test. The variance between the cohorts for the *number of rearings* are greater, but it should be taken into account that the last cohort in Figure 3.2 is of the C57BL/6N strain, whereas all the other cohorts are of the C57BL/6J strain.

None of the successive behavioural tests (Light-Dark-Box (LDB)- six hours post OF, Elevated Plus Maze (EPM) - 24 hours post OF, Forced Swim Test (FST) - 30 hours post OF) uncovered reproducible differences between groups (data not shown).

We also tested the EPM and the LDB as read-out tests directly after the 20 minute interval of the two hour stress duration but no significant differences could be observed between the control and stressed groups (data not shown).

		Distance travelled [cm]					Number of rearing [#]					Distance in centre [%]					Time in centre [5]				
Cohort	**Stress Duration**	05 minute bin	10 minute bin	15 minute bin	20 minute bin	**Total**	05 minute bin	10 minute bin	15 minute bin	20 minute bin	**Total**	05 minute bin	10 minute bin	15 minute bin	20 minute bin	**Total**	05 minute bin	10 minute bin	15 minute bin	20 minute bin	**Total**
1-BI6J	15minutes	ns	*↑	ns	ns	**ns**	ns	ns	ns	ns	**ns**	ns	ns	ns	*↓	**ns**	ns	ns	ns	ns	**ns**
9-BI6N	15minutes	*↑	ns	**↓	ns	**ns**	ns	ns	ns	ns	**ns**	ns	ns	*↓	ns	**ns**	ns	ns	ns	ns	**ns**
2-BI6J	50 minutes	T↑	ns	ns	ns	**ns**	ns	ns	*↓	ns	**ns**	ns	ns	ns	**↓	**ns**	ns	ns	*↓	**↓	**↓
3-BI6J	50 minutes	***↑	ns	ns	ns	**ns**	**↑	ns	*↓	ns	**ns**	ns	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**
4-BI6J	50 minutes	***↑	ns	ns	ns	*↑	**↑	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**	*↑	ns	ns	ns	**ns**
5-BI6J	2 hours	***↑	ns	ns	ns	*↑	**↑	ns	ns	ns	**ns**	ns	ns	*↓	*↓	*↓	ns	ns	*↓	*↓	*↓
6-BI6J	2 hours	***↑	ns	ns	T↓	**ns**	**↑	ns	ns	T↓	**ns**	ns	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**
7-BI6J	2 hours	**↑	ns	T↓	T↓	**ns**	**↑	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**
8-BI6N	2 hours	***↑	*↑	ns	ns	*↑	***↑	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**	ns	ns	ns	ns	**ns**

Table 3.1: Acute stress test: Open Field parameters in five minute bins and totals

↑- increase; ↓- decrease; Significances: ***- p<0.001; **- p<0.01; *- p<0.05; T- p<0.1; ns- not significant

		Arena		Periphery				Centre					
Cohort	**Stress Duration**	Resting time [s]	Average Speed [m/s]	Distance travelled [cm]	Resting time [s]	Permanence time [s]	Average Speed [m/s]	Distance travelled [cm]	Resting time [s]	Permanence time [s]	Average Speed [m/s]	Latency [s]	Number of Entries [#]
1-BI6J	15minutes	ns	ns	T↑	ns	ns	ns	ns	ns	ns	*↑	ns	ns
9-BI6N	15minutes	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
2-BI6J	50 minutes	ns	ns	ns	ns	**↑	ns	ns	*↓	**↓	*↑	ns	ns
3-BI6J	50 minutes	ns	ns	ns	ns	ns	ns	ns	T↓	ns	ns	ns	ns
4-BI6J	50 minutes	ns	*↑	*↑	ns	ns	*↑	ns	ns	ns	ns	ns	ns
5-BI6J	2 hours	ns	*↑	**↑	ns	*↑	*↑	ns	ns	*↓	*↑	ns	ns
6-BI6J	2 hours	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
7-BI6J	2 hours	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
8-BI6N	2 hours	ns	*↑	*↑	ns	ns	*↑	ns	ns	ns	T↑	*↑	ns

Table 3.2: Acute stress test: Open Field parameters for 20 minute bin

↑- increase; ↓- decrease; Significances: ***- p<0.001; **- p<0.01; *- p<0.05; T- p<0.1; ns- not significant

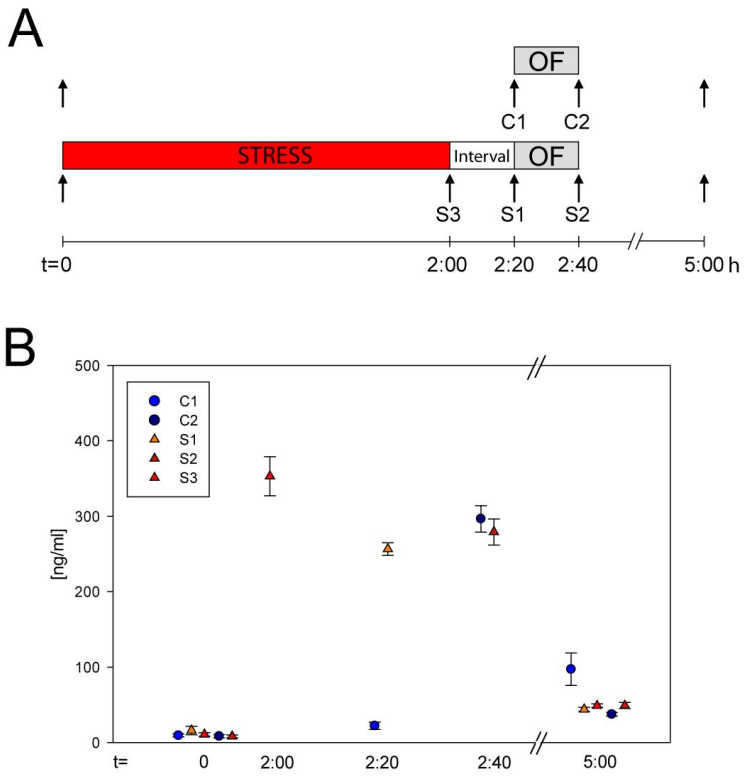

Figure 3.3: Corticosterone profile during the acute stress procedure
(A) Scheme of the experimental design. Arrows indicate time point of blood withdrawal. C1 and C2 are control groups. Stressed groups, S1 to S3, were exposed to a two hour restraint stress period. (B) CORT levels at the different time points of the different groups. At basal (t=0) no differences in CORT levels between groups can be seen. Directly after stress (S3), after the interval (S1) and after the OF (S2) CORT levels of the stressed animals are high. CORT levels of the control group (C1) are low at t=2:20, whereas they increase after the OF (C2). At t=5h all groups have low circulating CORT levels again. n=11-12 per group

Acute stress and corticosterone To verify that our restraint stress leads to a physiological stress response, we measured CORT levels in serum samples at different time points. Figure 3.3 depicts that baseline levels from all groups are equally low. After two hours of stress the expected rise in CORT levels in the stressed group can be observed (S3 in Figure 3.3), which stays elevated after the interval and even after the OF (cf. S1 and S2, respectively). Interestingly the CORT levels from the unstressed control group are also at high levels after the OF (cf. C2), suggesting that the OF exposure itself is a stressor. All CORT levels sink back to low levels five hours after the first blood withdrawal.

Acute stress and pharmacology For evaluation of the role played by CORT in the behavioural response to the two hour acute stress test, we inhibited the synthesis of CORT. Metyrapone was the drug of choice. It blocks the catalysing enzyme, the

steroid 11beta-hydroxylase. Thereby circulating CORT levels are dramatically reduced, thus eliminating negative feedback of CORT. We could show that CORT is responsible for the increased activity in the first five minutes of the OF. Figure 3.4 shows the interaction between the different treatment groups in the first five minutes of the OF in both the locomotor activity and *number of rearings*, indicating that CORT feedback is important in the behavioural outcome of the acute stress test (*Distance travelled*: Interaction: $F_{1,46}$=17.223, P<0.001; post hoc: Holm-Sidak: Vehicle: Con vs Stress: t=5.213, P<0.001; Metyrapone: Con vs Stress: n.s.; *Number of rearings*: Interaction: $F_{1,46}$=7.669 P=0.008; post hoc: Holm-Sidak: Vehicle: Con vs Stress: t=3.092, P=0.003; Metyrapone: Con vs Stress: n.s.). To control for CORT synthesis inhibition, blood was collected after the OF. CORT levels were significantly reduced in the Metyrapone-injected animals ($F_{1,47}$=365.509, P<0.001; data not shown). We also saw an interaction between injection and stress ($F_{1,47}$=13.935, P<0.001), demonstrating a stress-induced increase of CORT in the vehicle-treated group and no such effect in the Metyrapone-treated group (post hoc: Holm-Sidak: Vehicle: Con vs Stress: t=5.840, P<0.001; Metyrapone: Con vs Stress: ns).

To further investigate the mechanism by which the behavioural response is mediated, we blocked one of the two major CORT receptors, namely the GR, which has a lower affinity for CORT but is occupied during peak levels of CORT secretion (see subsection 1.2.2). RU486, also called Mifepristone, is a potent GR antagonist. There is no significant interaction between the vehicle-treated groups and the RU486-treated groups. Only a stress-effect can be observed in both groups (*Distance travelled*: Stress effect: $F_{1,56}$=17.010, P<0.001; *Number of rearings*: Stress effect: $F_{1,56}$=20.586, P<0.001).

Open Field after acute stress without the interval To evaluate the necessity of the 20 minute interval before the OF, we tested animals in the two hour stress test but placed them directly into the OF after stress had ended. As can be seen in Figure 3.5 A, stressed animals spend a considerably higher amount of time grooming themselves during the first five minutes of the OF (U=0.00, P<0.001). Over the course of 20 minutes in the OF grooming durations increase by a factor of 10 from control to stressed animals (Percentage of grooming: Con: median: 3,7 % ; Stress: median: 35,5 %; data not shown). The stress effect in the first five minutes of the OF in the *distance travelled* is still observable, but relatively small and the expected increase in *number of rearings* after stress is gone (see Figure 3.5 B; *Distance travelled*: t_{21}=-2.437, P=0.024; *Number of rearings*: ns).

Time-lag between start of stress and Open Field by modulating stress- and interval-duration To assess the possibility that not the stress duration but the time-lag between start of stress and start of behavioural measurements is of importance, we tested C57BL/6J male mice with differences in stress- and interval-durations. Both stress groups were stressed in 50 ml tubes for different durations:

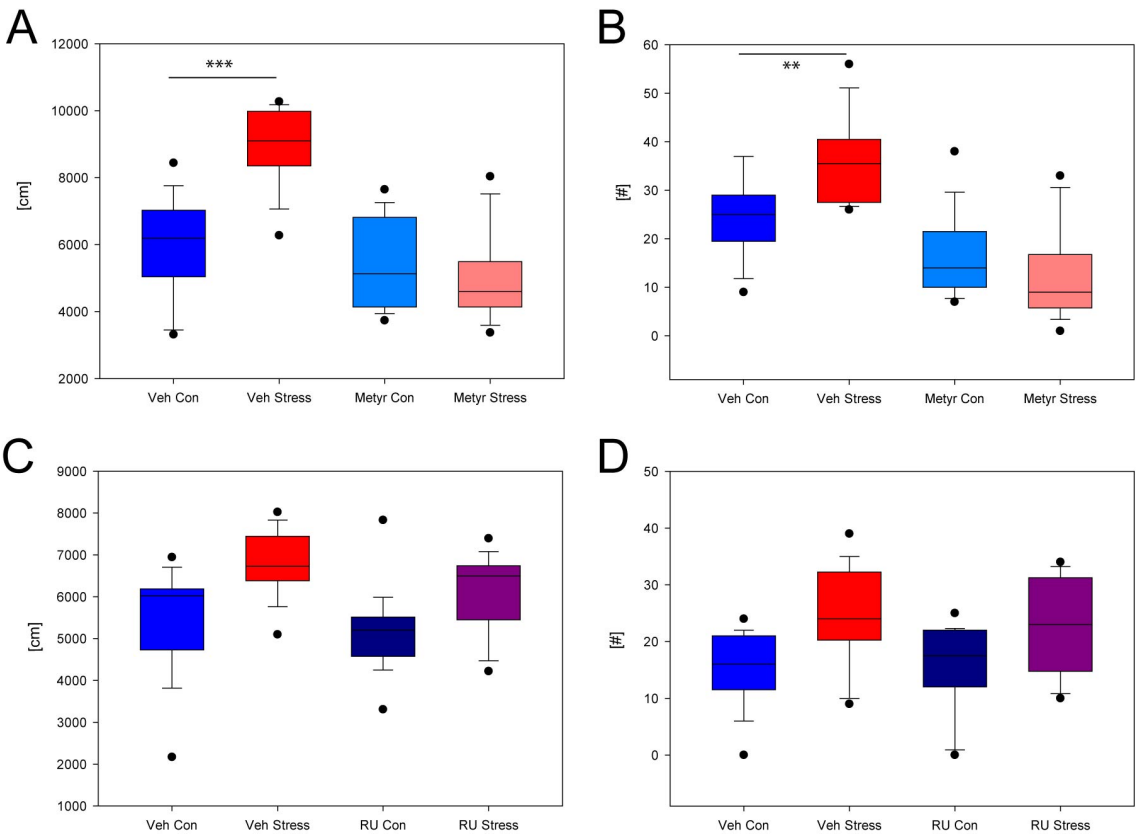

Figure 3.4: Pharmacological analysis of corticosterone feedback

Graphs (A) and (B) show the *distance travelled* and the *number of rearings* in the first five minutes of the OF with animals treated with Metyrapone and Vehicle. In both graphs statistics revealed a significant interaction and post hoc tests demonstrated that only in the vehicle-treated animals the expected increase in both parameters after stress occurred. Graphs (C) and (D) depict the *distance travelled* and the *number of rearing* in the first five minutes of the OF respectively with animals treated with either RU486 or vehicle. Vehicle-treated as well as RU486-treated animals show an increase in activity in both parameters after two hours of stress. Significances: ***- $p<0.001$; **- $p<0.01$; n=11-15 per group; Veh- vehicle; Metyr- Metyrapone; RU- RU486

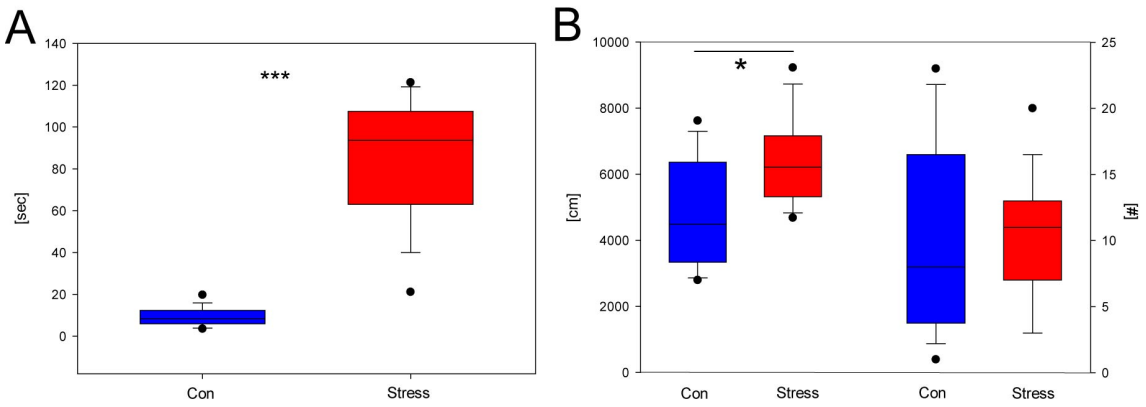

Figure 3.5: Effects of omitting the interval

Animals of the stress group were subjected to two hours restraint stress after which they were directly placed into the OF arena, without an interval. (A) *Grooming duration* and (B) *distance travelled* (left side) and *number of rearings* (right side) in the first five minutes of the OF. The highly significant increase in time spent grooming drowns the typically highly significant effect of stress on *distance travelled* and *number of rearings*. Significances: * - $p<0.05$; ***- $p<0.001$; n=11-12 per group

one group was stressed for two hours and the other stress group for 15 minutes (see Figure 3.6 A). Both groups received an interval, resulting in a period of 2:20 h from beginning of stress to the beginning of the OF. That is an interval period of 20 minutes for the two-hour stress-group and an interval period of 2:05 h for the 15 minute stressed group. Control animals were placed directly from their home cage into the OF arena. A One Way ANOVA revealed a significant difference between groups (H_2=13.525, P=0.001) and post hoc testing revealed a significant difference between the control and two hour stressed group (Q=3.634, P<0.05; Dunn´s-Method), but not between the control and the 15 minute stressed group in *distance travelled* in the first five minutes of the OF (see Figure 3.6). There was no difference between groups found at the level of *number of rearings* (data not shown).

Validation of the acute stress test with mutant mouse lines To validate our acute stress test for the use in mutant mouse lines, we used two mutant mouse lines which are known to differ in their stress-responsivity (see subsection 5.1.2). We stressed mice of the Cor26Nes line, where mutants overexpress CRH and are known to be more stress-reactive, and mice from the *CRH-R1*KO mouse line, where mutants are known to be more stress-resistant, with different stress durations. As can be seen from Figure 3.7 A and B, the CRH overexpressors show a clear response to the 15 minute stress test compared to their wild-type litter-mates (First acute stress (Figure 3.7 A): Interaction: $F_{1,37}$=4.217, P=0.048; post hoc: Holm-Sidak: WT: Con vs Stress: ns; Mut: Con vs Stress: t=1.722, P=0.094; Second exposure (Figure 3.7 B): Interaction: $F_{1,38}$=9.005, P=0.005; post hoc: Holm-Sidak: WT: Con vs Stress: n.s.; Mut: Con vs Stress: t=3.404, P=0.002).

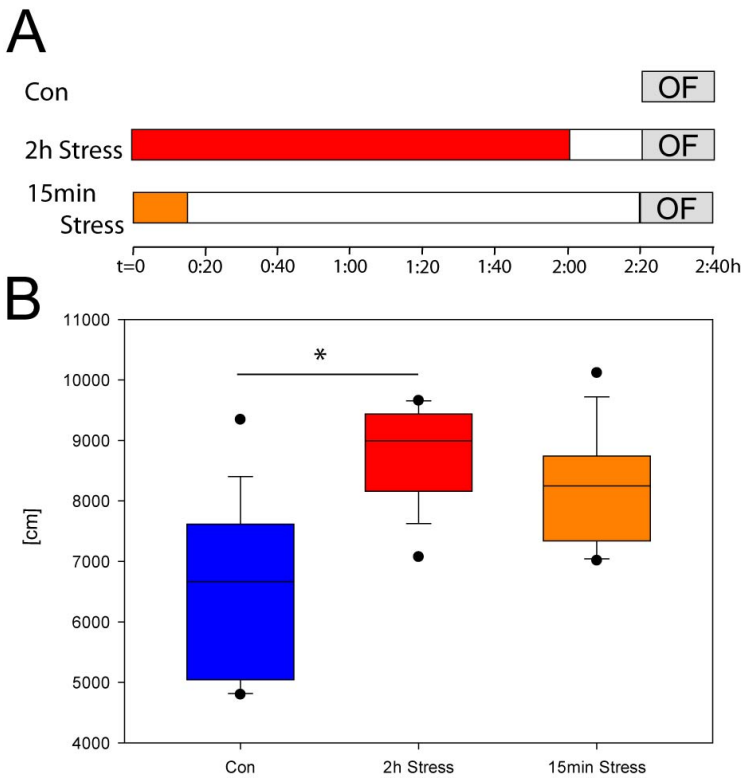

Figure 3.6: Time-lag between start of stress and start of OF: differences in stress and interval duration

(A) Scheme of the experimental design. 2h Stress (red): Animals were exposed to two hour stress period followed by a 20 minute interval and successive OF testing. 15min Stress (orange): These animals were stressed for 15 minutes followed by an interval duration of 2:05 hour. Thereafter they were placed in the OF arena. Controls (blue) were placed directly from the home cage into the OF arena. (B) *Distance travelled* in the first five minutes of the OF; Significances: * - $p<0.05$; Con and 2h-Stress: n=12 per group; n(Stress15min)=8; Red and orange bar represents stress duration, white bar represents the interval duration

The *CRH-R1*KO mutants, in contrast, do not respond to the 15 minute stress duration although the wild-types do (First cohort: Genotype effect: $F_{1,43}$=8.501, P=0.006; Stress effect: $F_{1,43}$=4.118, P=0.049; post hoc: Holm-Sidak: WT: Con vs Stress: t=2.875, P=0.006; Mut: Con vs Stress: n.s.). The second cohort of *CRH-R1*KOs strengthened the results from the first cohort by showing that even after two hours of stress mutants do not react to stress (see Figure 3.7; Interaction: $F_{1,17}$=6.728, P=0.021; post hoc: Holm-Sidak: WT: Con vs Stress: t=4.223, P<0.001; Mut: Con vs Stress: n.s.).

Acute stress and re-testing We further evaluated the reproducibility of the acute stress test within the same animals by re-testing them every second day for three times (Figure 3.8 A), twice within one week (data not shown) or throughout their lives (Figure 3.8 B). As can be seen from the different graphs, the stressed group always responds to the stress challenge with an increase in locomotor activity in the first five minutes of the OF (Stressed every second day: *Distance travelled*: Stress-day interaction: $F_{2,71}$=3.175, P=0.052; Stress effect: P<0.001; Day effect: $F_{2,71}$=79.954, P<0.001; post hoc: Holm-Sidak: Day 1: Stress effect: t=4.366, P<0.001; Day 2: Stress effect: t=5.611, P<0.001; Day 3: Stress effect: t=6.733, P<0.001; *Number of rearings*: Day-stress interaction: $F_{2,71}$=3.325, P=0.045; post hoc: Holm-Sidak: Day 1: Stress effect: t=2.121, P=0.039; Day 2: Stress effect: t=3.584, P<0.001; Day 3: Stress effect: t=4.900, P<0.001).

In cohort 12 (see Figure 3.8 B), animals were re-exposed to acute stress throughout their lives. As can be seen from the graph, stressed animals always showed the increase in locomotor activity (t-tests for the individual time points: Age 13-14 weeks: t_{22}=-5.396, P≤0.001; age 17-18 weeks: t_{21}=-7.270, P≤0.001; age 21-22 weeks: U=11.0, P≤0.001; age 24-25 weeks: t_{21}=-4.344, P≤0.001; age 29 weeks: t_{21}= -0.937, n.s.; age 34-35 weeks: U=11.0, P=0.001; age 37-38 weeks: t_{20} =-7.635, P≤0.001; age 95 weeks: t_{16}=-2.861, P=0.011; age 101-102 weeks: t_{16}=-4.104, P≤0.001) in the first five minutes of the OF. When analysing anxiety-related measures, i.e. percent of time spent in the centre, we do not get a stress-time interaction (data not shown). We tested the animals of the stress group for conditioning of the hyperactivity to the OF, by testing both stressed and control groups in the OF without a stress period before. No differences were found between the stressed and control group (see Figure 3.8 at the age of 29 weeks). Note that the animal numbers of cohort 12 declined over time. Throughout the experimental time of over two years one animal of the control group and five of the stressed-group died.

Acute stress and different settings Both OF systems tested: the infra-red beam break based Actimot, our standard system, and the video-based EthoVision system, revealed the stress-induced increase in locomotion within the first five minutes of the OF (see Figure 3.9). Absolute numbers are lower in the EthoVision System and the difference between the control and stressed group seems to be less strong (*Distance*

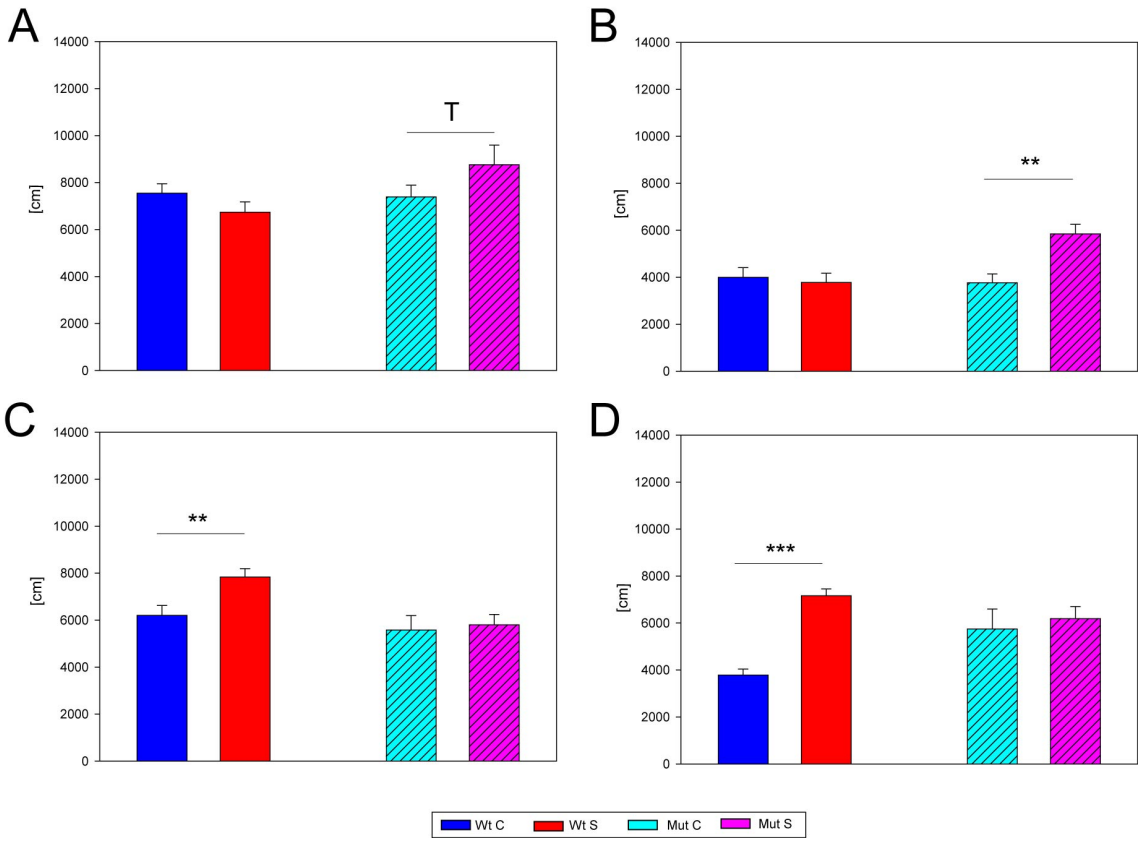

Figure 3.7: Validation of the acute stress test with mutant mouse lines
The acute stress test was validated with two mutant mouse lines, differing in their response to stress, due to alterations in the CRH system. In the Cor26Nes line, where mutants are known to be more stress-reactive, both wild-type and mutant litter-mates were exposed to a 15 minute restraint stress period. Compared to their unstressed controls, stressed wild-types do not show an increase in activity, whereas the stressed mutants do (A). After the second exposure (B), two weeks after the first acute stress, there is a significant interaction between groups. Again wild-types do not show a behavioural response to stress, whereas the mutants clearly do. For the animals from the $CRH\text{-}R1\mathrm{KO}$ line, where mutants are known to be less stress-reactive, the 15 minute restraint stress period only had an effect in the wild-types (C). This effect of reduced reactivity to stress in mutants is also evident in a second cohort of animals with a two hour exposure to restraint (D). Wild-types show an increased reactivity, whereas the stressed mutants do not show any difference compared to their unstressed controls. Error-bars shown as SEM; Significances: **- $p<0.01$; ***- $p<0.001$; n=7-13 per group in Cor26Nes mouse line and first cohort of $CRH\text{-}R1\mathrm{KO}$; n=4-6 in second cohort $CRH\text{-}R1\mathrm{KO}$

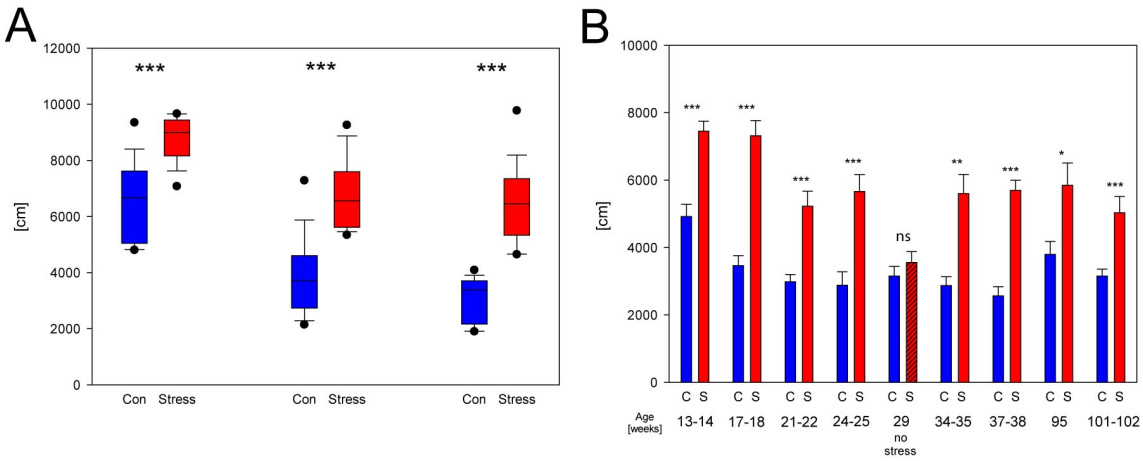

Figure 3.8: Repeated exposure to acute stress

When re-testing the same animals every second day for five days the increased activity in *distance travelled* (A) in the first five minutes of the OF persists and does not fade-out nor vanish. In graph (B) the *distance travelled* in the first five minutes of the OF after repeated exposure to the acute restraint stress test throughout life time is depicted. At the age of 29 weeks the stressed animals were tested for conditioned hyperactivity in the OF, by not stressing them, but placing them into the OF directly from the home cage, as done with controls. The lacking increase of activity demonstrates that the animals of the stress group do not respond to the environment they are placed into but to the actual stressor, namely restraint. Error-bars are shown as SEM; Significances: *- $p<0.05$; **- $p<0.01$; ***- $p<0.001$; n=12 per group, gradual decline in animal numbers in graph (B) to n(Con)=11 and n(Stress)=7 at the end of repeated stress exposure.

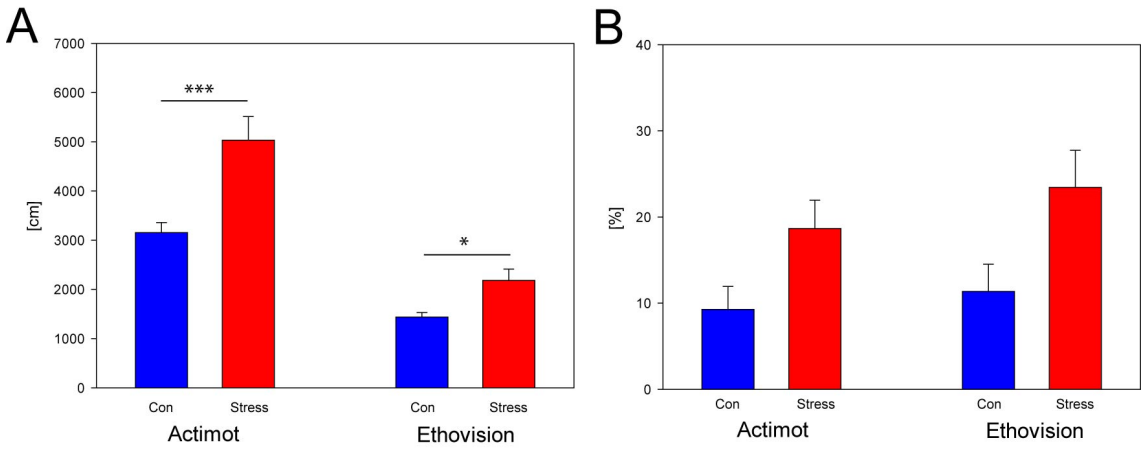

Figure 3.9: The Actimot versus the EthoVision system

C57BL/6J wild-type animals were tested in the two hours stress test and were exposed to the OF. While the Actimot system collected data via its infra-red beams, a camera mounted over the arena observed the animal via the EthoVision system. *Distance travelled* (A) and *time spent in the centre* (in percent) (B) was analysed for the two systems. Both with the Actimot and EthoVision system the increased activity of the stressed group can be observed, but absolute values differ between systems. No differences between the systems were observed for the percent of *time spent in the centre*. Error-bars are shown as SEM; Significances: *- $p<0.05$; ***- $p<0.001$; n(Con)=11; n(Stress)=7

travelled: Interaction: $F_{1,35}=5.005$, P=0.032; post hoc: Holm-Sidak: Actimot: Con vs Stress: t=5.253, P<0.001; EthoVision: Con vs Stress: t=2.089, P=0.045). Note that the percentage of *time spent in the centre* of the OF is similar between both settings (no significant interaction).

Testing animals in OFs which were placed in a bigger room, just being separated by plastic blinds from each other, did not reveal the expected behavioural differences in the first five minutes (data not shown).

Acute stress in animal holders Testing animals with higher body weight (i.e. old animals or obese mice, such as the db/db mouse line (see section 3.1.2)) urged us to test the possibility to restrain them in bigger tubes. For this purpose we used animal holders. Figure 3.10 shows the differences between the control and stressed group in the first five minutes of the OF test at the first and second stress exposure. At the first exposure only a difference in the *number of rearings* was seen (t-test: t_{20}=-2.131, P=0.046). At the second exposure, 14 days later, a clear difference between the stressed and control group can be seen in both vertical and horizontal activity (t-test: *Distance travelled*: t_{20}=-4.141, P≤0.001; *Number of rearings*: t_{20}=-5.078, P≤0.001).

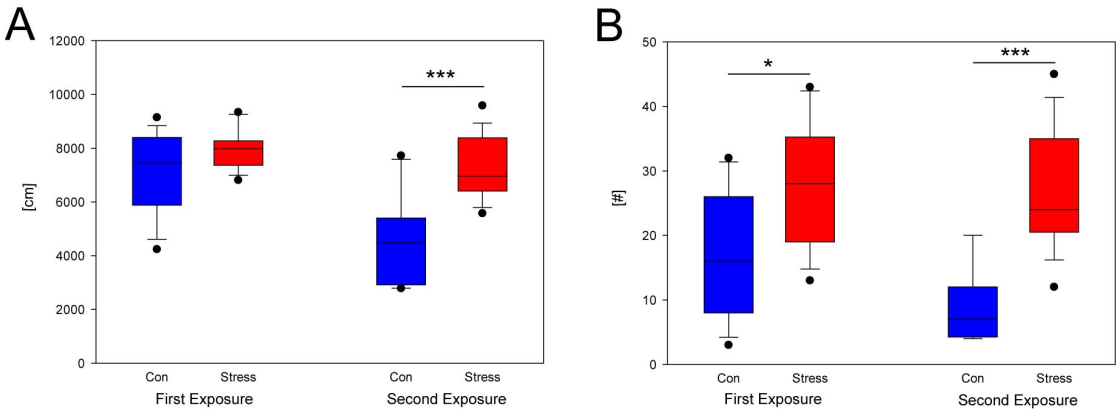

Figure 3.10: Acute stress in animal holders

Animals were restrained in animal holders for two hours. After the first exposure an increase in activity can only be seen in the *number of rearings*, whereas after the second exposure both *distance travelled* and *number of rearings* are significantly elevated. Significances: *- $p<0.05$; ***- $p<0.001$; n=11 per group

Acute stress with female and old male C57BL/6J mice, as well as inbred wild-type strains Further experiments were conducted to determine the response in young female and two year old male C57BL/6J mice, and in three other mouse strains (129Sv, BALB/c and C3H). Results can be seen in Figure 3.11 and Figure 3.12. Both female and aged male C57BL/6J mice show the expected increase in *distance travelled* in the first five minutes of the OF (t-test: Females: t_{21}=-3.000, P=0.007; Aged: U=9.000, P=0.006). As the females only showed a trend in *number of rearings* (t-test: t_{21}=-1.840, P=0.080) at the first stress exposure, they were exposed to the acute stress test for a second time. At this time point, when they reached body weights comparable with nine-weeks old males, they also showed the increase in *number of rearings* (t-test: *Distance travelled*: t_{21}=-5.048, P$\leq$0.001; *Number of rearings*: t_{21}=-2.495, P=0.021; data for *number of rearings* not shown). The aged males showed a stress-induced increase in *number of rearings* (t-test: *Number of rearings*: t_{16}=-2.257, P=0.038; data not shown).

As can be seen from Figure 3.12 all other strains tested have a lower locomotor activity compared to C57BL/6 (which is marked in Figure 3.12 with a light blue shade for controls and a light red shade for stressed animals). Both BALB/c and C3H show an increase in *distance travelled* after stress (*Distance travelled*: BALB/c: t_{22}=-3.678, P=0.001; C3H: t_{22}=-4.475, P$\leq$0.001). The 129Sv do not respond to the two hour acute stress with an increase in activity.

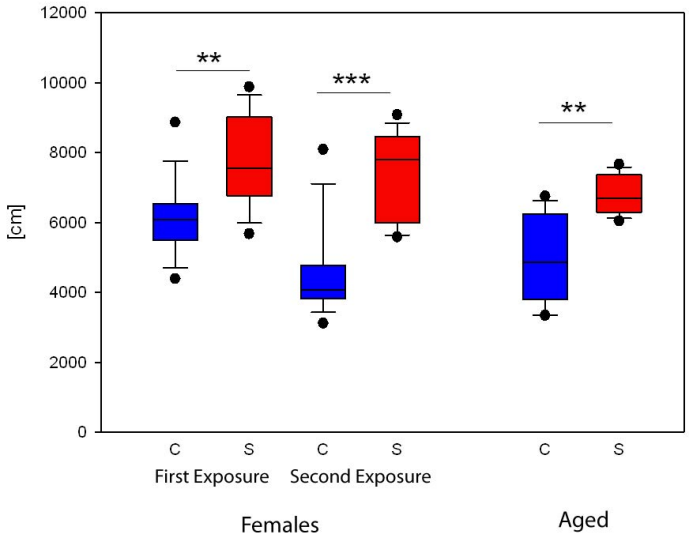

Figure 3.11: Female and two year old male C57BL/6J

Female C57BL/6J mice were tested with the two hour restraint stress test. In both exposures females show the expected increase in *distance travelled*. Aged C57BL/6J males also show the increase in *distance travelled*. Significances: **- $p<0.01$; ***- $p<0.001$; n=11-13 per group

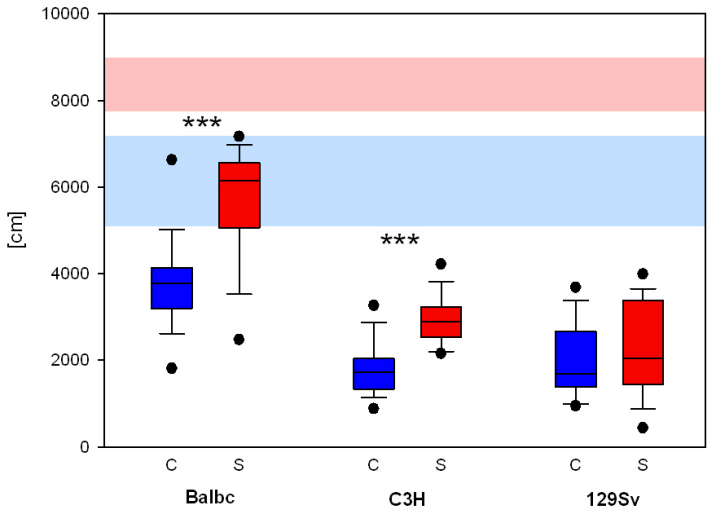

Figure 3.12: Acute stress test in different inbred strains

BALB/c, C3H and 129Sv wild-type animals were also tested with the two hours acute stress test. All of these strains show a lower locomotor activity compared to the C57BL/6 strain (horizontal shades). Both BALB/c and C3H show increased activity after stress. Only the 129Sv strain do not show the behavioural response after stress exposure. Horizontal shades: 25-75% Percentile of 4 cohorts (n: Con= 53, Stress= 49), Light blue: C57BL/6 control groups, Light red: C57BL/6 stress groups; Significances: ***- $p<0.001$; n=11-12 per group

3.1.2 Testing Mutant Mouse Lines

The AcStr01 mouse line The modified gene of this mouse line is neuron-specifically expressed and its protein is a member of the scaffold proteins of the postsynaptic density of excitatory synapses. Two cohorts of the AcStr01 mutant line have been analysed with the acute stress test. One cohort with a 15 minute restraint period and the other one with the two hour acute stress test. In Figure 3.13 graphs (A) and (B) show the parameters *distance travelled* and *number of rearings*, respectively for the cohort which has been stressed for 15 minutes. For *distance travelled* a genotype effect ($F_{1,33}$=23.441, P<0.001) as well as a stress effect ($F_{1,33}$=8.467, P=0.006) was observed. Post hoc testing revealed a difference within the wild-types (WT: Con vs Stress: $F_{1,17}$=20.466 P<0.001), whereas there was none in the mutants. Concerning the *number of rearings* after a 15 minute stress exposure we saw a genotype-sex interaction ($F_{1,33}$=5.171, P=0.03) as well as a genotype ($F_{1,33}$=6.445, P=0.016) and stress effect ($F_{1,33}$=25.692, P<0.001). After separating the data by sex, the males showed a stress effect ($F_{1,16}$=9.576, P=0.007), which is only significant in the wild-types ($F_{1,9}$=7.801, P=0.021). For the females a genotype ($F_{1,17}$=13.593, P=0.002) as well as a stress effect ($F_{1,17}$=17.226, P=0.001) was revealed. Mutant females showed lower *number of rearings* compared to wild-type females.

In the second cohort (graphs (C) and (D)), stressed with two hours of restraint, *distance travelled* showed a stress ($F_{1,69}$=62.091, P<0.001) and a genotype effect ($F_{1,69}$=165.125, P<0.001). In the *number of rearings* we see a stress-genotype interaction ($F_{1,69}$=10.871, P=0.002) and a stress effect ($F_{1,69}$=11.175, P=0.001). Post hoc testing revealed a significant stress effect ($F_{1,33}$=23.072, P<0.001) in the wild-types, whereas there is no difference in the mutants.

The AcStr02 mouse line The AcStr02 mouse line, a model for PD, was screened in the GMC within the Primary Screen, where only subtle genotype-differences were detected. These hinted to alterations in stress responsivity. To evaluate this we got a second cohort, consisting of female mice only. These animals were stressed for 15 minutes in the acute stress test. There was no interaction between genotype and stress condition, not in the *distance travelled* nor in the *number of rearings*. Only an increased locomotor activity in both stressed groups could be observed (Stress effect: $F_{1,43}$=25.965, P<0.001, post hoc: Holm-Sidak: WT: Con vs Stress: t=3.714, P<0.001; Mut: Con vs Stress: t=3.488, P=0.001). There was a genotype effect in the *number of rearings*, and post hoc testing revealed no significant differences between the wild-type control and stressed group, whereas in the mutants it did (Genotype effect: $F_{1,43}$=4.299, P=0.045; Stress effect: $F_{1,43}$=4.397, P=0.042; post hoc: Holm-Sidak: WT: Con vs Stress: ns; Mut: Con vs Stress: t=2.194, P=0.034). Because of these results the animals were tested a second time within the acute stress. Here again no genotype-stress interaction was observed. A clear stress effect was observed within the *distance travelled* (Stress effect: $F_{1,41}$=28.979, P<0.001). Still

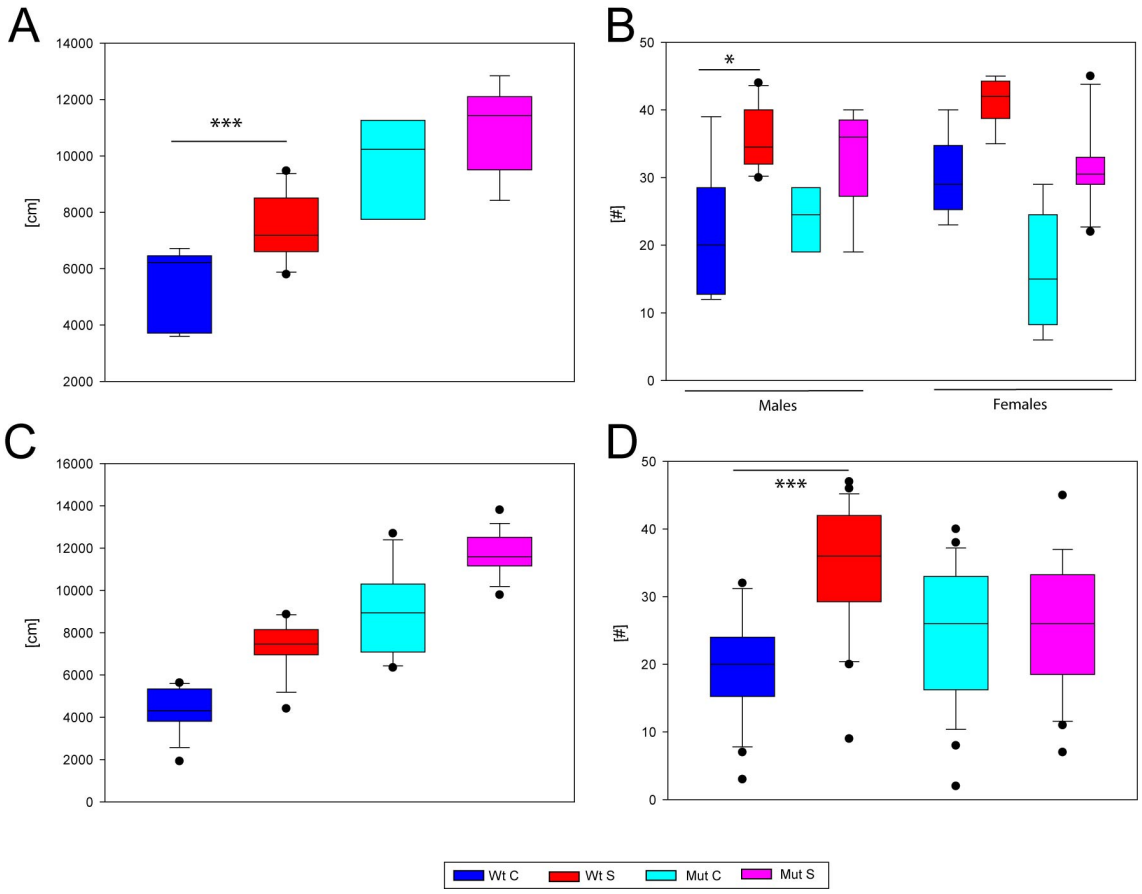

Figure 3.13: The AcStr01 mouse line

Animals from the AcStr01 mouse line were restrained for 15 minutes (A and B) and a second cohort for two hours (C and D) in the acute stress test. In the 15 minute acute stress test mutant animals show no increase in *distance travelled* (A) in response to stress, which is reflected in the male mutants in the *number of rearings* (B), where again post hoc testing revealed no difference in the mutants. For the two hours stress exposure, there are no differences in stress response in the parameter of *distance travelled* between the genotypes (C). Only in the *number of rearings* (D) post hoc testing revealed no difference in the mutants, in comparison to wild-types, where there is a significant difference. Note the overall genotype effect in *distance travelled*, in which the mutants show hyperactivity. Significances: ***- $p<0.001$; *- $p<0.05$; (A) n= 9-11 per group, (B) n=4-6 per group; (C) and (D) n=9-11 per group

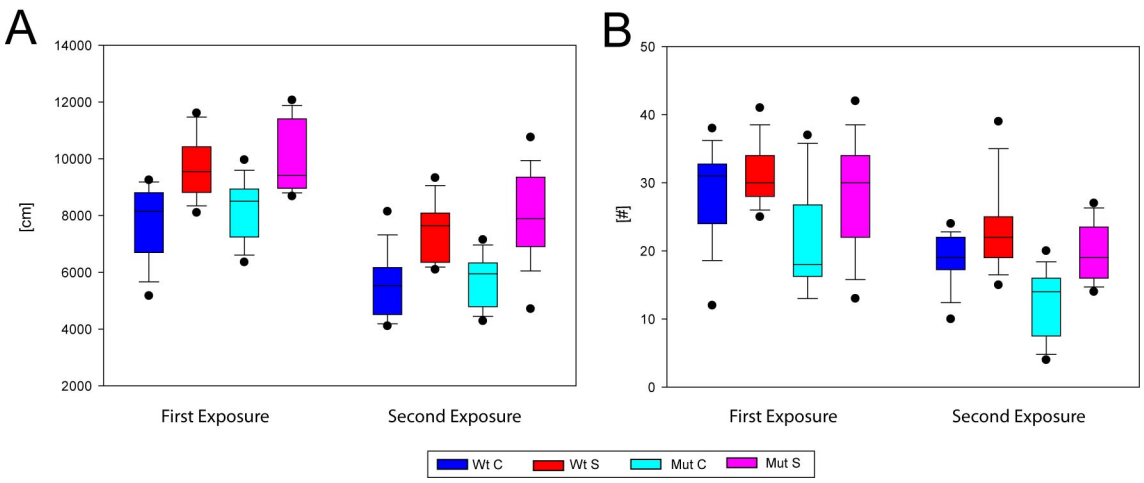

Figure 3.14: The AcStr02 mouse line

Females of the AcStr02 mouse line were tested with the 15 minute acute stress test. No interactions in *distance travelled* (A) and *number of rearings* (B) were revealed. The increase in *number of rearings* within both exposures was stronger in the mutants than in the wild-type litter-mates. n=10-12 per group

the effect on *number of rearings* was stronger within the mutants than in the wild-types (Genotype effect: $F_{1,42}$=9.522, P=0.004; Stress effect: $F_{1,42}$=13.827, P<0.001; post hoc: Holm-Sidak: WT: Con vs Stress: t=2.067, P=0.045; Mut: Con vs Stress: t=3.204, P=0.003).

The Emory mouse line The Emory mouse line, a model for progressive cataract formation, was tested because of the results from our and the Pathology Screen within the GMC Primary Screen, which hinted to a reduced stress-responsivity. In the two hours acute stress test animals showed a stress-induced increase in *distance travelled,* which was driven by the wild-types (Stress effect: $F_{1,51}$=8.396, P=0.006; post hoc: WT: $F_{1,21}$=6.829, P=0.016; Mut: ns; see Figure 3.15 A). For *number of rearings* we did not observe any increase, but there is a genotype effect ($F_{1,51}$=15.525, P<0.001) with mutants having a lower *number of rearings*. Four days after the acute stress exposure animals were re-exposed to the two hour restraint stress test and blood samples were taken at different time points (at basal, two hours post basal and recovery levels, see Figure 3.15 E and F). The higher CORT levels in mutant control groups can be clearly seen in both males and females. CORT levels over time have the typical sex difference, in that the females show higher levels. If we analyse males and females separately we observe a genotype effect in both sexes (Males: Genotype effect: $F_{1,28}$=7.703, P=0.01; Females: Genotype effect: $F_{1,28}$=5.879, P=0.023). The area under the curve (AUC, see Figure 3.15 D) was calculated for each group separately. Statistical analysis revealed several effects: the typical sex effect ($F_{1,53}$=48.494, P<0.001), a genotype effect ($F_{1,53}$=9.992, P=0.003) and a stress effect ($F_{1,53}$=37.525, P<0.001). When separating the two sexes and

analysing data independently a stress and genotype effect can be observed in both males and females (Males: Stress effect: $F_{1,28}$=43.087, P<0.001; Genotype effect: $F_{1,28}$=8.404, P=0.007; post hoc: WT: Con vs Stress: $F_{1,14}$=55.103, P<0.001; Mut: Con vs Stress: $F_{1,14}$=9.413, P=0.008; Females: Stress effect: $F_{1,25}$=13.514, P=0.001; Genotype effect: $F_{1,25}$=4.372, P=0.047; post hoc: WT: Con vs Stress: $F_{1,9}$= 9.507, P=0.013; Mut: Con vs Stress: $F_{1,9}$=4.447, P=0.051). As we see differences in basal CORT, we calculated the ratio between control and stress-group depicted in Figure 3.15 D. Mean values of the control groups were set at 100% and the change for the respective stress-group was calculated. Statistical analysis revealed a genotype-stress interaction ($F_{1,60}$=20.324, P<0.001). Post hoc testing revealed, in both wild-types and mutants, a significant difference (WT: Con vs Stress: t=8.617, P<0.001; Mut: Con vs Stress: t=2.887, P=0.005) and a significant difference between the stressed groups (Stress: WT vs Mut: t=6.393, P<0.001).

The Aphakia mouse line The *aphakia* mouse mutants have a severe lens phenotype, as mutants do not develop an eye lens (compare name of the line). This is due to a mutation in the promoter region of the *Pitx3* gene. It is a model of PD as these animals exhibit a loss of DA neurons in the ventral tegmental area (VTA) and substantia nigra (SN). These animals went through the Primary Screen of the GMC. During blood pressure measurements in the Cardiovascular Screen some animals died, thus the screeners posed the question, if these animals were more stress-reactive. We tested three different genotypes in the 15 minute acute stress test; wild-types, heterozygous and homozygous animals, but here we left out the heterozygous animals as they show intermediate or wild-type like behaviour. It should be taken into account that only the homozygous animals are blind. For the *distance travelled* in the first five minutes we only see a trend between the control and stressed mice ($F_{1,26}$=3.236, P=0.084). Post hoc testing revealed a significant difference in the wild-types ($F_{1,12}$=21.601, P=0.001) but not in the mutants. For the *number of rearings* in the first five minutes we observed a trend in genotype-stress-sex interaction ($F_{1,26}$=3.266, P=0.082), a stress-sex interaction ($F_{1,26}$=4.586, P=0.042), a stress effect ($F_{1,26}$=4.702, P=0.039) and a strong genotype effect ($F_{1,26}$=66.346, P<0.001). We then analysed males and females separately. In males we have a genotype effect ($F_{1,12}$=37.918, P<0.001), with mutants showing reduced *number of rearings*, and in females a genotype effect ($F_{1,12}$=28.903, P<0.001) and a stress effect ($F_{1,12}$=9.658, P=0.008). The female wild-types showed a stress-induced increase in number of rearings, whereas the mutants do not (WT: $F_{1,7}$=7.667, P=0.028).

The HMGN1 mouse line The *HMGN1* gene affects chromatin structure and epigenetic modifications. The mouse line provider, Dr. Bustin, found hypoactivity and less *distance travelled* in the centre and less entries in the centre in the OF test, which hint to possible increased anxiety-related behaviours. To test this we exposed these animals to the acute stress challenge of a two hour restraint duration. All stressed animals travelled more in the first five minutes of the OF ($F_{1,32}$=74.625,

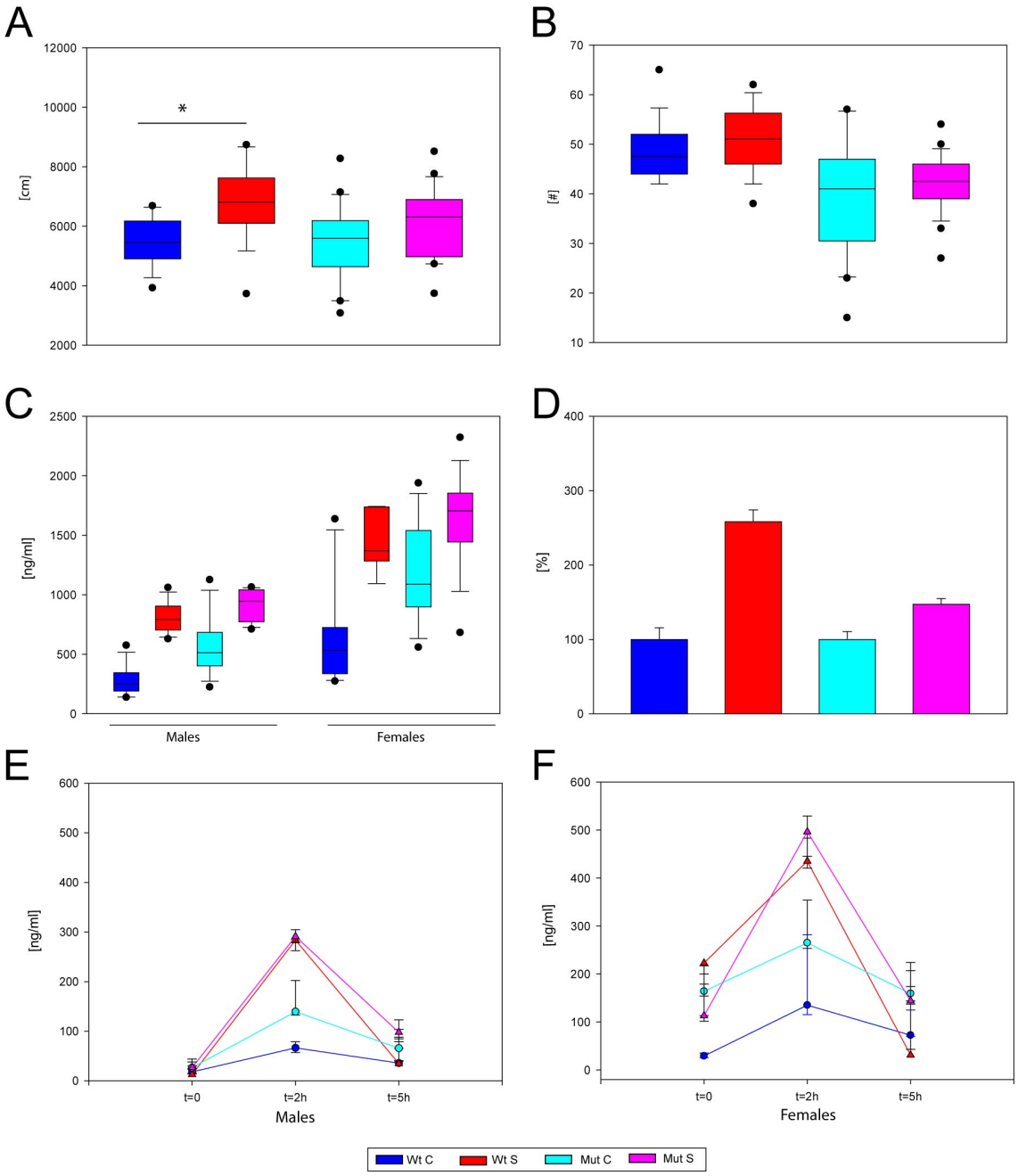

Figure 3.15: The Emory mouse line

Mice of the Emory mouse line were tested in the two hour acute restraint stress test. Stressed animals showed an increase in *distance travelled* in the first five minutes (A), which was driven by the wild-types. In *number of rearings* (B) we observed a genotype effect, with mutants rearing less. After another acute stress exposure for two hours blood was drawn and analysed for CORT levels (C and D). In the calculated AUC (C) differences between the sexes, the genotypes and the stress conditions can be seen. In (D) the AUC from controls was set at 100% and from this change in AUC (%) for the stressed groups was calculated. Data from males and females is pooled. The CORT profile over time for males and females is depicted in E and F, respectively. Note that the wild-types are no litter-mates. Significances: *- $p<0.05$; Males: n=8 per group; Females: n=5-10 per group

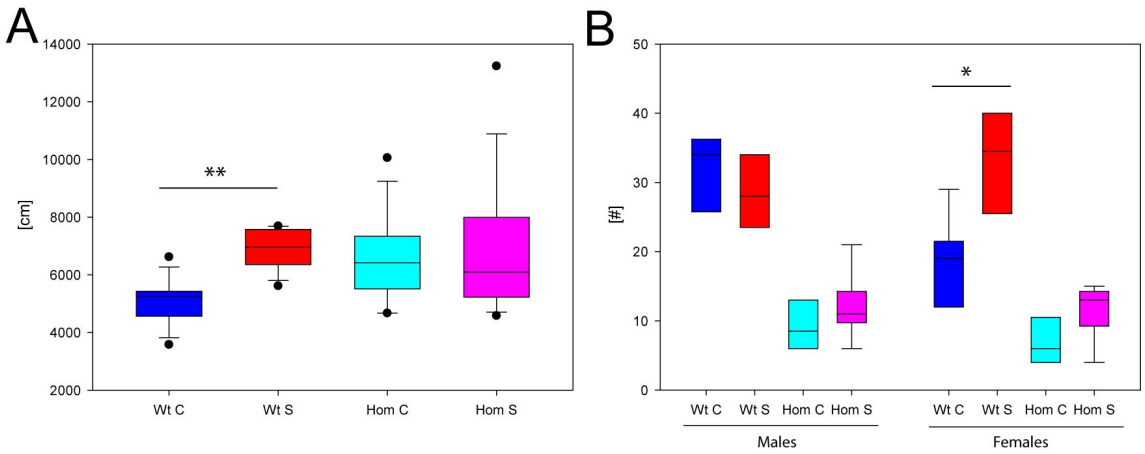

Figure 3.16: The Aphakia mouse line

Male and female mice from the *aphakia* mouse line were tested within the 15 minute acute restraint stress test. In *distance travelled* pooled data from males and females shows a stress effect in the wild-types (A). For the *number of rearings* (B) males and females are depicted separately. Males and females show a genotype effect only. Significances: *- p<0.05; (A): n=8-10 per group; (B): n=3-5 per group

P<0.001; post hoc: WT: Con vs Stress: $F_{1,16}$=31.250, P<0.001; Mut: Con vs Stress. $F_{1,16}$=44.104, P<0.001). In the *number of rearings* we observed a stress-sex interaction ($F_{1,32}$=4.152, P=0.05) and a trend in genotype effect ($F_{1,32}$=3.743, P=0.062). Dividing the two sexes and applying statistical analysis we could reveal a trend in stress effect in females ($F_{1,16}$=3.871, P=0.067). In males we saw a genotype ($F_{1,16}$=5.845, P=0.028) and a stress effect ($F_{1,16}$=45.953, P<0.001, post hoc: WT: Con vs Stress: $F_{1,8}$=13.323, P=0.006; Mut: Con vs Stress: $F_{1,8}$=37.532, P<0.001).

The HST014 mouse line This mouse line was found in the ENU-screen having increased plasma urea levels. In the Primary Screen of the GMC the HST014 mouse line showed high transfer arousal and hypoactivity in the modified Shirpa (**S**mithKline Beecham, **H**arwell, **I**mperial College, **R**oyal London Hospital, **p**henotype **a**ssessment) test of the Neurology Screen and reduced CORT levels in the Steroid Metabolism Screen. This posed the question of how these animals react to an acute restraint stress. Interestingly males and females reacted differently (*Distance travelled*: Genotype-stress-sex interaction: $F_{1,28}$=3.086, P=0.09) After separating by sex, we could not observe any stress nor genotype effect in either of the sexes.

For *number of rearings* in the first five minutes we find a trend in sex effect ($F_{1,28}$= -3.526, P=0.071) and a significant genotype effect ($F_{1,28}$=6.619, P=0.016). In the males there is no significant effect on stress nor genotype. In the females we could observe a genotype-stress interaction ($F_{1,18}$=8.182, P=0.01) and a trend in stress effect ($F_{1,18}$=3.783, P=0.068; post hoc testing did not reveal any differences within wild-type nor mutant mice).

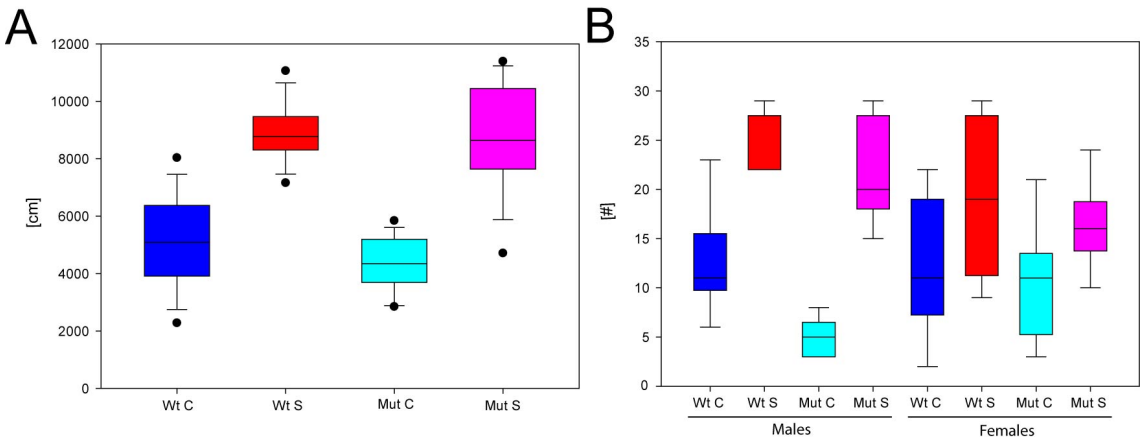

Figure 3.17: The HMGN1 mouse line

Animals from the HMGN1 mouse line were exposed to a two hour restraint stress period. In graph (A) the *distance travelled* and in graph (B) the *number of rearings* in the first five minutes of the OF are depicted. There are no genotype specific interactions. Male mutant mice have a lower *number of rearings* compared to wild-types. n=5 per group

Analysis of the CORT levels at basal and two hours after stress revealed no genotype-specific alterations (data not shown). We see an expected stress effect ($F_{1,27}$=15.895, P<0.001) and a typical sex effect ($F_{1,27}$=8.238, P=0.008). When we look at the sexes separately we see a stress effect in both males and females (Males: $F_{1,10}$=7.769, P=0.019; Females: $F_{1,17}$=12.054, P=0.003). When looking at the AUC we get no significant stress effect in the male wild-types but a significant stress effect in the mutants ($F_{1,5}$=8.346, P=0.034). For the females we get a trend in the wild-types ($F_{1,6}$=5.102, P=0.065) and a significant effect in the mutants ($F_{1,11}$=5.588, P=0.038).

The Sms1 mouse line *Sms1*, also known as *Tmem23*, is associated with AD in a GWAS study by Riemenschneider et al (unpublished). In the Primary Screen of the GMC this mouse line revealed a decreased anxiety-related behaviour in the female mice and increased *distance travelled* in the OF. The Steroid Metabolism Screen found a trend in increased CORT levels in the males and the Neurology Screen found hypoactivity in males and increased tail elevation in both sexes in the modified Shirpa. These results hint to possible alterations in stress-reactivity, thus we tested this mouse line in our acute stress test.

As shown in Figure 3.19 A, the animals display a significant stress-genotype interaction ($F_{1,39}$=5.54, P=0.024) in the *distance travelled* in the first five minutes of the OF. We see a genotype effect ($F_{1,39}$=4.744, P=0.036) as well as a genotype-sex interaction ($F_{1,39}$=6.088, P=0.018). After dividing the animals by sex, we do not get a genotype-dependent effect in the males, whereas in the females we get a genotype-stress interaction ($F_{1,21}$=5.699, P=0.026). Post hoc testing revealed a

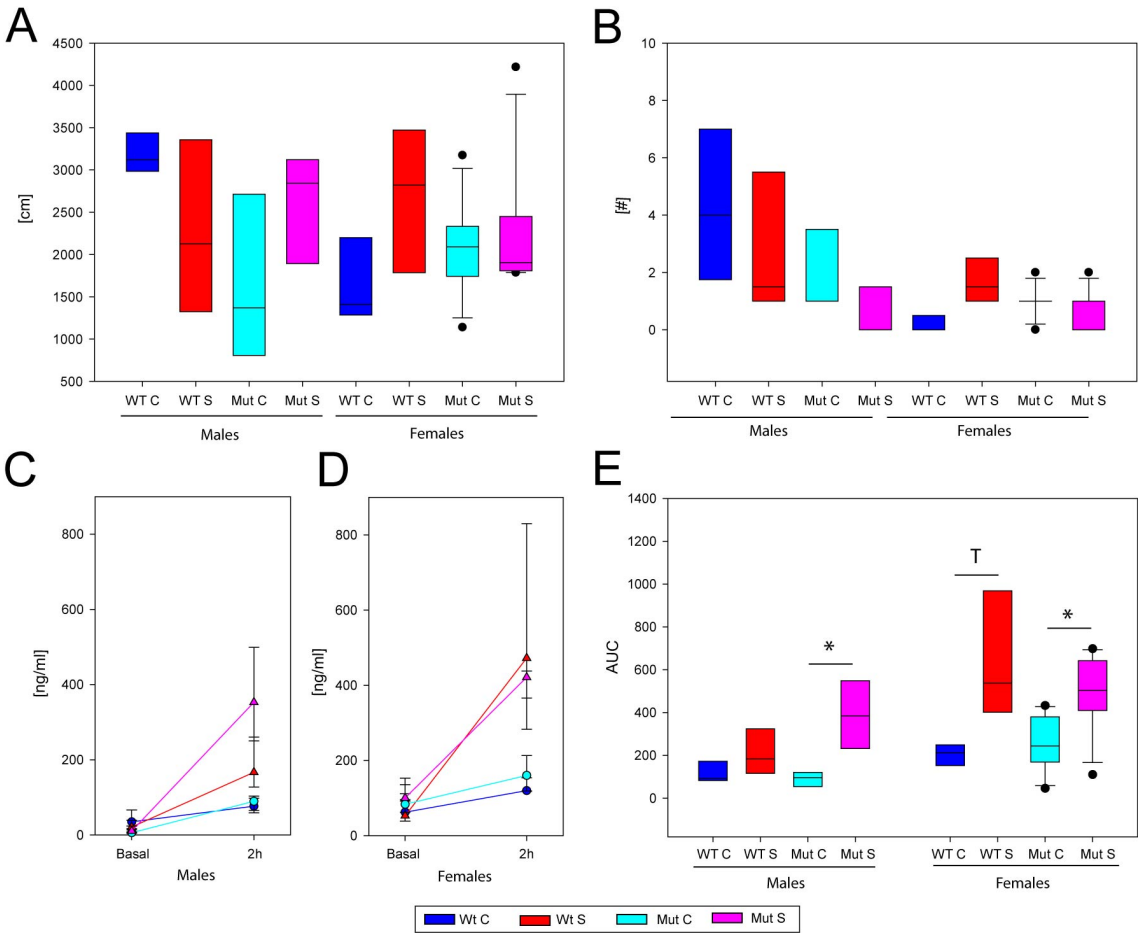

Figure 3.18: The HST014 mouse line

Mice of the HST014 mouse line were acutely stressed for two hours. There is a trend in genotype-stress-sex interaction in *distance travelled* (A). Post hoc testing did not reveal any differences in males or females. In the *number of rearings* (B) a trend in sex-difference could be observed, with females having a genotype-stress interaction. Post hoc testing in the females did not reveal significances within the two genotypes. Graph C and D show CORT levels of both males and females, respectively, at basal and 2h post basal. In graph E the AUC is depicted. Significances: *- $p<0.05$; T-$p<0.1$; Note that animal numbers are very low: Males: n=3-4; Females: n=4-7

stress effect in the wild-types but not the mutants (WT: $F_{1,13}$=10.985, P=0.006; Mut: ns). For *number of rearings* we get a stress-genotype interaction ($F_{1,39}$=5.121, P=0.029) and trend in sex-genotype interaction ($F_{1,39}$=3.496, P=0.069) as well as a trend in sex-genotype-stress interaction ($F_{1,39}$=3.941, P=0.054). After separating the two sexes we see no effect in the males. The females showed a genotype-stress interaction ($F_{1,21}$=8.631, P=0.008) with again an effect in the wild-types but not in the mutants (WT: Con vs Stress: $F_{1,13}$=5.6, P=0.034; Mut: Con vs Stress: ns).

One week after the first exposure to stress, animals were re-tested. This time blood samples were taken at basal (before stress) and at stress (after 15 minutes stress) levels. Control animals were bled simultaneously.

The third acute stress test was applied one week after the second exposure. This time animals of the stress group were restrained for two hours. In the first five minutes of the OF, both *distance travelled* (see Figure 3.19 B) and *number of rearings* showed a stress-genotype effect (*Distance travelled*: Interaction: $F_{1,39}$=8.746, P=0.005; post hoc: WT: Con vs Stress: $F_{1,24}$=37.111, P<0.001; Mut: Con vs Stress: ns; *Number of Rearings*: Interaction: $F_{1,39}$=13.694, P=0.001). As there also was a sex-genotype ($F_{1,39}$=4.353, P=0.044) and a sex effect ($F_{1,39}$=4.079, P=0.05) in *number of rearings*, this parameter was analysed in the sexes separately. Males showed a stress-genotype interaction ($F_{1,18}$=5.222, P=0.035), which was significant in the wild-types ($F_{1,11}$=48.950, P<0.001), but not the mutants. In the females we also got a stress-genotype interaction ($F_{1,21}$=8.986, P=0.007), in which again wild-types respond to stress ($F_{1,13}$=20.876, P=0.001) and mutants did not.

Homozygous animals of the Db/Db mouse line The *db/db* mouse line is used as a model of diabetes. Due to their high body weight (Median: 47.3 g) *db/db* mice were restrained in animal holders. After their first two hour acute stress these animals only showed a tendency in increased *distance travelled* (t_7=-2.200, P=0.064) and in *number of rearings* (U=2.000, P=0.063) in the first five minutes of the OF. After the second stress exposure they showed a clear effect in *distance travelled* (t_7=-3.221, P=0.015). Note that, overall, the amount of *distance travelled* as well as the animal number is very low. We only had the chance to test homozygous animals.

3.1.3 Voluntary Wheel Running and Acute Stress

Running causes an increase in CORT levels [5] but it is also known to be beneficial for stress-resistance or relieving stress. This posed the question of how voluntary running will affect acute stress-reactivity. For this, male C57BL/6 mice ran for four weeks after which they were exposed to the acute stress challenge for two hours of restraint. As can be seen from Figure 3.21 in both *distance travelled* and *number of rearings* in the first five minutes of the OF we see an interaction (*Distance travelled*: Interaction: $F_{1,39}$=6.515, P=0.015; *Number of rearings*: Interaction: $F_{1,39}$=9.598, P=0.004). For the *distance travelled* we can see a clear difference between the two

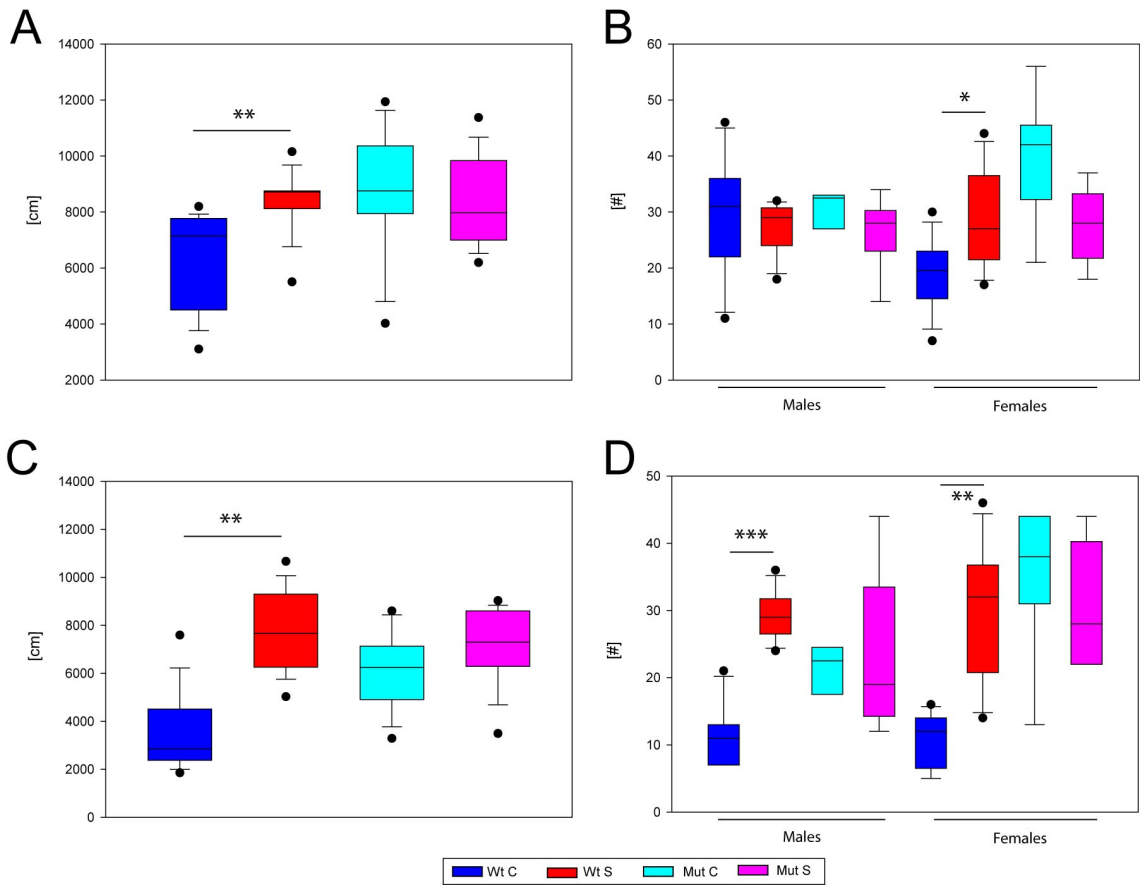

Figure 3.19: The Sms1 mouse line

Animals from the Sms1 mouse line were tested in the acute stress test for three times. The first two times animals of the stress group were restrained for 15 minutes. *Distance travelled* and *number of rearings* in the first five minutes of the OF of the first exposure is depicted in (A) and (B), respectively. The third time stress exposure lasted for two hours. *Distance travelled* and *number of rearings* in the first five minutes of the OF are depicted in (C) and (D), respectively. Significances: **- $p<0.01$; n= 9-14 animals per group

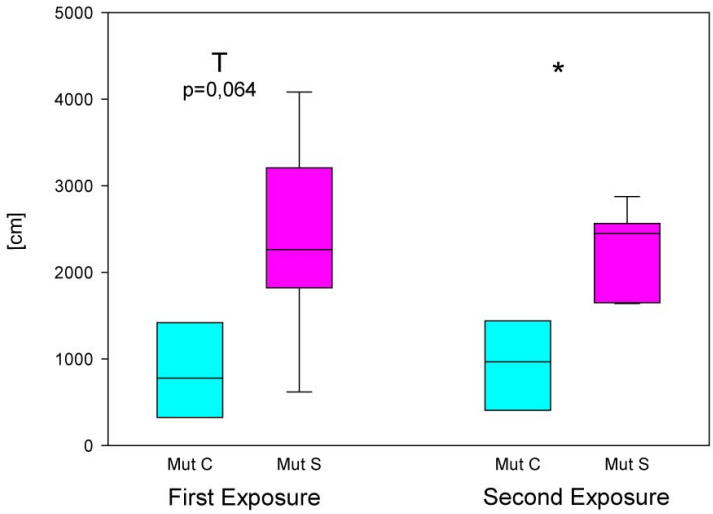

Figure 3.20: Homozygous db/db mutants

Db/db homozygous mutant mice were exposed to a two hour restraint period. Note the overall low locomotory activity in these mice. After the first exposure these animals showed a tendency towards an increased activity, which became significant in the second exposure. Significances: T- $p<0.08$; *- $p<0.05$; n(con)=4; n(stress)=5

control groups, where the runner controls have a lower *distance travelled* (post hoc: Holm-Sidak: t=3.890, P<0.001). Both non-runners and runners reach the same level of *distance travelled* after stress. For the *number of rearings* the runners do not respond to the stress with an increase in this parameter (Post hoc: Holm-Sidak: Non-runners: Con vs Stress: t=4.655, P<0.001; Runner: Con vs Stress: ns).

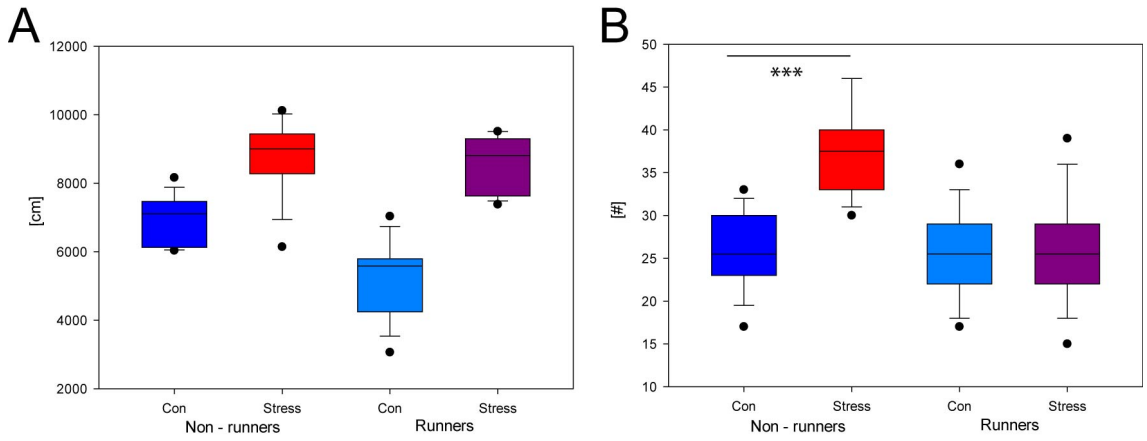

Figure 3.21: Voluntary wheel running and acute stress

After four weeks of running male mice show an altered stress response. Their baseline locomotor activity level is significantly lower than that of non-runners (A). Also the *number of rearings* is not increased after stress exposure, indicating an altered stress-response (B). Significances: ***-$p<0.001$; n=10 per group

3.2 Chronic Stress Challenge

3.2.1 Establishment of the Chronic Stress Challenge

C57BL/6N wild-type animals

Establishment of the chronic stress protocol For the first two experiments we used the method published by Kim and Han [93]. They showed that a chronic restraint stress for 14 days, two hours per day at 10 o´clock in the morning induced anxiety-related and depression-like behaviours as seen in the EPM and FST, as well as reduced body weight.

We applied the above mentioned method to two cohorts. Our stressed animals showed a reduction in body weight (AUC: Korea 1: t_{22}=6.031, P≤0.001; Korea 2: t_{22}=4.566, P≤0.001) but there were no significant differences between groups in any of the three behavioural tests (as there were EPM, OF, FST), except for an increase in *number of rearings* in the periphery of OF in both cohorts (data not shown). We did not see any changes in anxiety- or depression-related behaviours.

The subsequent experiments were conducted with an unpredictable chronic stress protocol, as it has been suggested, that unpredictable stress is more effective than a predictable one [45, 127, 141, 183]. Thereby we stressed the animals in a pseudo-random order changing between different stress conditions (for details see Figure 5.1). The first two cohorts of the unpredictable restraint stress (Unpred. 1 and Unpred. 2) showed a reduced body weight gain and an increase in anxiety-related behaviours, indicated by reduced *distance travelled in the centre* and *time spent in the centre* of the OF at day 22, eight days after the end of stress (see Figure 3.22) (Unpred. 1: *Total distance travelled in the centre*: t_{22}=2.499, P=0.020; *Total time spent in the centre*: t_{22}=2.388, P=0.026; Unpred. 2: *Total distance travelled in the centre*: t_{22}=2.431, P=0.024; *Total time spent in the centre*: t_{22}=2.382, P=0.026).

To investigate if the anxiety phenotype in the OF is even stronger one day after stress cessation (day 15) we interchanged the EPM and OF. We did not see any effects of stress on anxiety-related parameters in the OF or the EPM (Unpred. 3 and 4). Thus we changed OF testing back to its original time point at day 22. This time we left out the EPM, as it did not reveal any changes in the first two cohorts of unpredictable stress (Unpred. 1 and Unpred. 2) and would be only time consuming in up-coming testing of mouse lines. This protocol (Unpred. 4-6) revealed no increased anxiety-related behaviours in the OF.

Installing the EPM on day 15 again (Unpred. 8) did not restore the behavioural phenotype seen in the OF in the first two cohorts of unpredictable stress.

Concerning body weight changes over the stress period we reliably saw a reduction in all the stressed groups (see Figure 3.23).

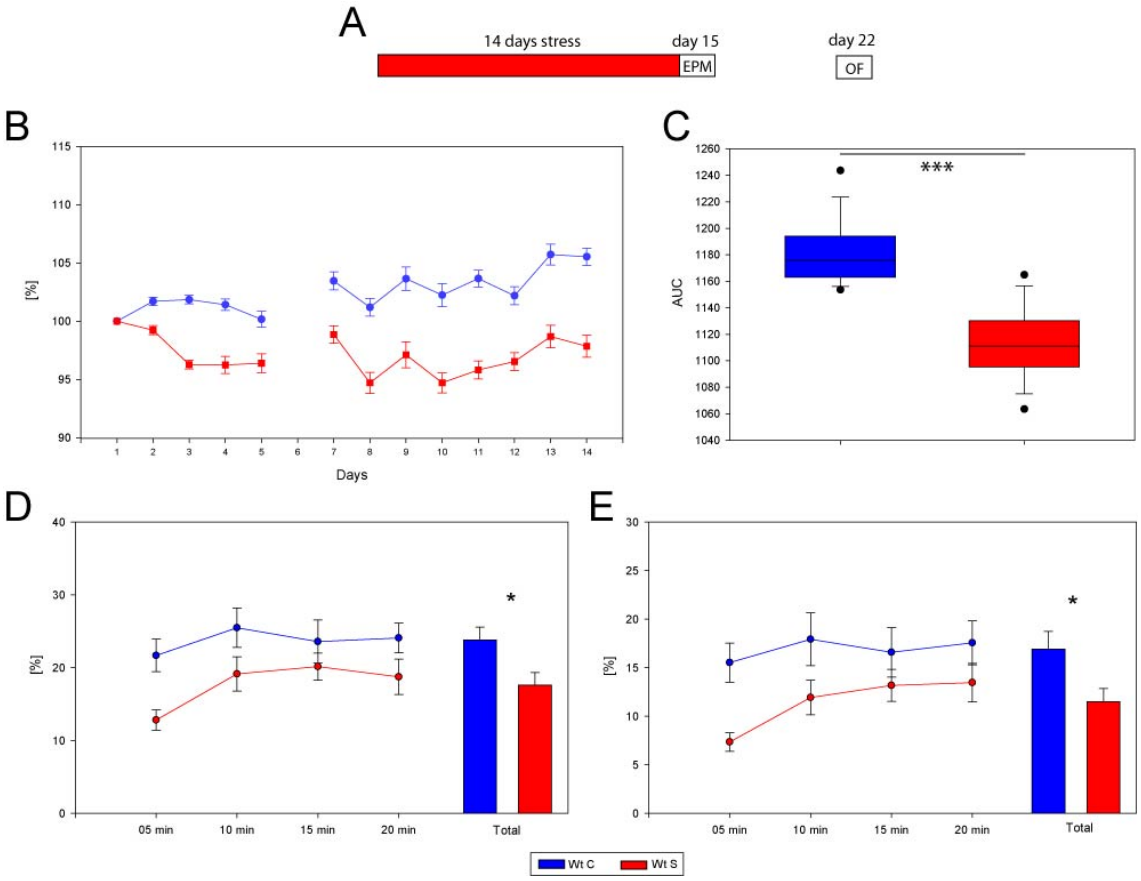

Figure 3.22: Chronic unpredictable stress in wild-type C57BL/6

Scheme of experiment conductance (A). Body weight gain during the two weeks of stress (B) and AUC of body weight (C). Eight days after the end of the two weeks unpredictable restraint stress period, stressed animals showed increased anxiety-related behaviours as can be seen in the reduced *distance travelled in the centre* (D) and *time spent in the centre* (E) of the OF arena (both in percent) in cohort Unpred. 1. Significances: *- $p<0.05$

Day	2	3	4	5	6	7	8	9	10	11	12	13	14
Korea 1	ns	**↓	*↓	*↓	*↓	**↓	**↓	**↓	***↓	**↓	**↓	**↓	**↓
Korea 2	T↓	**↓	*↓	**↓	**↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓
Unpred. 1	***↓	***↓	***↓	**↓	n.d.	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓
Unpred. 2	***↓	ns	ns	T↓	***↓	***↓	***↓	***↓	***↓	**↓	***↓	***↓	***↓
Unpred. 3	ns	**↓	**↓	*↓	ns	*↓	*↓	***↓	*↓	**↓	*↓	**↓	T↓
Unpred. 4	***↓	***↓	T↓	ns	**↓	**↓	*↓	**↓	**↓	**↓	*↓	*↓	ns
Unpred. 5	***↓	*↓	**↓	***↓	*↓	**↓	*↓	n.d.	**↓	*↓	T↓	T↓	***↓
Unpred. 6	*↓	**↓	ns	*↓	*↓	T↓	***↓	***↓	***↓	**↓	***↓	***↓	***↓
Unpred. 7	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓	***↓
Unpred. 8	***↓	***↓	**↓	**↓	***↓	**↓	***↓	***↓	***↓	***↓	**↓	***↓	***↓

Figure 3.23: Body weight from all chronically stressed cohorts

Percent body weight change measured every day and statistics of the applied t-test. ↓- decrease; Significances: ***- p<0.001; **- p<0.01; *- p<0.05; T- p<0.1; ns- not significant; n.d.- not determined

3.2.2 Mutant Mouse Lines

For further deciphering the chronic restraint stress, two mouse lines, which differ in their stress-reactivity, were chosen. These were the mouse lines Cor26Nes and *CRH-R1*KO (compare section 3.1.1 and subsection 5.1.2). From each of these two mouse lines we received two cohorts. In each case the first cohort was exposed to the same specifications and the second cohorts to different ones (see Figure 3.24). A third mouse line, the CK-X line, also a *CRH-R1* knock out mouse line, was tested with a three week long unpredictable stress protocol.

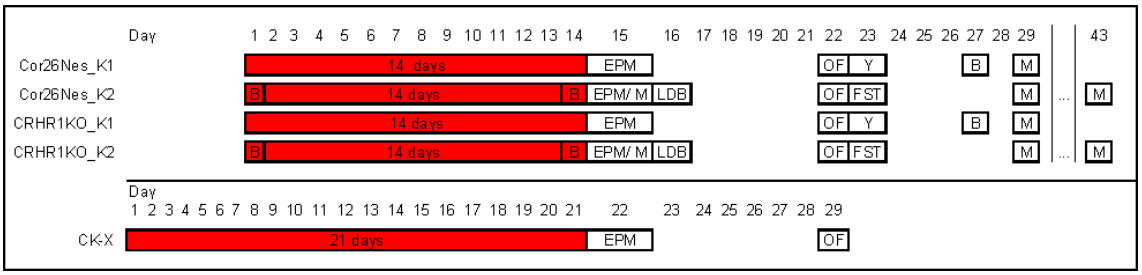

Figure 3.24: Scheme for chronic stress tests in different mutant mouse lines

The different protocols applied for mutant mouse lines, with alterations in the CRH-system. The first four lines were all tested with the 14 day long unpredictable chronic restraint stress protocol. Both corresponding cohorts of the Cor26Nes and the *CRH-R1*KO line were treated the same way. From animals of cohorts 2 blood was drawn at day 1 and day 14 of stress. The CK-X line was tested 21 days with the unpredictable restraint stress protocol. B- Blood withdrawal; EPM- Elevated Plus Maze; FST- Forced Swim Test; LDB- Light-Dark-Box; M- brains fixed for morphological analysis; OF- Open Field; Y- Y-Maze

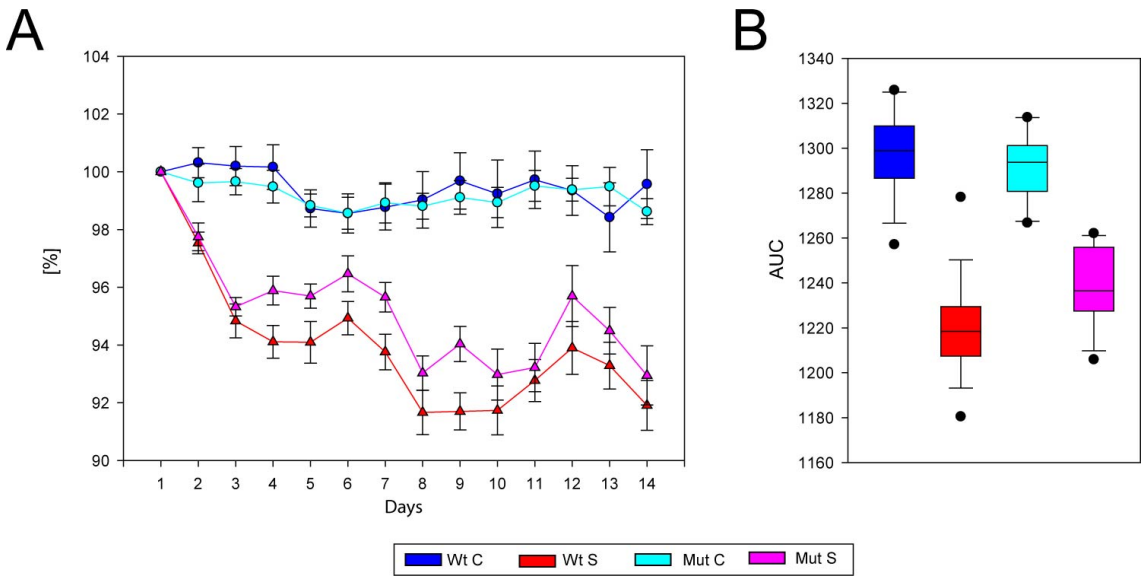

Figure 3.25: Cor26Nes mouse line: Body weight

Body weight changes throughout the two week stress period (A). Body weight at day 1 set as 100%. There is a day-stress interaction. In (B) the AUC was calculated, showing the reduced body weights in the stressed groups. Dark blue- wild-type control (n=12); light blue- Cor26Nes mutant control (n=8); red- wild-type stress group (n=11); pink- Cor26Nes mutant stress group (n=5).

The Cor26Nes mouse line

Body weight

In the first cohort of Cor26Nes mice, statistics revealed a significant stress effect ($F_{1,35}$=92.773, P<0.001) and a trend in genotype-stress interaction ($F_{1,35}$=3.071, P=0.088) (see Figure 3.25). This shows that the stressed groups reduce their body weight compared to controls. The second cohort of CRH overexpressors showed a stress effect ($F_{1,48}$=14.174, P<0.001). We further calculated the AUC (see Figure 3.25B) for both cohorts. Both cohorts showed a highly significant stress effect (Cohort 1: stress-genotype interaction: $F_{1,38}$=3.062, P=0.089; Stress effect: $F_{1,38}$=92.197, P<0.001; Cohort 2: Stress effect: $F_{1,50}$=13.679, P<0.001) and post hoc testing revealed a stress effect in both wild-types and mutants (Holm-Sidak: Cohort 1: WT: Con vs Stress: t=8.654, P<0.001; Mut: Con vs Stress: t=5.201, P<0.001; Cohort 2: WT: Con vs Stress: t=2.795, P=0.007; Mut: Con vs Stress: t=2.459, P=0.018).

Behaviour

EPM: *Distance travelled* in the arena, as well as in the Open Arms (OA), revealed a significant stress effect, which is driven by the wild-types (*Total distance travelled*: Stress effect: $F_{1,33}$=10.195, P=0.003; post hoc: WT: Con vs Stress: t=2.640,

P=0.013; Mut: Con vs Stress: t=2.000, P=0.055; *Distance travelled in OA*: Stress effect: $F_{1,33}$=11.458, P=0.002; post hoc: WT: Con vs Stress: t=3.203, P=0.003; Mut: Con vs Stress: t=1.81, P=0.08). Stressed animals spent more time in the OA (Stress effect: $F_{1,33}$=17.085, P<0.001; post hoc: WT: Con vs Stress: t=3.284, P=0.003; Mut: Con vs Stress: t=2.691, P=0.012) and had more entries into the OAs (Stress effect: $F_{1,33}$=8.860, P=0.006; post hoc: WT: Con vs Stress: t=1.993, P=0.055; Mut: Con vs Stress: t=2.223, P=0.034), which is an effect of the increased activity (see Figure 3.26 A and B). The second cohort of animals did not reveal this increased activity in the EPM, which was conducted under different illumination levels than the first cohort.

On day 22, when animals were tested in the OF, animals from cohort 1 and 2 showed the same results in *distance travelled* (see Figure 3.26 C; depicted is cohort 1). In the 20 minute OF period both cohorts showed a stress effect in the parameter of *total distance travelled*, which was driven by the mutants as revealed by post hoc tests (Cohort 1: Stress effect: $F_{1,38}$=5.764, P=0.022; post hoc: WT: Con vs Stress: n.s.; Mut: Con vs Stress: t=2.615, P=0.013; Cohort 2: Stress effect: $F_{1,39}$=7.610, P=0.009; post hoc: WT: Con vs Stress: n.s.; Mut: Con vs Stress: t=2.986, P=0.005). No changes were found in the *total time spent in the centre* and *total distance travelled in the centre* of the OF arena in any of the cohorts (see Figure 3.26 D).

Organ weights

Because animal numbers are very low (n=4 per group) no statistics was calculated, but still the expected effects of stress on both of these organs could be detected. Relative adrenal weight was increased and relative thymus weight was reduced after stress on day 15. These effects were gone at days 29 and 43 (see Figure 3.27).

Morphology

Morphological analysis of pyramidal cells in the CA3 region of the hippocampus was done by impregnating the brain hemispheres in a Golgi-Cox solution. Four brains per group were analysed at the different time points. Both apical and basal dendritic trees were traced, but only changes in the apical tree are shown here. Depicted in Figure 3.28 is the length of the apical dendrites from brains of the second cohort. Sholl analysis revealed a stress-day-genotype interaction ($F_{2,2204}$=28.870, P<0.001). When applying statistics to the different days, we get significant genotype-stress interactions at day 15 and day 43 (day 15: $F_{1,988}$=6.531, P=0.011; day 29: ns; day 43: $F_{1,646}$=93.009, P<0.001). At day 29 we saw a trend in stress ($F_{1,570}$=3.025, P=0.083), which was driven by the mutants as demonstrated in post hoc testing (WT: Con vs Stress: ns; Mut: Con vs Stress: $F_{1,456}$=7.450, P=0.007). For *total apical length* (Figure 3.28 B) no significant differences can be

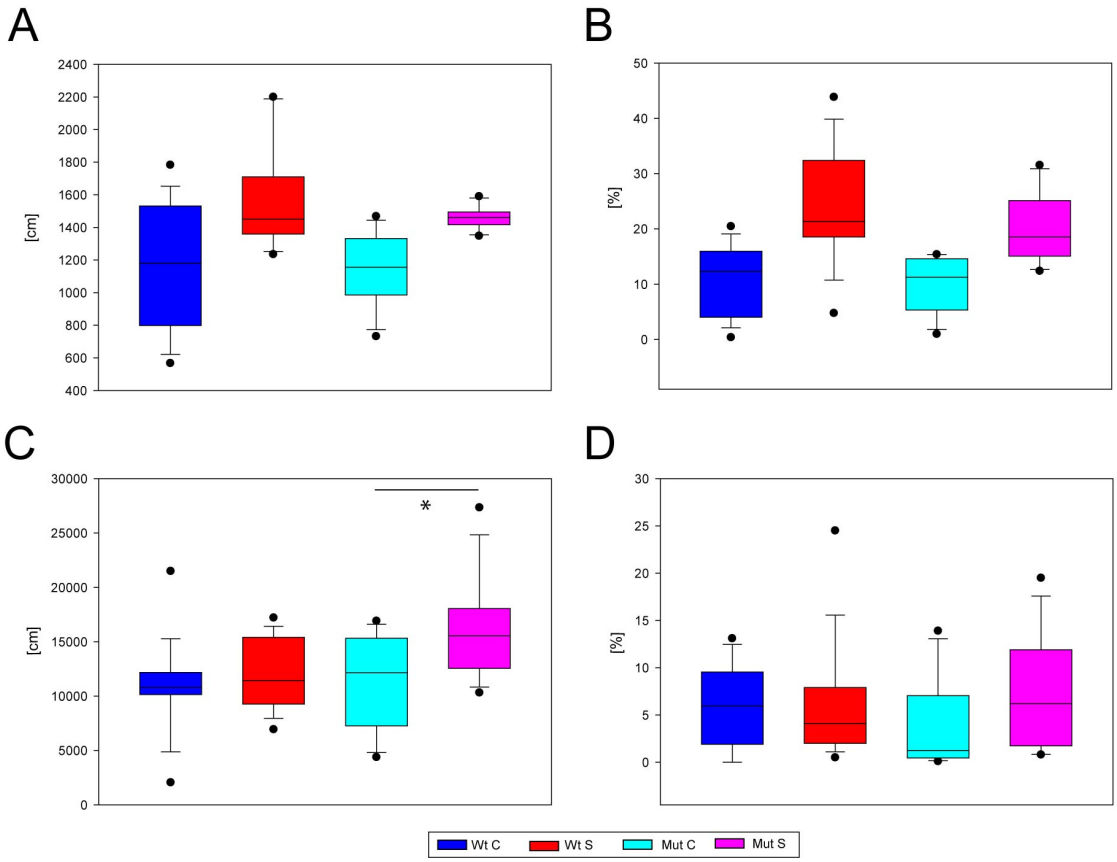

Figure 3.26: Cor26Nes mouse line: Behaviour

After two weeks of unpredictable restraint stress stressed animals of both genotypes showed an increase in *distance travelled* (A) and *time spent in the open arms* (B) of the EPM one day after stress cessation. Eight days after the end of the chronic stress increased *distance travelled* (C) driven by the mutants could be observed in the OF. No changes were found in anxiety-related behaviours, like *time spent in the centre* of the OF arena (D). EPM: n=6-11; OF: n=8-12

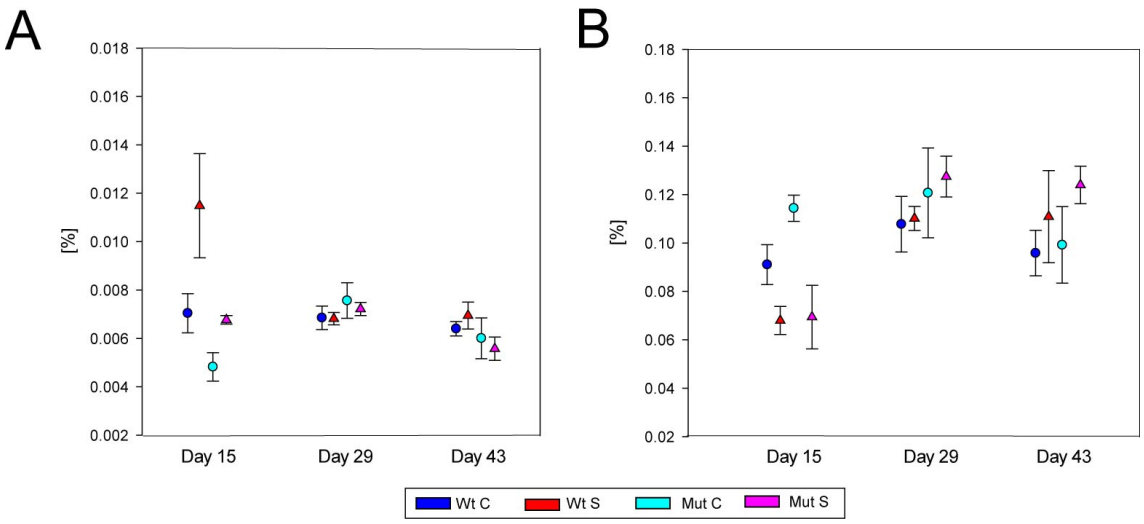

Figure 3.27: Cor26Nes mouse line: Relative organ weights

Cohort 2 of the Cor26Nes line were sacrificed at different time points after stress cessation. Both adrenal weight (A) and thymus weight (B) was calculated as percentage of body weight. On day 15 adrenals are increased in weight in the stressed groups and thymus weights are reduced at that time point. At the other time points no differences between groups were observed.

observed at day 15 and 29, whereas at day 43 a stress-genotype effect ($F_{1,16}$=8.703, P=0.009) occurred. At day 43 we saw no difference in the wild-types, but a trend in the mutants (Mut: Con vs Stress: $F_{1,10}$=4.907, P=0.051).

The CRH-R1KO mouse line

Body weight

The first cohort of the *CRH-R1*KO mouse line revealed a significant day-genotype interaction ($F_{12,372}$=2.278, P=0.008) and a highly significant stress effect ($F_{1,31}$= 11.748, P=0.002) on body weight change. Analysing the two genotypes separately revealed a significant stress effect only in the wild-types ($F_{1,17}$=20.036, P<0.001) with no effect in the mutants. Also in the second smaller cohort the effect of genotype-day interaction ($F_{11,165}$=5.178, P<0.001) as well as stress effect ($F_{1,15}$=12.157, P=0.003) could be recapitulated.

In the AUC we can observe a clear stress effect, which was present in both cohorts (Cohort 1: Stress effect: $F_{1,34}$=11.620, P=0.002; Cohort 2: Stress effect: $F_{1,18}$=11.671, P=0.004). Post hoc testing revealed that the stress effect was driven by the wild-types (t=3.409, P=0.002), whereas there was no difference in the mutants in cohort 1 (see Figure 3.29).

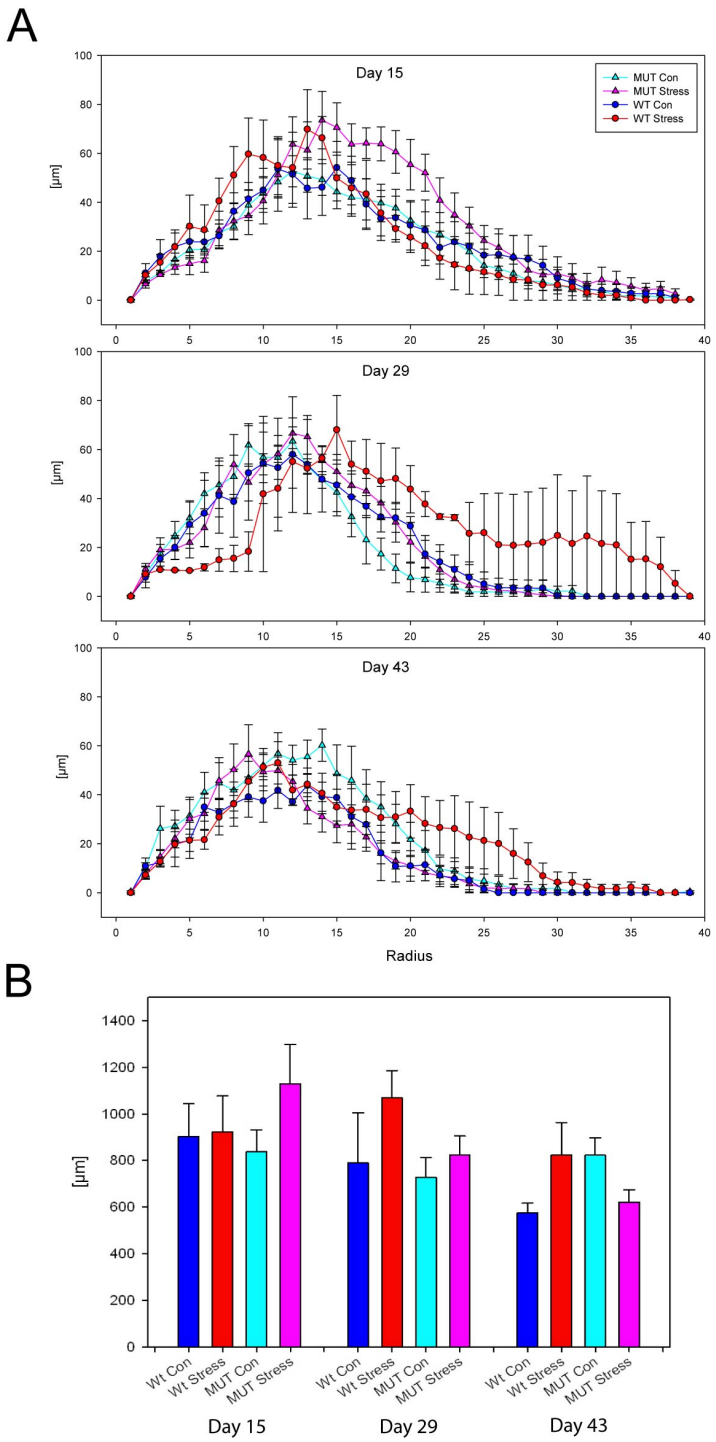

Figure 3.28: Cor26Nes mouse line: Morphological analysis of the CA3 region
Shown in (A) is the apical dendritic length at the different days in a Sholl analysis. (B) depicts the total apical dendritic length.

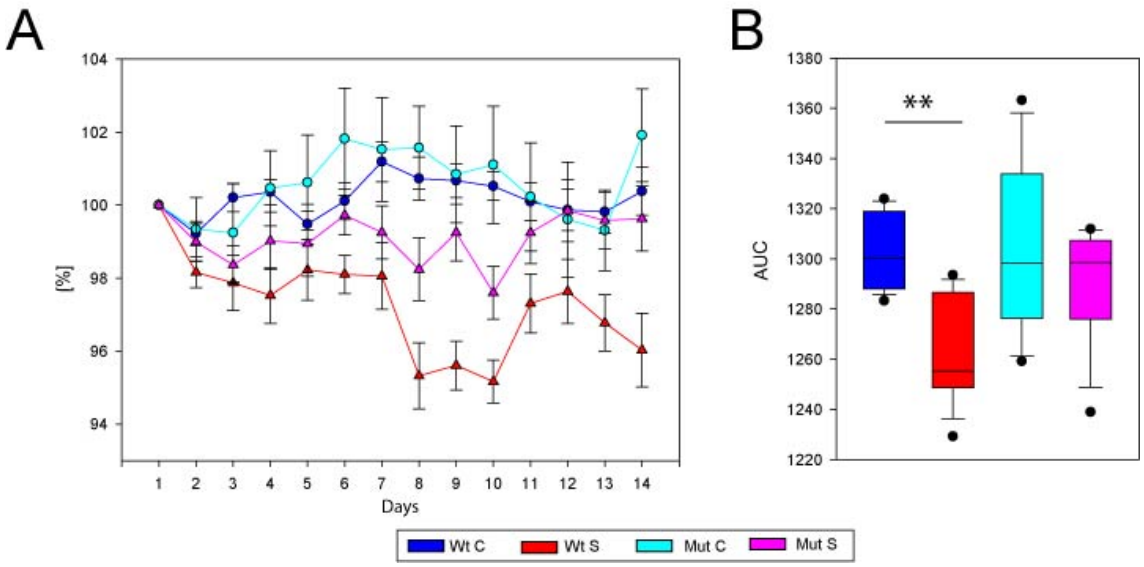

Figure 3.29: CRH-R1KO mouse line: Body weight

Body weight changes throughout the two week stress period (A). Body weight at day 1 set as 100%. AUC (B) demonstrates the stress effect, which is significant in the wild-types. Dark blue- wild-type control (n=10); light blue- *CRH-R1*KO mutant control (n=7); red- wild-type stress group (n=9); pink- *CRH-R1*KO mutant stress group (n=9).

Behaviour

EPM: In the *CRH-R1*KO line we saw an activating effect of chronic stress: both stressed groups had an increased *distance travelled* (Stress effect: $F_{1,34}$=15.216, P<0.001; post hoc: WT: Con vs Stress: t=2.697, P=0.011; Mut: Con vs Stress: t=2.819, P=0.008; see Figure 3.30), spent more time in the OA (Stress effect: $F_{1,34}$=8.794, P=0.006; post hoc: WT: Con vs Stress: t=2.855, P=0.008; Mut: Con vs Stress: ns) and had increased mean velocities (Stress effect: $F_{1,34}$=5.662, P=0.024; Genotype effect: $F_{1,34}$=3.491, P=0.071; post hoc: WT: Con vs Stress: t=2.378, P=0.024; Mut: Con vs Stress: ns, data not shown).

OF: Also in the OF this increase in activity could been seen. Here we saw an increase in *total distance travelled* (Stress effect: $F_{1,34}$=9.150, P=0.005; post hoc: WT: Con vs Stress: t=1.716, P=0.096; Mut: Con vs Stress: t=2.529, P=0.017) as well as *total number of rearings* (Stress effect: $F_{1,34}$=4.447, P=0.043; Genotype effect: $F_{1,34}$=7.209, P=0.012; post hoc: WT: Con vs Stress: ns; Mut: Con vs Stress: t=2.331, P=0.026, data not shown) after chronic stress. Post hoc testing for the *number of rearings* revealed that the difference was mainly produced by the stressed mutants. We did not observe any significant differences in the parameters *time spent in the centre* and *distance travelled in the centre*.

Organ weights

Animal numbers are very low so that no statistical analysis was conducted. Nevertheless it is interesting to see the differences between the groups. The *CRH-R1*KO mutants had a lower adrenal weight than the wild-type controls both at day 15 and 29. Also the effect of stress on thymus weight was reduced in the mutants, whereas you can see the difference in the wild-type groups at day 15.

The CK-X mouse line

Body weight

The CK-X mouse line is also a *CRH-R1* knock-out, comparable with the *CRH-R1*KO mouse line mentioned above, although the mutation is in a different exon (see subsection 5.1.2 for details). This mouse line was chronically stressed for three weeks with the unpredictable restraint stress protocol. From Figure 3.32 one can clearly see the differences in body weights. There was a significant day-stress-genotype interaction ($F_{17,578}$=1.841, P=0.021). This day-stress interaction was present in the wild-types, but not in the mutants (WT: $F_{17,374}$=4.040, P<0.001, Mut: ns).

The AUC demonstrates that stress affects both genotypes (Stress effect: $F_{1,37}$=16.966, P<0.001), post hoc testing revealed a stronger effect in the wild-types (t=4.226, P<0.001) and only a trend in mutants (t=1.956, P=0.059).

Behaviour

EPM: In the EPM one day after the end of stress, statistics revealed a significant genotype ($F_{1,37}$=4.752, P=0.036) and stress effect ($F_{1,37}$=7.124, P=0.012) in *total distance travelled* (Figure 3.33 A). Post hoc testing showed that the stressed mutants did not react with increased *distance travelled*, whereas the wild-types did (WT: Con vs Stress: t=3.414, P=0.002; Mut: Con vs Stress: n.s.). The same holds true for the parameter of *percent time spent in the OA* of the EPM (Figure 3.33 B). Again mutants did not show the stress-induced increase the wild-types did (Genotype effect: $F_{1,37}$=12.609, P=0.001; Stress effect: $F_{1,37}$=8.432, P=0.006; post hoc: WT: Con vs Stress: t=3.280, P=0.002; Mut: Con vs Stress: n.s.). It is interesting to note that the mutants did show an increased *time spent in the OA* compared to their wild-type controls (Con: WT vs Mut: t=3.273, P=0.002), illustrating the effect of basal reduced anxiety.

OF: In the OF, one week after the EPM, no differences between the groups could be detected in *total distance travelled* (see Figure 3.33 C). For the parameter of *percent time spent in the centre* of the OF, a difference between the genotypes could be

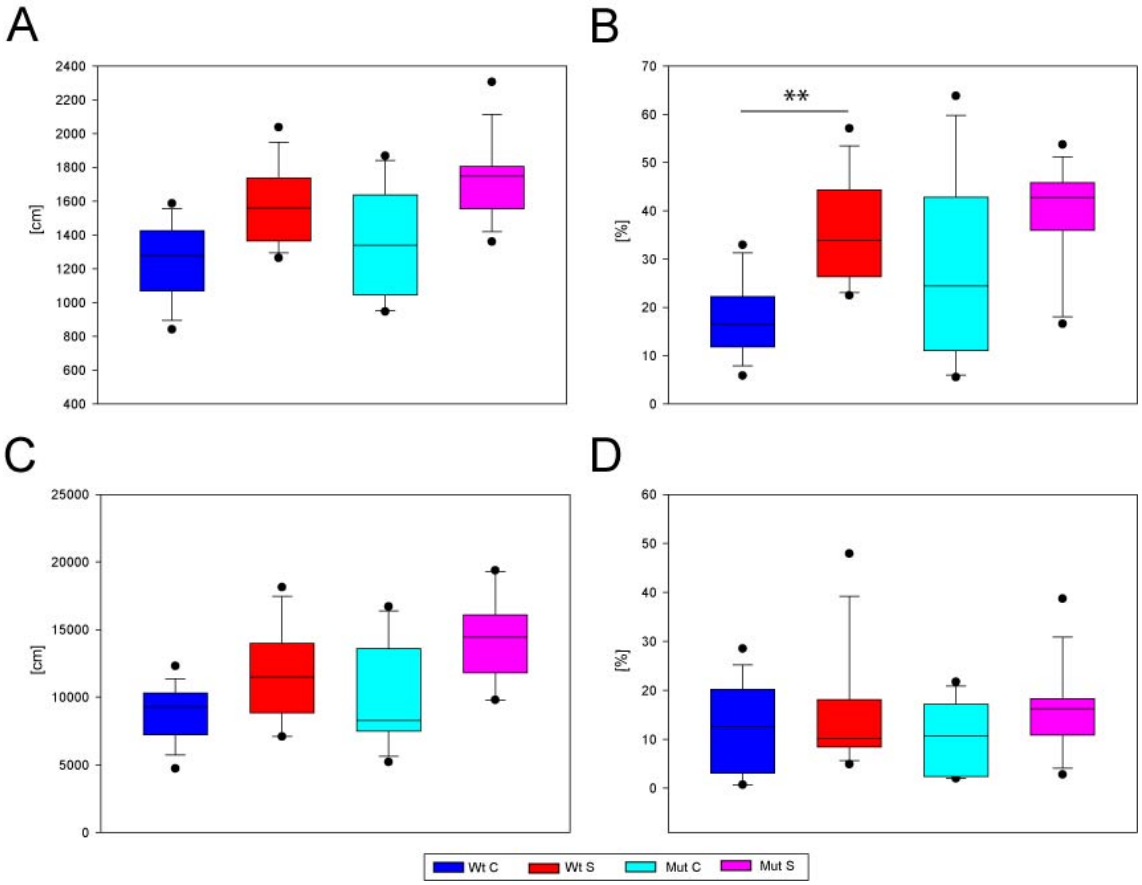

Figure 3.30: CRH-R1KO mouse line: Behaviour

After two weeks of unpredictable restraint stress, stressed animals showed an increase in *distance travelled* (A) and *time spent in the open arms* (B) of the EPM one day after stress cessation. The stress effect seen in the *time spent in the OA* was driven by the wild-types. Eight days after the end of the chronic stress increased *distance travelled* (C) mainly driven by the mutants could be observed in the OF. No changes were found in anxiety-related behaviours, like *time spent in the centre* of the OF (D). n=7-10

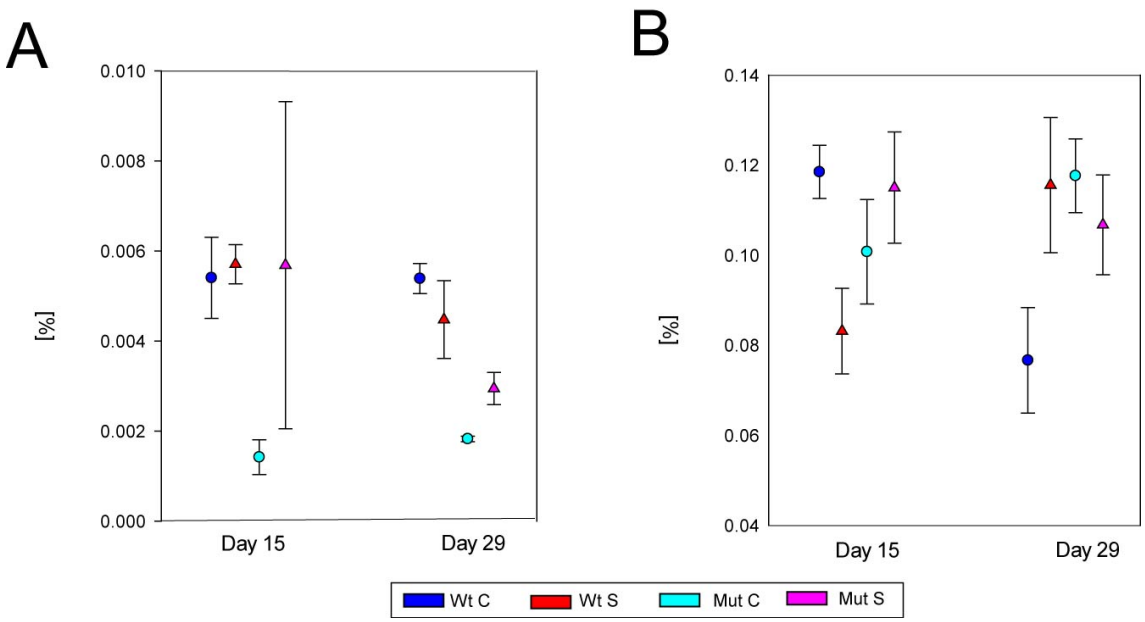

Figure 3.31: CRH-R1KO mouse line: Relative organ weights

Cohort 2 of the *CRH-R1*KO line were sacrificed at different time points after stress cessation. Both adrenal weight (A) and thymus weight (B) was calculated as percentage of body weight. On day 15 and 29 adrenal weights are lower in the mutant group. Thymus weights are reduced in the wild-type stressed animals at day 15.

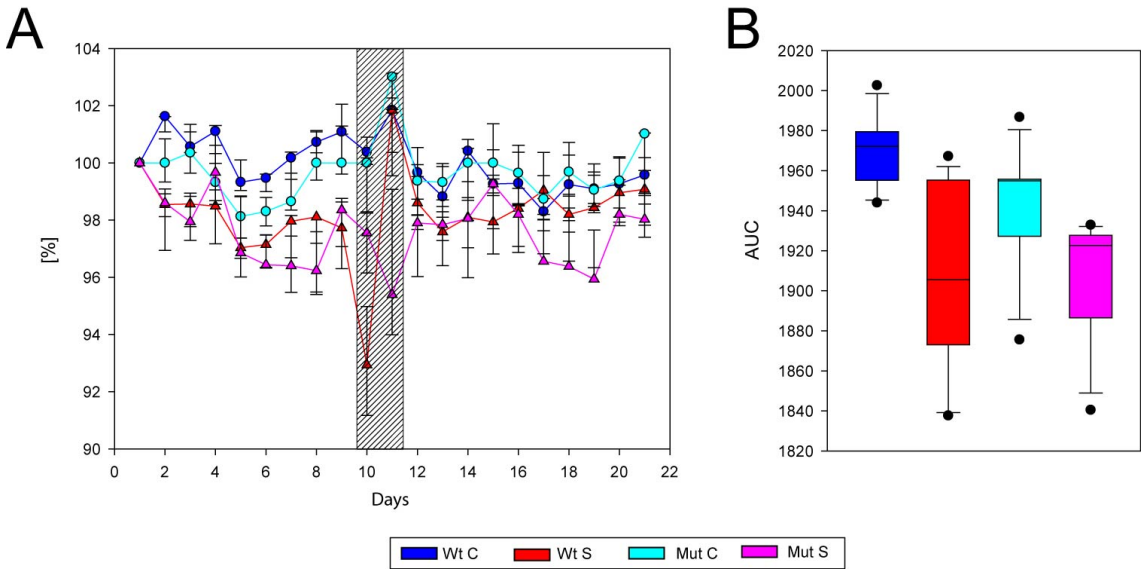

Figure 3.32: CK-X mouse line: Body weight

(A) Body weight changes throughout the three week stress period. Body weight at day 1 set as 100%. AUC shows the stress effect, which is stronger in wild-types (B). Dark blue- wild-type control (n=12); light blue- CK-X mutant control (n=7); red- wild-type stress group (n=12); pink- CK-X mutant stress group (n=7); Shadow at day 10 and 11: body weight from only half of the animals was taken.

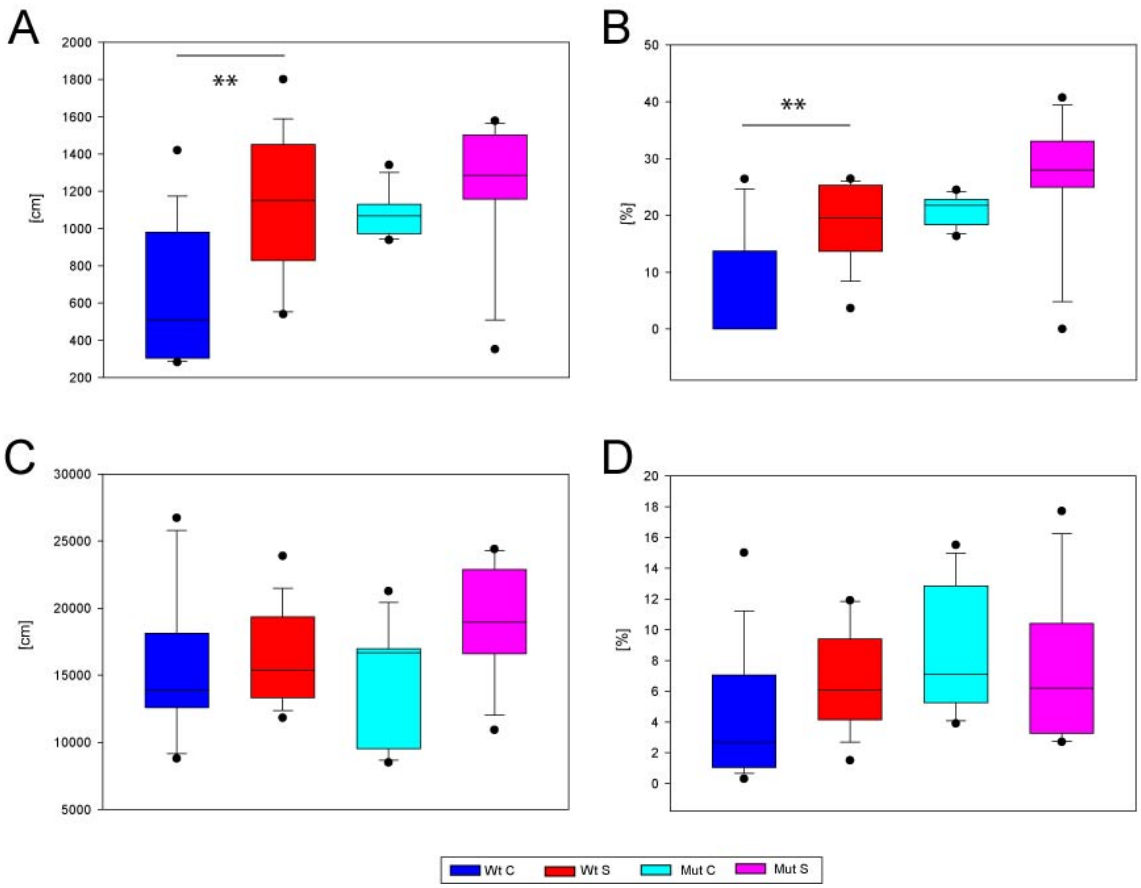

Figure 3.33: CK-X mouse line: Behaviour

Behaviour of the CK-X mouse line. Graphs (A) and (B) depict *distance travelled* and percent *time spent in the open arms* of the EPM respectively. In both parameters the wild-types show a stronger stress effect. Below in (C) and (D) parameters from the OF are shown; *total distance travelled* and percent of *time spent in the centre*, respectively. No differences, except a small genotype effect in *time spent in centre*, was observed. n=7-12

revealed; the mutants tended to spend more time in the centre (Genotype effect: $F_{1,37}$=3.983, P=0.054), especially when comparing the two control groups (Con: WT vs Mut: t=2.277, P=0.029) (see Figure 3.33 D).

Organ weights

Only the adrenals were dissected and weighed. Statistical analysis revealed a trend in interaction (Interaction: $F_{1,25}$=3.180, P=0.088) and a significant genotype effect was observed (Genotype effect: $F_{1,25}$=9.477, P=0.005), with mutants having lower relative adrenal weights.

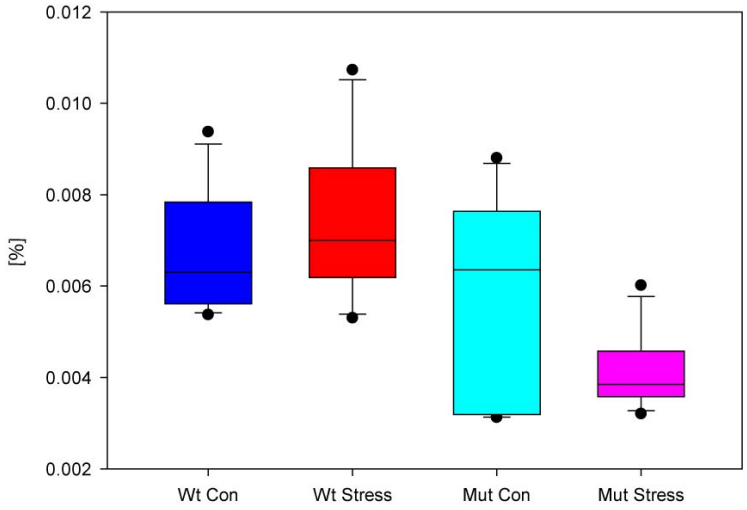

Figure 3.34: CK-X mouse line: Relative adrenal weights
Relative adrenal weight, measured eight days after end of chronic stress, in the mutant mice are lower compared to their wild-type litter-mates. N=6-7

3.2.3 Two Year-Old C57BL/6N Mice

C57BL/6N male mice were tested at the age of two years in the chronic stress paradigm. They were subjected to two weeks of unpredictable restraint stress.

Body weight

The body weight change over time revealed a day-stress interaction ($F_{12,207}$=2.607, P=0.003). When looking at the calculated AUC, statistics revealed a difference between the two groups (t_{13}=2.908, P=0.012). Animals of the stressed group showed a lower AUC, indicating an overall stronger weight reduction due to stress.

Behaviour

EPM: In the EPM, in which all animals were tested, the stressed animals clearly showed an increase in *distance travelled* (t_{15}=-5.276, P<0.001) as well as a trend in increased *percentage of time spent in the OA* (U=16.0, P=0.06).

LDB and OF: In the LDB and the OF, where only a subset of animals (n=4/5 controls and stressed respectively) were tested, again an increase in activity could be detected. Interestingly, the stressed animals showed an increase in *distance travelled* within the first five minutes of the OF (data not shown).

Organ weights

Adrenal weights at day 15 hinted towards a slight increase within the stress group. At day 29 these weights did not differ between the groups any more. Thymus weights

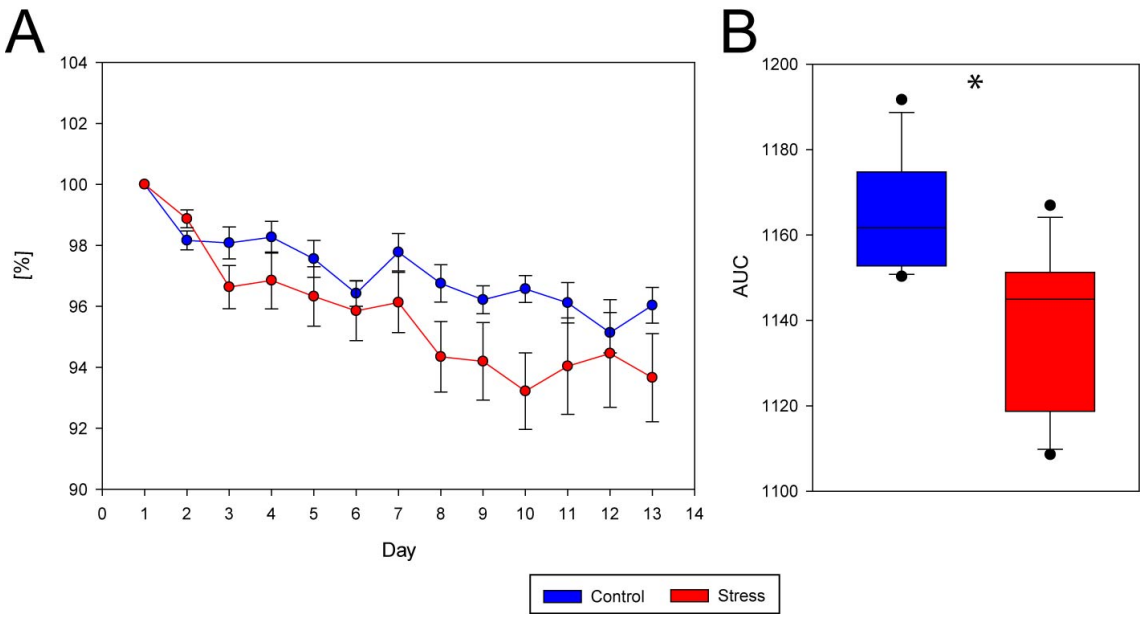

Figure 3.35: Aged mice: Body weight
(A) Body weight changes throughout the two week stress period. Body weight at day 1 set as 100%. (B) Body weight over time calculated as AUC. Stressed group shows a smaller AUC; Significances: *-p<0.05, Dark blue- control (n=8); red- stress group (n=9)

at day 15 appeared to be lighter in the stress group, but the thymus in these aged mice did not seem to regenerate. At day 29, thymic weights were still lower in the stressed animals.

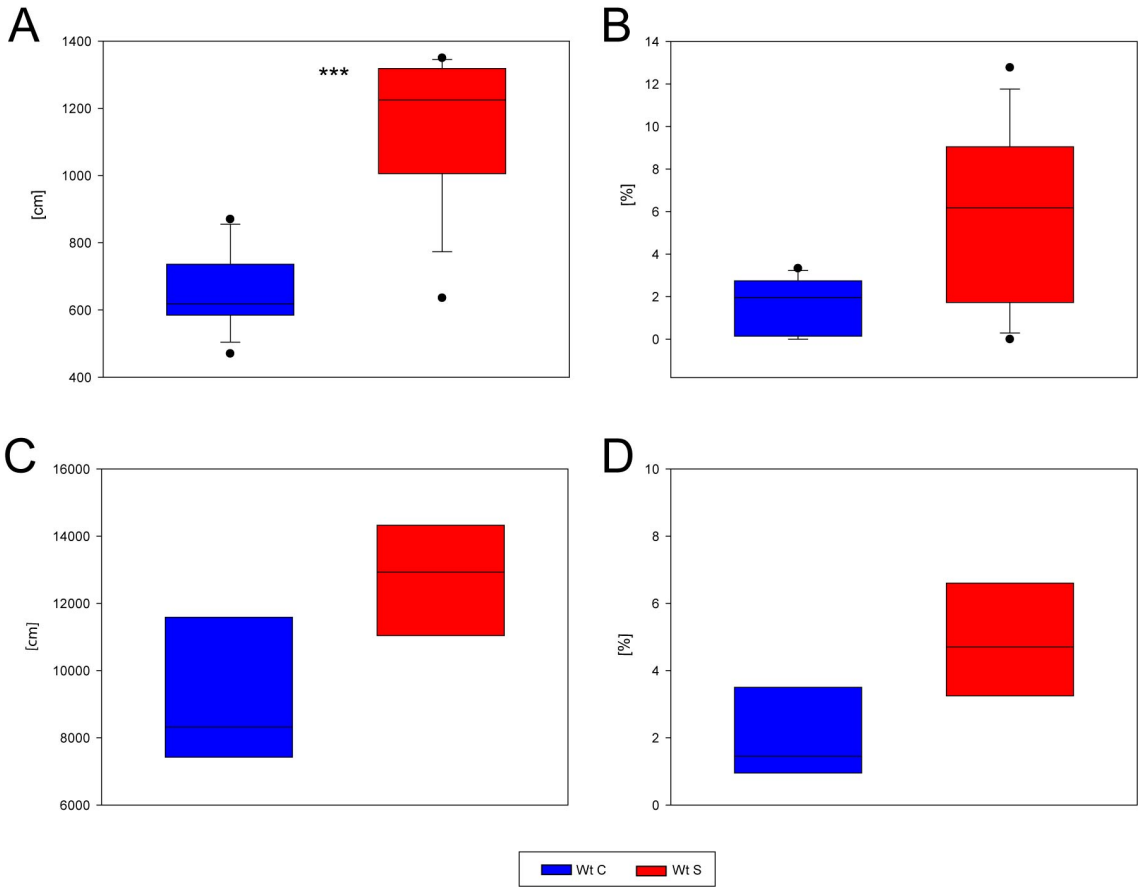

Figure 3.36: Aged mice: Behaviour

Graphs (A) and (B) depict *distance travelled* and percent *time spent in the open arms* of the EPM respectively. Below in (C) and (D) parameters from the OF are shown; *total distance travelled* and percent of *time spent in the centre*, respectively. n(EPM)=8-9; n(OF)=4-5

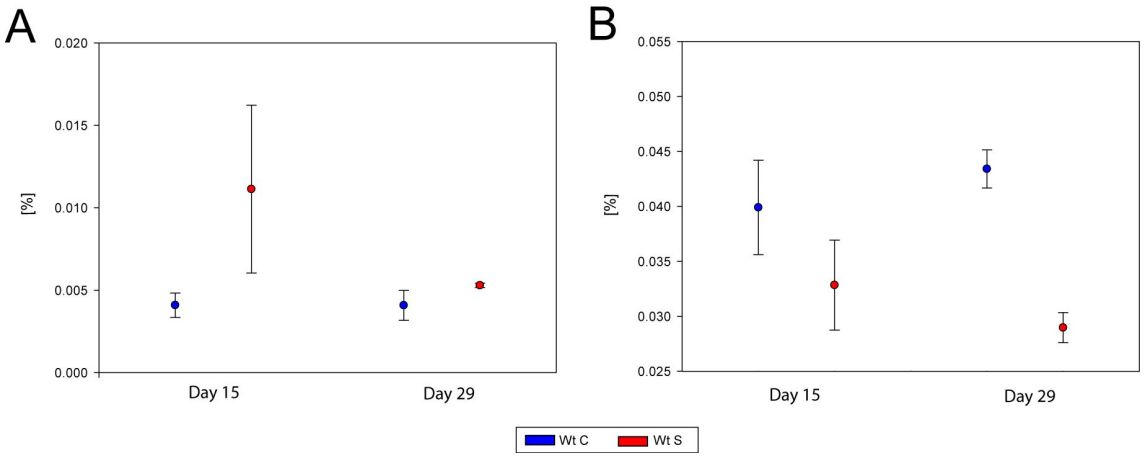

Figure 3.37: Aged mice: Relative organ weights

Relative adrenal (A) and thymus (B) weights in old animals as percentage of body weight. Organs were taken at different time points after stress. n=2-4

4 Discussion

For the establishment of the stress challenges C57BL/6 mice were used, because of their growing importance as a background strain in genetically modified mice. Large-scale mutagenesis programs like the International Knockout Mouse Consortium[1] (IKMC) aim to mutate all protein-coding genes in C57BL/6N embryonic stem cells. This consortium includes the EUCOMM/ EUCOMM tools, the Knockout Mouse Project of the USA, the North American Conditional Mouse Mutagenesis Project (NorCOMM) and Texas A&M Institute for Genomic Medicine (TIGM). Many of the animals from the EUCOMM project are phenotyped within the GMC, hence we established our tests with this mouse strain, although C57BL/6 are known to be less stress-reactive than for example the BALB/c strain [59, 131, 155].

4.1 Acute Stress Challenge

4.1.1 Establishment of the Acute Stress Challenge

We could show that an acute restraint stress for two hours leads to reproducible changes in the first five minutes in the OF applied 20 minutes post-stress. The effect can not be seen in any other behavioural test we conducted, namely the EPM and LDB, 20 minutes post-stress, nor at later stages (i.e. six hours post-OF, 24 hours post-OF nor 30 hours post-OF). This is in accordance with the literature, where also no behavioural changes were observable 24 hours post-stress [34]. The stress-induced effect we see in the OF is increased activity, in both *distance travelled* and *number of rearings*, which are very consistent over different cohorts of C57BL/6 male animals, even when looking at absolute values (c.f. Figure 3.2). Restraint is routinely used to activate the HPA-axis. A rise in CORT levels in different restraint methods has been shown in several studies [19, 34, 51, 107, 126]. We could show that the two hours restraint stress leads to an increase in CORT level, which continues to be elevated throughout the interval and the OF. This demonstrates that the HPA-axis is activated by our acute restraint stress test. The OF, as a new environment, has a CORT elevating effect, as shown by the second control group (see Figure 3.3), thereby acting as a stressor itself.

Observations during the interval period, between stress and OF, showed that, within these 20 minutes, animals spent time grooming, which is not surprising due to the

[1] http://www.knockoutmouse.org

fact that most of the animals relieve themselves within the restraint tube. To test whether this interval is essential, we stressed the animals for two hours after which they directly went into the OF, without the 20 minute interval. It could be clearly shown that stressed animals spent approximately 28 % of the time grooming within the first five minutes of the OF (compared to controls which spent only 12 % of the time grooming) and therefore hamper the other measures (see Figure 3.5). We have demonstrated that the interval is essential for correct interpretation of the resulting measures. We could further show that the duration of the stress itself, but not the time-lag between the beginning of the stress period and the OF, independent of stress duration, is necessary to induce activity (c.f. Figure 3.6).

To validate our protocol we took two mutant mouse lines, which have alterations in the CRH-system and thus have been shown to respond differently to stress. The Cor26Nes line, a CRH overexpressing line, was shown to have a hypersecretion of CORT and ACTH after a ten minute restraint period (only in male animals) but no differences under basal conditions [107]. We could show that these mice respond to our 15 minute stress with an increased activity, validating our protocol for detecting animals with a higher stress-reactivity.
The *CRH-R1*KO mutant mice have been shown to have no differences in plasma ACTH under basal conditions, but, after a five minute forced swim stress, both ACTH and CORT levels were significantly lower [172] compared to wild-type animals. Also, in our acute stress test (in the 15 minutes and the two hours), the *CRH-R1*KO mutant mice showed no response in contrast to their wild-type litter-mates. Results from both mouse lines verify that altered HPA-axis activity is reflected in *distance travelled* and *number of rearings* in the first five minutes of the OF after acute restraint stress.

Release of CRH activates the HPA-axis with its final secretagogues CORT. Many publications have shown a connection between CRH and locomotion. Intracerebroventricular injection of low doses of CRH, which resemble stress levels, induce an increase in locomotion in the OF [101]. Two studies showed a transient increase in locomotion after restraint stress, which was only apparent in the first few minutes of the OF [100, 146]. We find the stress-induced difference in *distance travelled* and *number of rearings* only within the first five minutes of the OF, thereafter the difference subsides in the subsequent five minute bins. Therefore we think that the already elevated CORT levels of the stressed group is the physiological mechanism mediating the observed behaviour. NA would be another alternative, although these levels increase within seconds, and as the OF acts as a stressor itself, this possibility seems less likely. To test whether the increased activity is driven by elevated CORT levels, we blocked CORT production by injecting Metyrapone. Metyrapone blocks the 11beta-hydroxylase, the CORT synthesising enzyme. The Metyrapone-injected animals did not show an increase in activity after two hours of stress, demonstrating the importance of circulating CORT for the behavioural response to restraint stress. For further examination we blocked the possibility of CORT to feedback on the GR by injecting a GR-antagonist, RU486 (also known as Mifepristone). We saw that

blocking the GRs did not lead to an elimination of the increased activity through stress, which reveals that GRs are not necessary for the behavioural effect. Taken together the results of the pharmacological experiments demonstrate the importance of CORT on the behavioural outcome, but also showing that its effects are not mediated via the GR, but probably through the MR, which should be tested. The MR has long been considered a "dull" receptor, as Jöels et al [86] put it, as it is nearly constantly occupied by CORT, through its high affinity for it. In the past few years it has become clear that the MR plays a major role in stress adaptation. There is considerable evidence that there is a lower-affinity MR, transmitting non-genomic actions of CORT. Karst et al [88] showed that CORT can rapidly alter hippocampal signalling, which is mediated via a non-genomic but MR-dependent mode (as shown by mouse mutants and GC receptor agonists and antagonists). Also this effect was only significant when higher (i.e. 10 nM CORT) concentrations were injected and not with lower concentrations, indicating a lower-affinity receptor. The BSA-CORT conjugate, which cannot cross membranes, showed an effect, demonstrating that the receptors mediating these effects are on the cells membrane. Dorey et al. found that non-genomic effects of acute stress on memory retrieval are dependent on membrane MRs [54]. How CORT mediated its effects in our acute stress test has still to be evaluated. As stress-induced locomotion appears shortly after stress (in our case 20 minutes after the end of stress) it seems to exclude the possibility of genomic actions, which need hours from stimulating gene expression and leading to changes on protein levels [56]. To separate between genomic versus non-genomic actions, one could use protein synthesis inhibitors and/or transcription inhibitors to evaluate the genomic effects of CORT. As shown by Sandi et al. [147], a single CORT injection induced locomotor activity within 7.5 and 15 minutes and could not be blocked by RU486 nor cycloheximide, a protein synthesis inhibitor, demonstrating a non-genomic, GR-independent mechanism. This mechanism might be the same for our acute stress test.
Now it is accepted that a delicate balance of MR and GR is necessary for physiological stress reactivity. The MR is thought to play a major role in maintaining viability and setting a threshold for the stress response (probably through the genomic actions of the high-affinity MR) and in enhancing the brains activity, specifically in limbic areas, inducing the early response to a stressor (via the non-genomic actions of a membrane bound MR). The GR is thought to be important for the shut-down of the HPA-axis as well as the preservation and encoding of acquired information during stress exposure [86].

In the literature repeated stressing is often found to decrease the response to it over time, as measured by CORT levels [31, 66]. Repeated homotypic stressing with our protocol, within a week or even two days, such a habituation to the stressor is not evident in the behavioural response. It might even increase the contrast between control and stressed groups, as can be seen in Figure 3.8 where the first response to the stressor is less pronounced than on the second or third exposure. Both groups habituate to the test arena, but without fading of the stress effect. Even throughout

the life time, animals do not habituate to the stressor (see Figure 3.8). Together with the results of the pharmacological experiment it suggests that we repeatedly induce a CORT response to stress. Also there is no time-dependent increase in percent of *time spent in the centre* in the animals tested throughout life time, demonstrating that there is no increased nor decreased anxiety-related behaviour obvious in the stressed group. At the age of 29 weeks these animals were tested in the OF without a stress period to control for conditioning to the OF. As we saw no increase in activity in the originally stressed group, this experiment demonstrates that the OF environment does not become a conditioned stimulus inducing a conditioned behavioural response.
These findings are very important and beneficial. It makes it possible to re-test animals with the restraint stress test and analyse different read-outs, for example to draw blood from the re-tested animals to analyse CORT levels, or one could think of pharmacological manipulations and treatments (c.f. acute stress test in different mouse lines, e.g., Emory, HST014 and *Db/db*).

To evaluate differences between OF test settings, we performed an experiment under different conditions. Two OFs were placed in a room, separated by plastic blinds, to mimic OF set-ups as they are frequently used in other labs. The expected increase in locomotor activity and *number of rearings* in response to stress was not observed, illustrating the necessity to test within our small chambers. The question why this is so remains. Maybe the surrounding inside the small chambers is less stimulating than in an experimental room. The small chambers only include one OF arena and nothing else, so that each mouse is tested in isolation. In contrast the bigger experimental room has shelves on the walls and different equipment is stored, which is being investigated by the mouse, not to forget the auditory and olfactory contact the tested animals have between each other during the testing duration.

As for testing with different OF systems (Actimot versus EthoVision) we see in both systems investigated the stress-induced difference in *distance travelled*, although there is an interaction between system used and stress effect. As the *percentage of time spent in the centre* of the OF is similar between both settings, it points towards differences in calculating the *distance travelled*. The video-based EthoVision system calculates the centre of gravity from the observed body of the mouse, and with this recognizes movements. The Actimot system is based on infra-red-light-beam breaks where the animals body is estimated by the different beam breaks. Therefore this system is more sensitive to smaller movements, e.g. of just the nose reaching back and forth thereby breaking light beams, contributing to the *distance travelled* measure. These small movements do not lead to big changes in the body´s centre of gravity in the EthoVision system and therefore the *distance travelled* here is much lower. In that sense the term *"distance travelled"* used by the Actimot system might be misleading, and a term like "activity" might be more appropriate. Another factor adding to the differences could be the different frequencies by which changes are recognized; the Actimot system runs on up to 100 Hz (with 52 Hz in our set-up), whereas the EthoVision runs with up to 30 Hz (12.5 Hz in our set-up). Taken

together, the Actimot system is more appropriate for our testing, because it senses also small movements, and thus is more sensitive.

Stressing heavier mice in animal holders showed that restraint in these bigger tubes also increased horizontal and vertical activity. Of course one always should keep in mind that the more space an animal has inside the tube, which depends on both body weight of the mouse and size of the tube, the less it will be restrained and therefore stressed. This might account for the more subtle effects and higher variability in young females and old or obese mice (c.f. Figure 3.20).

We could also show that the two hour stress test works for female and for very old (over two years of age) male C57BL/6J mice. Other strains like the BALB/c and the C3H are not as active as the C57BL/6 strains, which is known [39, 50, 104, 114], but they also show the increase in activity after a two hour stress period. The C3H line was interesting to analyse, because these animals are blind by the time of weaning (approximately at three weeks of age). Although the animals were blind at the time of testing, they showed the increased activity, demonstrating that the acute stress test is suitable for blind mice as well. The only strain we tested and did not lead to the, by now expected, differences was the 129Sv strain. This shows the importance of testing litter-mates in parallel to the mutants, for different background strains can lead to differences in the stress-response in the OF. Genetic background is an important factor to keep in mind. This is illustrated by Holmes et al. who showed the influence of genetic background on phenotypes [74]. They demonstrated that a null mutation in the serotonin transporter leads to differences in anxiety-related behaviour when bred on different genetic backgrounds. The mutants on a C57BL/6 background showed increased anxiety-related behaviours, whereas the one bred on a 129S6 background did not, although these showed alterations in the serotonin transporter function. There are different explanations for this. First, it could be that the anxiety-related phenotype was not observable due to a "ceiling effect" in the 129S6 strain, as they already have higher levels compared to C57BL/6. The second explanation could be that flanking regions influence behaviour. If a mutation is brought into an animal, the flanking regions of the mutated site are often of another genetic background than the animal itself, thereby it is possible although minimal, that these regions have an influence on the phenotype [185]. This effect can be minimized if animals are back-crossed for several generations. Thirdly, the different genetic make-up, can lead to gene-gene interactions, thereby modifying the phenotype [38].

4.1.2 Testing Mouse Mutant Lines

The AcStr01 mouse line The modified gene is specifically expressed in the brain as a scaffold protein at the post synaptic density of excitatory synapses. Together with other scaffold proteins, such as members of the Shank-family, they link trans-membrane proteins such as receptors, and signal transduction components to the

microfilament network of the cell [95]. Defects in scaffold proteins can lead to different neurological defects. For example, loss of SAPAP3 has been shown to model obsessive-compulsive disorder [184] and Shank1 alters anxiety [78]. This leads to the assumption that this gene might also have an impact on the HPA axis, as alterations of the axis have been implicated in neuropsychiatric disorders, and therefore changes in behaviour and/or response to stress.

The AcStr01 mutants show no stress-induced increase concerning the *distance travelled* within the 15 minute stress test. Also in the *number of rearings* the male mutants show no significant increase. This hints to a possible higher threshold in stress reactivity compared to wild-type litter-mates. Analysing the data from the two hour stress test, we did not see a difference between genotypes in post hoc testing for *distance travelled,* but still in *number of rearings.* Mutants do not show a stress-induced increase in *number of rearings.* These two independent cohorts demonstrate that there is a subtle difference in stress-responsivity at low stress levels, with mutants being more stress-resistant.

Although the mutants exhibit extremely high levels of locomotion this is not the reason for observing no effect in the mutants in the 15 minute stress test; animals show, within the two hour challenge, an increase in this parameter, which exceeds the one seen after the 15 minute stress test. In the Steroid Metabolism Screen no differences between the genotypes was observed at basal CORT levels, which support the theory of only subtle changes in stress reactivity.

The AcStr02 mouse line The mutation of this mouse line causes a loss of function in the gene, which is associated with recessively inherited early-onset PD, which occurs in 1-2 % of PD patients. Mutations in this gene are linked to familial PD, characterized by an early onset, slow progression and good response to pharmacological treatment with levodopa. Interestingly, it is known that PD patients with this specific mutation show psychiatric alterations, namely depression and anxiety [151]. Neuropsychiatric disorders are not exclusively present in PD patients with such a mutation but are a prevalent symptom in all PD patients. Co-morbidity between PD and anxiety is estimated to be up to 40% [182]. The gene-trap induced null mutation was generated at the HMGU in the Institute of Developmental Genetics. This mouse line was screened by the GMC within the Primary Screen, where only subtle differences were detected between wild-type and mutant mice. One of these genotype-specific differences was found in the Neurology Screen with the modified Shirpa protocol. The modified Shirpa protocol is applied to rapidly test mouse mutant lines for abnormal phenotypes, especially in muscle function. Mutants were hyperactive during a short period in this new environment. This hyperactivity was not observed within the OF of the Behaviour Screen. Therefore the effect in the Shirpa protocol was interpreted as a very brief and transient stress reaction to a new environment.

In the female cohort we could recapitulate, at least in part, the higher stress-

reactivity in the mutants. The effects seen in the 15 minute acute stress test in the mutant mice hint on possible changes towards an increased stress-reactivity. A reduced body weight in the mutant animals could abolish the possibility that mutants were more stressed than wild-types due to increased body weights and less space within the restraint tubes. We also checked body weight gain of these animals within the two weeks after the first stressor. Results here point towards a possible higher stress sensitivity, as the mutants do not gain weight, whereas the wild-type controls do (data not shown). Together with the results from the Neurology Screen we have evidence of an increased stress-reactivity. It would be interesting to test these mice in a chronic stress test and look at different endpoints. A combination of several challenges, for example chronic stress and diet, would be promising as these mutants show a metabolic phenotype and the protein is implicated in oxidative stress and inflammatory responses. As only females were tested it would be interesting to analyse males as well and to evaluate the CORT profile of these animals.

The Emory mouse line The Emory mouse line is a model of senile cataract formation, which occurs at six to eight months of age. The Emory mice were tested because of interesting results from the Primary Screen. In the OF mutants were less anxious compared to wild-type animals. In the Pathology Screen female mutants showed a reduced diameter of the adrenal cortex and a vacuolisation of the adrenal medulla. The atrophy of the adrenal cortex hints to a reduced capability to produce and release CORT and therefore a possible impairment in the stress response. But also the thymus of the mutant animals was affected. Here some animals showed atrophy of this organ. These findings suggest an altered stress system. At that time the Steroid Metabolism Screen did not measure CORT levels, so that there was no data available for basal CORT levels in these animals.

To test the hypothesis of an altered stress system we acutely stressed these animals in a two hour restraint stress. It revealed a stress-reaction only in the wild-types. The mutants did not react to the acute stress with an increase in *distance travelled* nor *number of rearings*. This fits to the reduced anxiety-like behaviour we found in the Primary Screen in the OF.

To check if there is a difference in CORT levels, we conducted a second restraint stress on these animals and took blood samples at several different time points. The Steroid Metabolism Screen of the GMC measured these samples using the LC-MS/MS technology. All animals showed an increase in CORT levels in response to stress. Mutants showed increased basal CORT levels when comparing the two unstressed controls. This fits nicely to the atrophied thymus the Pathology Screen found in the Primary Screen. The atrophied thymus affected both males and females, whereas not all of the mutants were equally affected. Elevated CORT levels have been shown to reduce thymus weight [18, 169]. When looking closer at the actual increase in CORT, compared to the respective controls, we can see that the mutants show a smaller increase in CORT levels in response to stress than the wild-types. It could be due to a ceiling effect, although in both males and females the

stress-induced CORT levels do not seem exceptionally high. This again strengthens the stress-resiliance hypothesis. But how do increased basal CORT levels fit with reduced stress-reactivity? We can only speculate at this point, but seemingly there are compensatory mechanisms occurring or have occurred during development. Compensatory mechanisms are often found when the mutation is already expressed in the zygote. One of the compensatory mechanisms could be a change in the ratio of MR to GRs. GR expression levels have been shown to be reduced after chronic stress or elevated CORT levels. We know from our pharmacological experiment that the behavioural effect is not mediated via the GR, so that changes at the level of the MR seem more probable in the Emory mutants. Further support comes from a mouse model overexpressing the MR and showing reduced anxiety-related behaviours. In this respect it would be interesting to evaluate MR expression in the Emory mouse line and look at MR-GR ratios in different brain areas.

Also the adrenal glands show genotype-specific alterations in the females. Here the Pathology Screen found a vacuolization of the adrenal medulla and an atrophy of the adrenal cortex, both reflecting an altered stress system. It has been shown that under chronic stress the adrenals enlarge, due to hypertrophy of the medulla and zona fasciculata [174]. In another study chronic glucocorticoid treatment lead to adrenal atrophy [87]. Although the adrenal cortex is thinner in the mutants, it does not necessarily reflect insufficient CORT production nor secretion, as shown by comparable stress-induced CORT levels. Possibly the total enlargement of the adrenals, due to the vacuolization of the medulla, also increases the volume of the cortex, thus making it possible to reduce the cortical diameter at no expense of CORT production.

All of these genotype-specific changes have to be carefully interpreted, as the wild-types are not litter-mates. Also, one should keep in mind that these mice are bred on a CFW background, which is an out-bred strain. This might also be the reason for not seeing effects in the *number of rearings*, as this strain may react differently, in behaviour terms, to the stressor, to the C57BL/6 strain, with which we established the acute stress test.

The Aphakia mouse line The *aphakia* mutants carry two mutations in the *Pitx3* gene causing a defective eye lens development and loss of DA neurons in the SN [157]. As some animals of the *aphakia* line died during cardiovascular measurements in the Primary Screen, the screeners asked for a stress-reactivity test. The animals were exposed to a 15-minute restraint period. The *aphakia* mutants appear to have an altered stress response, as they do not react to the stressor with increased *distance travelled* nor increased *number of rearings*. Our results were supported by the Primary Screen, where homozygous mutants showed less anxiety-related behaviour in the OF (reflected in increased *time spent in the centre*) of the Behaviour Screen and a reduced startle response in the modified Shirpa protocol, conducted by the Neurology Screen. Interestingly, the Steroid Metabolism Screen found elevated CORT levels in the female mutants. It would be worthwhile testing these

mutants with a bigger cohort of animals in the acute stress test to reproduce our results and analyse CORT levels at different time points. Maybe compensatory mechanisms for the higher basal CORT secretion occurred at the level of receptors, rendering them less responsive to mild stressors. Comparing the obtained results with the EMORY mouse line shows some eye-catching parallels in phenotypes. Both lines show increased basal CORT levels, a reduced stress-responsivity and reduced anxiety-related behaviour, hinting to similar underlying mechanisms (c.f. section above of the Emory mouse line).

The HMGN1 mouse line *HMGN1* (high mobility group nucleosomal binding domain 1) is known to bind to nucleosomes and can therefore affect chromatin structure and epigenetic modifications as well as transcription, replication and DNA repair. As the gene is located on chromosome 16 (which corresponds to chromosome 21 in humans) it might play a role in Down Syndrome [136]. Although the mouse provider saw changes in the OF, we could not recapitulate these in our screen. This could be due to differences between test set ups and conduction.

In the acute stress test we did not see any genotype-specific differences in *distance travelled,* but in the *number of rearings*. For the last mentioned parameter we analysed males and females separately, due to significant sex differences. Males showed a stress-induced increase in *number of rearings*. We could reproduce the genotype-specific decrease in *number of rearings* in the mutants seen in the Primary Screen. For the females we only saw a slight stress effect. When stressing these animals for two hours we did not see any genotype-stress interactions. In the Primary Screen of the GMC the females showed an inhibition of the acoustic startle reaction, which is not due to hearing defects (Click-box test of the Dysmorphology Screen revealed no hearing deficits). As increased acoustic startle is associated with increased anxiety, the results from this line do not point towards an increased anxiety phenotype. Further, the Steroid Metabolism Screen showed in the mutant females a significant reduction in CORT levels. These results hint to a subtle change in the stress-system, which might be revealed in an acute stress test of 15 minutes restraint and a bigger cohort.

The HST014 mouse line Animals from the HST014 line show impaired kidney function, including increased plasma urea, creatinine and potassium levels as well as increased water intake. The function of the candidate gene *Kctd1* (Potassium channel tetramerisation containing domain 1) protein is yet unclear, although it seems to be involved in transcription, proliferation and ion channel assembly, only to mention a few [98].

Behavioural changes within the Primary Screen in the Neurology Screen (increased transfer arousal and hypoactivity) and reduced CORT levels in the Steroid Metabolism Screen hinted to an altered stress response. After a two hour restraint stress duration, the males and females showed different stress reactions. On that account,

data from both sexes cannot be pooled although animal numbers are low. The males show no differences in *distance travelled* and in *number of rearings*. For the females, no effect can be observed in the *distance travelled,* whereas they do show a stress-genotype effect in *number of rearings* and a subtle stress effect, hinting to reduced stress-reactivity. To sum up, one can say that the HST014 mutants do not show an increased stress response. Together with the Steroid Metabolism Screen we measured CORT levels pre- and post-stress, but they did not indicate a strong genotype-specific alteration. For CORT analysis and the behavioural test, animal numbers were small. Due to that we suggest testing a new cohort to increase animal numbers, thus strengthening statistics. There might be subtle changes in stress-reactivity but these differences have to be confirmed with a larger cohort.

The Sms1 mouse line Through the loss of the sphingomyelin synthase 1 (Sms1) the mutant animals show a reduction of poly-unsaturated fatty acids, sphingomyelins and cholesterol. Sphingomyelins and cholesterol are both known to directly influence gamma- and beta-secretases and thereby A-beta production [71]. The mutation is implicated in AD (Wittmann et al, in prep). This mouse line showed several alterations in the GMC´s Primary Screen, which hinted to changes in the stress-response system. These were reduced anxiety in females in the OF, increased tail elevation in males within the modified Shirpa protocol and a trend in increased CORT levels in the males measured by the Steroid Metabolism Screen. We tested these mice in the acute stress test three times. Twice with a 15 minute restraint period, the first time for evaluation of the behaviour in the OF and the second time we took blood samples for a CORT profile. The third time the animals were stressed for two hours. Already in the 15 minutes restraint stress test, mutant animals did not show a response, whereas the wild-types did. This effect was still apparent in the two hours stress test. Here again mutants did not reveal any stress-induced increases in locomotion nor rearings, whereas the wild-types did. Taken together, the mutants of the Sms1 mouse line clearly show a reduced stress-reactivity and analysis of the blood samples will give further insight into possible underlying alterations of CORT levels.

Homozygous animals of the Db/Db mouse line The homozygous mutants have a point mutation in the gene encoding the leptin receptor. They are a model for obesity and diabetes. We tested the homozygous mutant animals with our acute restraint stress, for examining the effect of restraint in hypoactive and obese mice in animal holders. Although the mutant *db/db* mice are hypoactive compared to C57BL/6 mice (*Distance travelled* in the first five minutes of the OF: *db/db* controls: <1000 cm; C57BL/6 controls: ~6000 cm), they do respond to the stressor with an increase in locomotor activity. No conclusions in respect to altered stress-reactivity can be drawn, as only mutants and no wild-type litter-mates were available. Comparability between mutants and wild-types would be difficult as the mutants have significantly higher body weights, and therefore have to be restrained in animal

holders and not as the wild-types in 50 ml tubes. This dramatic difference in body weight could be a confounding factor in the interpretation of the behavioural results obtained for this mutant line.

Running and stress After voluntary wheel running for four weeks animals respond differently to stress compared to non-runners. Runners show an increase in *distance travelled* after stress, but in the *number of rearings* they do not show an increase. When looking at baseline levels in the controls at *distance travelled,* we see that the runners show lower activity.

4.1.3 Conclusion

The non-invasive acute restraint stress test is established and it is an excellent method to measure stress-reactivity in mice. It can be used as a first elucidating test on stress response and can be repeated with the same animals many times, enabling the evaluation of stress responsivity at different stress durations, at different points in life, or with different end-measurements, e.g. blood withdrawal for further analysis (e.g. hormone measurements). The measured locomotor behaviour reflects secretion of CORT and its feedback mechanisms, but not through the GRs. We hypothesise that the stress-induced activity depends on non-genomic, MR-mediated mechanisms. For precise delineation of how CORT influenced behavioural outcome, further (pharmacological) tests will have to be done.

We analysed several mouse lines with our acute stress test, which in part show strong changes in stress reactivity. It is interesting to note the different patterns appearing in some mouse lines. For example mutants from both the Emory and the *aphakia* mouse line show reduced stress-reactivity with an increase in basal CORT levels as well as reduced anxiety-related behaviours within the OF under basal testing conditions (see Figure 4.1). At the beginning, it seemed possible that the differences in the Emory mouse line relate to the fact that wild-types were not litter-mates. But with the same kind of pattern appearing in the subsequently tested *aphakia* mouse line, it would be of interest to test if the underlying mechanisms are the same in both of these lines. Therefore it would be important to get an increase in animal number, especially for the *aphakia* mouse line, also to reproduce the findings. In all of the models where baseline CORT levels were altered, a closer look at thymus and adrenals seems worthwhile, as alterations in CORT levels have been associated with changes in adrenal and thymic weights [18, 169, 174]. Interestingly, in these mouse lines where increased basal CORT levels were measured, body weight is reduced.

The two parameters reproducibly altered in our acute stress test are locomotion and *number of rearings*. Rearing and locomotor activity are thought to be independent of each other [3, 25, 77, 103, 137]. Experiments with rats selected for high rearing activity and low rearing activity showed that although these animals differ in their rearing activity they do not show significantly different levels of locomotion

Mutant Mouse Line	Stress reactivity		Results from other tests
	15 min stress exposure	2 hour stress exposure	
AcStr01	Subtle ↓	Subtle ↓	SS: Cort: No differences
AcStr02	Subtle ↑	*Not tested*	NS /BS: Hyperreactive in modified SHIRPA (but not OF) No BW gain after stress exposure
Emory	*Not tested*	↓	BS: OF: Anxiety ↓ SS/ BS: Cort profile: Baseline ↑ PS: Thymus atrophy
Aphakia	↓	*Not tested*	BS: OF: Anxiety ↓ SS: Cort: ↑ (females) NS: Startle ↓
HMGN1	*Not tested*	=	BS: ASR: Females ↓ SS: Cort: Females ↓
HST014	*Not tested*	?	Animal number too low, due to significant sex differences
Sms1	↓	↓	BS: OF: Anxiety ↓ (females) NS: Tail elevation ↑ SS: Cort: Trend in males (↑)

Figure 4.1: Summary: Mouse Lines in the Acute Stress Test
ASR- Acoustic Startle Reaction; BS- Behaviour Screen; BW- body weight; Cort- corticosterone; NS- Neurology Screen, OF- Open Field, SS- Steroid Metabolism Screen, PS- Pathology Screen

[137]. Also in our experiments, we could not reveal correlations between rearings and locomotion after a two hour restraint stress in the first five minutes of the OF. Experiments from Cornish et al [37] showed that locomotion and rearing might be regulated via different mechanisms, as they saw distinctive effects of drugs on both of these behaviours. CNQX, a glutamate receptor antagonist, injected into the VTA caused a dose-dependent biphasic effect on rearing without having an effect on locomotion. The second evidence comes from OFQ (orphanin FQ- nociceptin), which antagonized the AP5[2]-induced rearing without changing locomotion. OFQ inhibits DA cells [128], thus hinting to the involvement of DA transmission in rearing behaviour. In that respect it is interesting to note that a genotype-specific reduction in *number of rearing* in animals with disturbances in the DA system, i.e. the AcStr02 and *aphakia* mouse lines, occurred. In the AcStr02 mutants a 6 % reduction of TH-positive cells in the VTA is apparent. TH is the rate limiting enzyme in catecholamine synthesis, converting l-tyrosine to L-Dihydroxyphenylalanine (L-DOPA). In the *aphakia* mouse line a loss of DA neurons in the SN and the VTA occurred [157]. Also here reduced *number of rearings* were observed. Another mouse line with a mutation in the *Pitx3* gene, the *eyeless* mouse line, have also a severe distortion of TH-positive cells in the SN and show reduced rearings as measured by the Behaviour Screen in the modified Holeboard [145].

[2]AP5 is a selective NMDA receptor antagonist

The VTA, as a main site of DA cells, is involved in regulating movement, especially in novel environments [77]. It is interesting to note that the mesolimbic pathway, with the VTA as a central player, might act as an integrative centre between limbic structures and motor circuits. It has been shown that the VTA has projections to the hippocampus, amygdala and PFC, including the motor cortex and via the nucleus accumbens and ventral pallidum to the SN and the basal ganglia. The importance of the mesolimbic system in novelty-induced locomotion was shown by Hooks and Kalivas [77]. They compared naive versus habituated rats in the OF and injected, amongst others, DA antagonists into the nucleus accumbens, which inhibited the novelty-induced locomotion. To show that it is not a general DA effect, they also blocked DA receptors in the SN, where they could not detect any differences between groups.

During stress, several different systems, e.g. the CRH-, NA-, and DA-system, are activated and modulated. These systems do not act independently, but work in concert. It has been shown that the CRH- and NA-system are interconnected, influencing each other (see subsection 1.2.3). Also, interactions of the NA- and DA-system have been shown in several regions ([170, 173] reviewed in[58]). For example, Grenhoff et al. showed that NA-neurons in the LC regulate activity of the midbrain DA neurons [67]. Acute stress has been shown to increase DA turnover and release [171, 2, 52] and these effects can be modulated by CRH. Immobilization stress-induced increase in DA, epinephrine and TH mRNA in the LC have been shown to be effected by CRH [83]. Other evidence for an interaction between the CRH- and the DA-system comes from the acoustic startle response. The BNST, as a major player in this reflex, is also the integrative centre for information from the PFC, amygdala and hippocampus to the PVN, pointing to its role in the stress response. The BNST is rich in CRH-containing neurons, thus it is not surprising that i.c.v. injections of CRH can modulate acoustic startle. Meloni et al. showed that blockade of the D1-receptor, by systemic injection of SCH23390, inhibited the CRH-increased startle reaction [121] and that cells from the major DA areas (A8-10), including the SN and VTA, project to the BNST. Interestingly it has been shown that CRH-containing neurons receive direct input from DA neurons [138]. We are still at the beginning in trying to understand the complex cross-talk between these systems and future research will give insight to their interconnections and contributions to the acute stress response.

Our acute stress test uncovers differences in stress reactivity, i.e. stress-resilience and stress-sensitivity. It is non-invasive, cheap in costs, easy and fast to apply and, above all, the behavioural read-out is highly reproducible, when tested in OFs located in individual small chambers. The acute stress test is therefore suitable for high-throughput phenotyping, as done by the GMC. Depending on the endophenotype a mouse line exhibits in the acute stress test, it might be more or less prone to develop or increase the severity of the disease modelled, after being subjected to chronic stress. Therefore the acute stress test can be used as an indicator for the usage of a specific mouse line in the chronic stress challenge.

4.2 Chronic Stress Challenge

Restraint stress is frequently applied, not only for acute elicitation of a HPA response but also as a chronic stressor. In the past, different methods for applying restraint have been used and behavioural testing after chronic stress varied in respect to test and time point applied. Animals were restrained most frequently in plastic tubes but also so-called immo-bags or decapicones were used. Tying the animal´s limbs, taping the animal to a board or table, placing the animal in a wire mesh restrainer or wrapping it into a cloth are also methods used. Even restricting activity of an animal to a small corner of a cage or restraining the animal in a tube plus immersing it into water are applied. All of these procedures differ in severity and sometimes the distinction between restraint (restricting movement to a minimum; e.g. in restraint tubes; less severe) and immobilization (no movement possible at all, e.g. taped on a board, more severe) is being made. Also the duration of the procedure differs dramatically: from a few minutes to several hours. For chronic stress, repetition of the stressor is also quite variable. Frequency of the exposure are between once a day for five days up to 40 days. These are only some of the differences between chronic restraint protocols. Other factors, like differences in behavioural testing, impact on the results as well. Here the point in time of testing after chronic stress, as well as the test applied, influences the outcome and interpretation. Also the conditions under which the behavioural test is conducted (e.g. testing during light vs dark phase, testing apparatus, see subsection 4.1.1) has consequences on the read-out. All these differences make it difficult to interpret results and to evaluate the best protocol.

A paper by Kim and Han [93] compared different restraint protocols by behavioural and endocrinological read-outs. C57BL/6J males were restrained in 50 ml tubes for two hours or eight hours a day for 14 days or six hours for ten days. They could show that their chronic restraint lead to reduced body weight gain in all three cohorts. For the cohort stressed for two hours per day, they analysed CORT levels at different days and saw a reduced CORT response on day 14 compared to day 1 after stress, with levels being approximately 69 % of those on day 1. Two days after stress cessation, animals were tested in the EPM. Here, animals stressed for 14 days showed an increased anxiety-related behaviour as seen in reduced levels of entering and spending time in the OAs. In the OF, tested on day 18 (four days post-stress), these groups did not differ from their controls. On day 28 mice were exposed to the FST and again only the 14 day stressed animals showed an increase in floating, demonstrating an increase in depression-related behaviour. They also tested two hours per day for seven days and two hours per day for ten days but here no differences were found. In conclusion, it was shown that two hours restraint per day for 14 days resulted in an increased anxiety- and depression-like phenotype.

4.2.1 Establishment of the Chronic Stress Challenge

We applied the chronic restraint stress protocol suggested by Kim and Han [93] (two hours per day for 14 days) in two cohorts of mice but could not replicate all of the above mentioned findings. The factor reproducibly affected was the body weight, which is reduced in stressed animals. We could not replicate the behavioural phenotypes observed in EPM, OF nor FST after 14 days of restraint stress. We thought that timed restraint stress periods for two hours each morning are foreseeable for the mouse and thus do not lead to high stress. Therefore we changed the protocol, making it unpredictable for the animals, adding another psychological factor. We varied the stress protocol in different parameters (see also section 5.2):

- the severity of restraint (without or with middle tubes of different sizes for tighter restraint),

- the restraint duration (15 minutes up to six hours),

- light conditions (in total darkness to highly illuminated or stroboscopic light),

- time points of stress (different times of day),

- and once in a while a stress-free day.

Unpredictable stress for 14 days led to reduced body weight gain and an increase in anxiety-related behaviours in the OF eight days after stress cessation (day 22), but not in the EPM on day 15 in a first cohort. We could reproduce these findings in another cohort of animals. This demonstrated that the unpredictable chronic restraint stress leads reproducibly to an increase in anxiety-related behaviours eight days after the end of a 14-day long unpredictable restraint stress period.

We interchanged the EPM on day 15 and OF on day 22 to see if we get stronger anxiety-related effects, when we test animals in the OF earlier (see Table 5.1). But this experiment did not reveal any behavioural differences between stressed and unstressed animals. We interpreted the results as time dependent effects seen in the OF, and switched back to the "old" protocol with the OF on day 22. Again we tested animals with the OF at day 22, but this time no EPM on day 15. No anxiety-related behaviour was detected in the OF. This hinted to the necessity of the EPM at day 15, although no differences between groups were present in this test. But when we tried to reproduce the protocol a third time, we did not observe any anxiety-related behaviours. Why this third cohort did not reveal increased anxiety-related behaviour in the OF after chronic stress remains ambiguous. On the underlying factors responsible for the discrepancies, one can only speculate. One factor might be the temporal conductance of the tests; the first two cohorts were tested temporally close to each other, whereas the third trial was conducted over half a year later. It seems counter-intuitive that animals housed in the laboratory under controlled conditions experience seasonal changes and reflect those in behaviour. But several studies have found seasonal changes in lab animals in different parameters tested [15, 123, 129]. Another factor might be differing life history affecting whole

cohorts. This could be due to changes in the surrounding environment affecting early life phases, thus changing the "hard-wiring" of underlying pathways. This has been shown for early life stress, which alters stress-reactivity later in life [122, 135, 143, 144]. That stress during pre- and post-natal life has consequences in adulthood is mainly accepted, thus stress to the mother and pups is avoided when breeding animals. Less caution in interpreting results is taken when comparing cohorts of animals that came from different mouse breeding facilities. Olfe et al. [131] showed that animals from a vendor compared to in-house bred animals of the same strain differ in their stress-response in adulthood. Yet it is impossible to control for each of these factors or include them in an analysis. The most probable confounding factor could have been an acute stress. Strekalova et al. [165] proposed that higher light conditions induce acute stress during testing and therefore induce hyperlocomotion, which acts as a confounding factor for detecting anxiety-related behaviours. In our two cohorts, which demonstrated increased anxiety-related behaviour, locomotion is not changed or it is even reduced after chronic stress. In the third cohort, where we tried to replicate these findings we saw an increase in locomotor activity, which might have superseded the anxiety-related behaviour. In our case we did not change any illumination setting between OF testing but one factor might have had an impact: We had to heat the OF chambers during testing the third cohort, with a fan heater. Here, possible burning of dust on the heating spiral might have acted as a stressor, thus eliciting hyperlocomotion in these animals. That an acute stressor has a different impact on chronically stressed animals was shown by several publications [10, 43]. The response to an acute heterotypic stressor even enhances stress response in chronically stressed animals.

Throughout all of the chronic stress experiments we always saw a significant difference in body weight gain due to stress. This was not due to inaccessibility to food, as we evaluated the body weight changes in a control group, which was deprived of food during the stress periods of the restraint group, and still significant differences existed (data not shown).

4.2.2 Mutant Mouse Lines

As behavioural results of the OF from the unpredictable chronic stress applied in wild-type C57BL/6N were not consistent, we decided to use mouse mutants, in which stress responsivity was altered. To this end we conducted the chronic unpredictable restraint stress with EPM on day 15 and OF on day 22 with the Cor26Nes and the *CRH-R1*KO mouse line. Both mouse lines have been shown to react differentially to acute stress. Mutants of the Cor26Nes mouse line show increased stress-reactivity, whereas mutants from the *CRH-R1*KO mouse line have a reduced stress-reactivity (see section 3.1.1, [107] and [172]).

The Cor26Nes mouse line For the Cor26Nes mouse line, the two weeks of unpredictable stress seem to affect the wild-types and mutants at the same intensity as no genotype differences in body weight can be observed. This could be due to a ceiling effect, i.e. stress was similarly severe in both genotypes so that the maximal body weight reduction was reached in both. Although acute stress response is altered in the mutant mice (see section 3.1.1), compensatory mechanisms might have occurred, by which allostatic load through chronic stress is not increased compared to wild-types. That stressing these animals worked is not only reflected in reduced body weight but also in changes of organ weights: both stressed groups show higher relative adrenal weights and lower thymic weights on day 15. It has been shown previously that increased CORT levels and/or chronic stress reduce thymus weight, leading to its atrophy and having the opposing effect on the adrenals [18, 169, 174]. Two weeks after stress cessation, weights of both organs have already regenerated and do not differ from control animals any more. This is in accordance with other studies. Blomgren et al. showed that the thymus needs approximately 14 days to recover and normalize its cell composition and immunological reactivity [18]. Behaviour in the EPM revealed an activating effect of stress, which is driven by the wild-type animals. In the OF, the increased activity in the stressed group is driven by the mutants. This effect in the OF was reproduced in the second cohort of Cor26Nes mice.

One finding, which is often found in chronically stressed animals, is the retraction of apical dendrites in the hippocampus [20, 119, 186]. We could not observe this in our animals at any of the three time points. Our results even point towards another direction: Dendrites of the apical tree have longer dendrites after stress-exposure. This difference was seen in two independent experiments. In contrast to the basal dendrites, the apical ones show the same pattern throughout these experiments. The question remains, why there is no retraction of the dendrites. The group of Bruce McEwen showed that, after a three week stress period with six hour restraint a day, apical dendrites retract [112, 186, 117]. One could speculate that this quite dramatic and severe stress leads to the excessive release of glutamate [60] and, in synchrony with increased CORT levels, even enhance its neurotoxic effects. Increases in extracellular glutamate levels have been shown to be neurotoxic, probably due to excessive stimulation of the N-methyl-D-aspartate (NMDA) receptor leading to increases of calcium. Calcium is a potent stimulator of several different pathways including degeneration processes, increasing ROS and therefore oxidative stress. Even CORT itself can modulate NMDA receptor-mediated calcium influx [168]. All these effects can finally culminate in neuronal death. To prevent this, the dendrites retract, saving the neuron from dying. It has been shown that blocking the NMDA receptors prevents stress-induced dendritic retraction [112]. Also lesioning of the entorhinal cortex, with its strong glutamatergic input into the hippocampal CA3 region, protects from stress-induced neuronal damage [166]. When looking at the Sholl analysis and the different time points, one could speculate that the stress-sensitive region could be at the distal part of the apical dendrites, which

also receive glutamatergic input from the entorhinal cortex and indirectly from the PFC, which has also been shown to be affected in chronic stress. Although also the basal dendrites receive glutamatergic input, they seem less sensitive to stress. This might be due to inhibitory GABA-ergic input. It has been shown that there is a heterogeneous distribution of GABA-receptors on dendrites [132].
The chronic stress we applied is less severe and lasts only for two weeks. Probably here the neuron can cope with glutamate and CORT levels by increasing receptors and glutamate re-uptake transporters [140]. Due to this, the neuron is not forced to retract its dendrites from the neurotoxic insults. The effect of moderate stress on dendritic morphology can also be seen in running. Running is known to increase CORT secretion [5, 57]. Yau et al. showed that running causes apical dendrites to increase in their length [187].
The Morphology of the CA3 region of the dorsal hippocampus has changed through chronic stress. The lack of satisfactorily stained neurons was one of the major problems encountered. Due to this circumstance, results should be interpreted with care.

In conclusion, the 14 days of unpredictable chronic restraint stress leads to subtle genotype-specific changes in behaviour, body weight, organ weights and hippocampal morphology. One explanation might be that the stress procedure was too severe to produce different effects in the genotypes. This might depend on a more stress-susceptible background strain, as the Cor26Nes were generated on a mixed background and subsequently back-crossed to C57BL/6, thus potentially still including non-C57BL/6 sites (see sec. 4.1.1). Or the stress procedure is not severe enough so that compensatory mechanisms of the mutants come into play and can counterbalance the detrimental effect of moderate stress. This last hypothesis is strengthened by the results obtained from hippocampal morphology, also suggesting that our chronic stress is not as severe as in other studies, where dendritic retraction has been observed.

The CRH-R1KO mouse line Body weight data from the *CRH-R1*KO mutants does not show a stress-induced decline. We can speculate that the reduced stress-responsivity in the mutant *CRH-R1*KOs is reflected in the less severe reduction in body weight. Reduced body weight after stress has often been reported in studies using restraint as the stressor of choice. In the EPM, stress causes an increase in activity, which is genotype-independent. *Time in OA* is increased in the stressed animals, but this effect is mainly driven by the wild-types. In the OF, an increase in both horizontal and vertical activity can be observed. These effects are driven by the mutants. Organ weights for the *CRH-R1*KO line show, at unstressed levels, the expected reduced adrenal size in the mutants, which stays over time, and nicely depicts reduced CORT levels. In thymic weight we see a decrease in wild-types at day 15, with a possible overcompensation at day 29. Thymic weights of mutants do not differ between control and stressed group, not on day 15 and not on day 29.

This reflects the missing negative influence of CORT on the thymus.

When comparing the behaviour of the two different mutant lines, the Cor26Nes and the *CRH-R1*KO mouse line, we can see that stress induces an increased activity in both lines. After two weeks of stress, the increased activity in the EPM is stronger in the wild-types than in the mutants, independent of the mutant line. In the OF conducted one week later, the pattern changes in that way that the mutants drive the stress effect seen in activity. Again, one could argue that the illumination levels might have been too severe, thereby inducing anxiety and thus corrupting anxiety-related behaviours [165]. The exposure to chronic stress might have triggered changes in other systems (possibly the NA system) thus inducing active-coping in response to a novel stimulus, namely the EPM and OF. Why both lines show a stress-induced increase in locomotion remains to be investigated. Possibly adaptive responses are triggered to compensate and to ensure that the animal is still able to react appropriately to a novel stressor. This has been shown for repeated homotypic stressing, where the stress-induced response declines over time, but when exposed to a novel (heterotypic) stressor the stress-induced response is even greater. Also in humans with elevated CORT levels, adaptations occur. Here, receptors are down regulated, making them less CORT-responsive and diminishing negative impact of CORT on the cells. At the same time, other stress-responsive systems come into play, thus rendering the organism able to react to an acute stress [4].

Taken together the behavioural read outs do not seem appropriate in evaluating the stress effect. Unknown variables seem to influence behavioural outcome making it unreliable and therefore renders this kind of read-out as inappropriate. In the literature many different stress protocols have been proposed but none of them seemed applicable and reliable enough for the integration into the GMC II. Not only did results vary between labs but also within the same groups. The only reproducible parameter emerging in this field is the change in body and organ weights. The reduction of body weight due to chronic immobilisation stress is consistent in our experiments and in the literature [32, 80].

The CK-X mouse line We received another *CRH-R1* knock out mouse line, the CK-X mouse line, and subjected it to three weeks of unpredictable restraint stress. Analysis of the AUC for the body weight over time revealed only a trend in stress-induced body weight loss in the mutants, whereas we saw a significant loss in the wild-types. Again, changes in body weight seem to depend on different HPA axis function and therefore stress-responsivity. In the behavioural tests, this line showed a stress and genotype effect in the EPM on day 15. The mutants showed no increased activity nor *time spent in the OA* in the EPM in response to stress. Interestingly, in this mouse line we get basal differences between control wild-types and control mutants with respect to anxiety-related behaviours, with a reduction in the mutants depicted as an increase in *percent of time spent in the OA*. This is in accordance

with their lower stress reactivity and reduced anxiety-related behaviour measured by Dr. Deussing and his group. In the OF, the reduced anxiety-related behaviour is still apparent between the two control groups. The effects seen on relative adrenal weights were recapitulated in the *CRH-R1*KO line; mutants show lower adrenal weights compared to wild-types.

Seemingly also three weeks of unpredictable stress is not strong enough to induce clear stress-genotype interactions and increased anxiety-related behaviours in the wild-types. We do not know if this is due to the duration of stress or if restraint is not severe enough to consistently induce increased anxiety-related behaviours.

4.2.3 Two Year-Old C57BL/6N Mice

The two year old wild-type animals show a small but significant difference in the AUC of body weight. When comparing the body weight curves to younger animals one can see that also the old control group reduced their body weight, which is not found in control animals of a younger age. This could be due to the increased susceptibility to stress in old animals, which might already be elicited by the handling procedures each day within the controls. The stress susceptibility might come from age-dependent hypercortisolism [149]. The old animals show a stress-dependent activational effect in the behavioural tests. Another difference we saw compared to younger animals is in relative thymus weights. The thymus weight at day 29 is still lower in the stressed animals and seemingly has not regenerated as it is seen in younger mice. Maybe this is due to the age of the mice. It has been shown that the thymus atrophies with ageing [90]. These results demonstrate that old animals should be analysed with care, due to the underlying physiological changes, which make the controls susceptible to relatively mild stressors, such as handling.

4.2.4 Conclusion

Reduction of body weight has been repeatedly shown in chronic restraint stress paradigms [10, 32, 69]. This parameter is also reproducible within all of our chronic restraint stress experiments. The underlying mechanisms of how chronic stress influences body weight remains the objective of further research. It has been shown that CRH has an impact on body weight. Smagin and colleagues [156] showed that injection of a CRH-antagonist i.c.v. prevented the stress-induced body weight loss. Our experiment with the *CRH-R1*KO supports the influence of the CRH-system on body weight control. Leptin does not seem to play a major role in inhibiting food intake during stress. This was shown by Harris et al. [68] who demonstrated that repeated stress reduced body weight as well as leptin levels, although the latter was delayed in onset of decline. Body weight reductions are also seen in depressed patients and after stress exposure. But also the opposite occurs. In chronic stress paradigms employing social defeat as the stressor of choice, weight gain of the defeated animal

is observed. It can be speculated that social stressors and psychological stressors (such as restraint) have a different quality [13, 120] leading to divergent activation of different pathways and systems thus leading to different effects on body weight.

Using behavioural tests as a read out for chronic restraint stress challenges was shown to be an unreliable parameter, because behaviour of an animal in the test arena depends on many factors. One is the timing of the behavioural test and which test will be applied. As seen from our chronic stress experiments, this is not trivial. Depending on the pre-exposure to another behavioural test, results from the subsequently tested one can be altered. Also, the abandoning of a test changes the outcome in the following. Different testing conditions impact on the animals behaviour while testing. It has been shown that different light conditions alter locomotion, and this was proposed to corrupt anxiety-related behaviours [165]. Behavioural read out tests at the end of chronic stress seems quite susceptible to confounding factors, thus we suggest to employ them as additional tests and not as the defining measure of successful stressing.

Although 14 days of unpredictable restraint stress leads to increased anxiety-related behaviours in the OF, eight days after stress cessation in two cohorts, none of the subsequently tested cohorts could reproduce these results. Still, the underlying reasons remain unclear. The literature search showed that a reliable and reproducible chronic stress protocol is quite difficult to establish. We could show that a stress-induced reduction of body weight is the most robust measurement, beside an increase in relative adrenal weight and a reduction in relative thymic weights. This is in accordance with the literature [32, 80]. In conclusion, we can say that our chronic unpredictable stress protocol for 14 days does stress the animals. For a measure of stress, alterations in body weight, as well as thymic and adrenal weight, can be reliably used.

5 Methods

5.1 Animals

After arrival, the animals were left undisturbed for at least one week before testing started. Mice were housed in groups (if not mentioned otherwise) in IVCs (individually ventilated cages, Greenline, Tecniplast) under a 12h light/dark cycle (lights on at 7:00 o´clock) with ad libitum access to food and water. Room temperature was kept constant at 22 °C ±1 °C and a humidity of ~55 %. Experiments with wild-type C57BL/6 animals began at the age of about 9 weeks (for the age of specific cohorts see Table 5.2 and Table 5.3). Age of the different mutant lines varied and is specified in Table 5.3. If not mentioned otherwise, experiments were conducted with male mice (also see Table 5.2 and Table 5.3). All acute stress tests and all behavioural tests were conducted in the morning until one o´clock latest.

All experiments were approved by the Government of Upper Bavaria, Germany (Regierung von Oberbayern, Deutschland).

5.1.1 Wild-type animals

C57BL/6 wild-type mice were obtained from Charles River (Sulzfeld, Germany) or came from in-house breedings. Other wild-type lines (i.e. 129S2/SvPasCrl, BALB/cAnNCrl and C3H/HeNCrl (referred to as 129Sv, BALB/c and C3H respectively)) and female C57BL/6J were purchased from Charles River.

5.1.2 Mutant Mouse Lines

Mutant mouse lines were imported into the GMC from different facilities; for specifications see subsection 5.1.2.

The Cor26Nes mouse line CRH overexpressing mice were obtained from the Max-Planck-Institute (MPI) of Psychiatry (Munich, Germany), where they were generated and bred. Briefly, mice were generated by using homologous recombination under the *ROSA26* locus and flanked by *loxP* sites. Homozygous mice were then crossed with transgenic *Nes-cre* mice, so that CRH overexpression was restricted to the CNS, under the nestin promoter, only (referred to as Cor26Nes). For detailed

information see Lu et al. 2008 [107]. We received two cohorts of animals consisting of males only. The first cohort was subjected twice to a 15 minute acute stress duration (Inter-trial interval of two weeks), six weeks later they were stressed for two hours. The second cohort was first tested in the OF under basal conditions but with different illuminations and one week later they were tested in the 15 minute acute stress. After the acute stress experiments, both cohorts were run through the chronic unpredictable stress test.

The CRH-R1KO mouse line The *CRH-R1*KOs were also generated at the MPI of Psychiatry (Munich, Germany). For generating these mice the region encoding the transmembrane domains 4-7 in the *CRH receptor 1* gene was replaced by a *neomycin*-cassette. This led to a deletion of the coding sequence of the transmembrane regions V, VI and VII, which resulted in a truncated version of the protein, unable to transmit any ligand-induced signals. Chimeras were bred to CD1 or 129/Ola mice. For detailed description see Timpl et al. 1998 [172]. We got two *CRH-R1*KO cohorts from the MPI in Martinsried (Germany), which consisted of males only. The first cohort underwent, in the same order as the first cohort of the Cor26Nes, the acute stress (twice 15 minute stress, then once a two hour stress), the second cohort underwent a single two hour stress and then both cohorts were subjected to the chronic stress. Note that the second cohort was very low in animal number (Mutants: n=8; wild-type: n=11).

The CK-X mouse line The here referred to as CK-X mouse line was generated at the MPI of Psychiatry (Munich, Germany). $Crh1^{loxP}$ mice, which have exons 9-13 (which encode transmembrane domains 4-7 in the *CRH receptor 1*) flanked by *loxP* sites (for details see Müller et al. 2003 [126]), were bred to a *deleter-cre* mouse line. This resulted in the deletion of these exons ubiquitously. We got one cohort of male animals, which were subjected to the unpredictable chronic stress test for three weeks and subsequently were analysed for their behaviour (cf Figure 3.24).

The AcStr01 mouse line As this mouse line has not been published yet the name of this line has been blinded and a random name (AcStr01) assigned. A gene trap vector was inserted into this gene, which leads to a knock-out. The loss of both RNA and protein expression has been shown via Northern and Western blot analysis by the collaborative partner. Mice from this line have been bred on a C57BL/6J background for more than seven generations. Two cohorts (both males and females) were tested with the acute stress protocol. One cohort went through the normal Primary Screen of the GMC and at the age of 14 weeks these animals were tested in the acute stress for two hours. The second cohort came to the GMC exclusively for behavioural screening. These animals underwent several behavioural tests under basal conditions (EPM, FST, Object Recognition, Social Discrimination

and Y-Maze) after which they were then tested in the 15 minute acute stress test (at the age of 21-24 weeks).

The AcStr02 mouse line The knock-out mouse model for recessive early-onset Parkinson´s disease was generated by a gene-trap induced null mutation at the HMGU in the Institute of Developmental Genetics. Animals were back-crossed for several generations to C57BL/6 mice. Female mice were subjected to the acute stress test with a stress duration of 15 minutes. Results from the first acute stress suggested a differential effect of stress on the genotypes, thus a second 15 minute long acute stress duration was applied.

The Emory mouse line The Emory mutation was observed in a CFW breeding colony at the Emory University in Atlanta (GA, USA) [97]. Animals show cataracts occurring at around six to eight months of age. Linkage analysis suggest two genes affected in the Emory mouse line, but the exact region has not been identified until now. This is why the mice have to be bred separately so that the wild-types are not litter-mates. Mice were imported to the HMGU in 2006 from the Jackson Lab and bred at the HMGU by the group of Prof. Dr. J. Graw (head of the Eye Screen in the GMC). He reported that mutant mice are more aggressive. A cohort was tested with the two hour stress test (both males and females). Animals were stressed a second time for two hours and blood was collected at different time points (basal: t=0; post stress: t=2h and 3h post stress: t=5h) to measure CORT levels. The measurement was done by the Steroid Metabolism Screen (head: Prof. Dr. Jerzy Adamski) of the GMC via LC-MS/MS technology. For calculating the AUC the trapezoid rule was applied.

The Aphakia mouse line This mouse line was established after a spontaneous mutation leading to a defective eye lens. It was published in 1968 by Varnum and Stevens [181]. The *aphakia* mutants have two deletions in the regulatory region of the *Pitx3* gene. The homozygous mutants are blind due to defective embryonic development which leads to missing lenses. Also these mutants show a loss of DA neurons in the SN, which is a key finding in PD patients. These animals are bred on a C57BL/6J background. We tested three genotypes; wild-type, homozygous and heterozygous littermates (both males and females). These animals came from Prof. Dr. Graw, head of the Eye Screen from the GMC. Note that animal numbers of the different groups are quite low.

The HMGN1 mouse line The *HMGN1* (high mobility group nucleosomal binding domain 1) knock-out mouse was generated by replacing a part of intron 1, exons 2 and 3, and a part of exon 4 with a neomycin-resistance expression cassette (for detailed description see [17]). The animals have been back crossed for over ten generations with C57BL/6J. Animals have been generated by Dr. Bustin and colleagues

at the National Cancer Institute in the Lab of Metabolism in Bethesda (MD, USA). We tested these animals with the two hour acute stress test.

The HST014 mouse line Through the phenotype-driven ENU mutagenesis program this mouse line was established at the HMGU. Homozygous animals die after birth. The candidate gene *Kctd1* (Potassium channel tetramerisation containing domain 1) was found via linkage and sequence analysis, revealing a point mutation leading to an amino acid change, which is dominantly inherited [98]. The animals of this line were kept on a C3H background, causing blindness due to progressive retinal degeneration. Animals tested within the GMC Primary Screen were all blind, as shown by the Eye Screen with the Optical Drum test. In the Neurology Screen the mutant animals showed more transfer arousal and hypoactivity and the Steroid Metabolism Screen found reduced CORT levels. This lead to the assumption that these mutant mice might be altered in their stress-responsivity, which was then to be tested within the two hour acute restraint stress test. The animals were exposed to a second two hour stress test for analysing CORT levels. Blood was drawn at t=0h, before the restraint stress, and at t=2h after stress. Blood was taken from controls in parallel. Samples were analysed by the Steroid Metabolism Screen.

The Sms1 mouse line Mice were generated at the IDG by inserting a gene trap vector into intron 5 of the *Sms1* (aka *Tmem23*) gene. These animals have been back crossed to C57BL/6J for five generations (Wittmann et al, in prep). As we saw alterations in the Primary Screen in the Behaviour and Neurology Screen hinting on altered stress reactivity, we got a cohort for secondary screening, which included acute stress. Animals were acutely stressed for 15 minutes. A week later animals were again subjected to a 15 minute long stress duration, but this time blood was withdrawn from the tail vein at before stress and 15 minutes later. Another week later animals were subjected a third time to the acute stress. This time restraint lasted for two hours with a subsequent interval and an OF read-out.

Homozygous animals of the Db/Db mouse line Mice were purchased from the Jackson Laboratory[1] and offspring were achieved through heterozygous matings, which was done by the Diabetes Group (head: Dr. Susanne Neschen) of the Institute of Experimental Genetics (HMGU) within the GMC. These mutant animals have a deficiency in the leptin receptor through a point mutation. Nine male homozygous animals were taken to assess possible changes in these hypoactive animals to an acute stress of two hours. Animals were restrained in animal holders, because of high body weights. After five days animals were re-tested in the acute stress test.

[1] http://jaxmice.jax.org/strain/000697.html

5.2 Stress

Stress was applied through restraining animals in well-ventilated conical 50 ml tubes. When indicated mice received a 3 or 4 cm long middle tube, which was slipped over their tails to further restrict movement within the restraint tubes. Animals were always stressed in a separate room from where behavioural tests were conducted and control mice stayed.

For the acute stress, all animals were transferred to the testing room at least 30 minutes prior to the start of stressing or behavioural testing to acclimatize. Animals of the stress group were restrained for different durations, thereafter transferred into a clean animals housing cage for a 20 minute interval, and subsequently went into the behavioural test arena (see Figure 3.1). For testing in the OF or LDB both control and stressed animals were each put into one test arena in parallel, for we have two set ups available for these tests. For the EPM or the FST, animals from the different groups were alternately tested. Body weight was measured before stress or behavioural testing in stress- and control group respectively. For specifications of testing conditions see Table 5.2, Table 5.3 and Table 5.4.

For the chronic stress test, mice were weighed each day before the stress procedure. Mice of the stress group were stressed in well-ventilated 50 ml tubes under different conditions according to the protocol (see Figure 5.1).

5.2.1 Acute Stress

Establishment of the Acute Stress Test

Acute stress and different behavioural tests: Mice of the stress group of the first nine cohorts were stressed for different durations (15 minutes, 50 minutes and two hours) in the restraint tubes under boxes. After the 20 minute interval, animals were tested in the OF; six hours post stress animals were tested in the LDB; 24 hours after stress animals underwent the EPM and another six hours later were tested in the FST (see Table 5.2 for differences in post stress behavioural testings).

Acute stress and other post-interval behavioural tests (EPM and LDB): Four animal cohorts (cohort 10-14) were tested in the two hour acute stress protocol (see Table 5.2) with the EPM as the behavioural read-out test. The protocol varied in duration of the interval and EPM testing conditions (EPM conditions: cohorts 11 and 13 in total darkness and for cohorts 10 and 12 with the centre illuminated with 100 lux).

Another cohort of animals, cohort 12, was taken to analyse the LDB as the read-out test.

Acute stress and corticosterone: Naïve male C57BL/6J animals were single-housed (cohort 14). Blood samples from each animal at basal (t=0), post stress (t=2:00, 2:20 or 2:40) and recovery (t=5:00) levels were taken. Blood was taken twice from the tail vein and for the last sample trunk blood from the sacrificed animal was collected. Animals were divided into five groups: Control 1 (post stress blood sample was taken at t=2:20); Control 2 (post stress sample taken at t=2:40, after OF); Stress 1 (post stress sample taken at t=2:20); Stress 2 (post stress sample taken at t=2:40, after OF); Stress 3 (post stress sample taken at t=2h, before the interval and OF). Stressed animals were restrained for two hours in absolute darkness, had an 20 minute interval, and Control 2, Stress 2 and Stress 3 went through the OF (see Figure 3.3). Blood was collected in Microvettes (Sarstedt, Germany) left 24 hours to coagulate, centrifuged and the supernatant (serum) was collected and stored at -20 °C until further processing. CORT was measured by radioimmunoassay at the MPI of Psychiatry.

Acute stress and pharmacology:

CORT synthesis inhibition:

48 C57BL/6J males were purchased from Charles River Sulzfeld and were single-housed upon arrival. Animals were divided into four different groups, 12 animals per group. A vehicle group and metyrapone-injected group (metyrapone is a 11beta-hyrdoxylase inhibitor, which blocks the conversion of 11beta-deoxy-corticosterone to CORT), both groups were subdivided into control (unstressed) and stress groups. Animals received two injections at a volume of 0.007 ml/g body weight. The first injection 12 hours prior to stress (150 mg/kg body weight) and the second injection directly before stress (100 mg/kg body weight). Control animals were injected in parallel. Metyrapone was dissolved in propyleneglycol and saline (40:60 %; Sigma-Aldrich, St. Luis, USA).

GR-Antagonist:

Upon arrival from Charles River (Sulzfeld, Germany) C57BL/6J males were single-housed and divided into four groups: Vehicle-injected controls, vehicle-injected stressed, RU486-injected controls and RU486-injected stressed animals. All animals received an i.p. injection one hour pre-stress or in case of the controls 3:20h pre-OF. Stressed groups underwent a two hours stress (with 3 cm middle tube under a box) after which they were put into a clean cage for 20 minutes and thereafter were placed in the OF for 20 minutes. RU486 (Tocris Biosciences, Missouri, USA) was injected at 25 mg/kg body weight, at a volume of 0.003 ml/g body weight. As RU486 was dissolved in DMSO vehicle-injected animals received DMSO alone.

Open Field after acute stress without the interval Stressed animals of cohort 15 were subjected to a two hour acute stress period, but did not undergo the 20 minute interval. Instead, they were placed directly after stress into the OF. Behaviour

of both control and stressed animals was recorded, and grooming was scored with a hand-held computer (Psion Teklogix WorkaboutPro and The Observer, Noldus Information Technology, The Netherlands) from the recorded video files.

Time-lag between start of stress and Open Field by modulating stress- and interval-duration C57BL/6J male mice were divided into three groups: a control, a 2h Stress and a 15minute Stress group. The 2h Stress group received a two hour stress period, followed by a 20 minute interval period and subsequent OF testing. The 15minute Stress group was exposed to a 15 minute restraint stress followed by a 2:05h long interval duration after which they were tested in the OF. Control animals were placed directly from their home cage into the OF arena 8 (see Figure 3.6).

Acute stress re-testing Repeated testing was undertaken with animals of two different cohorts. One cohort (cohort 23) was re-tested every second day for three times at the age of nine weeks for a two hour acute restraint period. The other cohort (cohort 12; see Table 5.4) was re-tested throughout their lives, partly under different conditions, for example at the age of 24/25 weeks animals were tested in the afternoon (from 13:00 til 17:00), at the age of 29 weeks no stress was applied, but both groups were subjected to the OF (to check the possible conditioned response stressed animals might show when put into the OF) and from the age of 37 weeks onwards animals were restrained in animal holders (see section 5.2.1) due to high body weights.

Acute stress with different settings For comparison between two automated systems, the Actimot and the EthoVision system, each animal of cohort 12 was placed into the OF (Actimot system, see below) with a monochrome camera over it tracing the mouse via the EthoVision system (Version 3.1.16, Noldus Information Technology, The Netherlands; 12.5 Hz).

For testing in different set up surrounding conditions, we placed the OF arenas in a small room, separating the two set ups only by opaque plastic walls (cohort 22).

Acute stress in animal holders For these experiments, animals were restrained in bigger tubes (acrylic animal holders (ENV-263B) from Med Associates Inc, Vermont, USA). Animals of cohort 19 had higher body weights (First acute Stress: mean: 35,28 g). Mice of the stressed group were subjected to two acute stress sessions for two hours each with 14 days between testing (see also Table 5.2).

Acute stress and C57BL/6J females and two-year old males and other strains The response to a two hour stress was further evaluated in different groups/strains: female C57BL/6J (cohort 16), old male C57BL/6J (101 weeks old; cohort 18), 129Sv males, C3H males and BALB/c males.

Acute Stress and mouse lines
For applied protocols see Table 5.3 and subsection 5.1.2.

Acute Stress and voluntary wheel running Single housed C57BL/6 mice, 20 of them with access to a running wheel in their home cage and 20 controls, which did not have a running wheel, were tested after four weeks in the two hour acute stress test.

5.2.2 Chronic Stress

Two cohorts of the C57BL/6J strain underwent the "Korean Protocol" [93] (Korea 1 and Korea 2), where animals were restraint for two hours a day starting at 9 o´clock (at an illumination level of 160 lux for Korea 1 and under a box for Korea 2) over a period of 14 days (see Table 5.1). On day 16, animals were subjected to the EPM, on day 18 to the OF and on day 28 and 29 to the FST. Every day of stressing and on behavioural testing days the body weight from each animal was taken.

For all other cohorts (C57BL/6N) of chronic stress (Unpred. 1-8) a different protocol was applied (see Figure 5.1). For making stress unpredictable for the animals this protocol was chosen. Behavioural tests differed in their order and testing date (see Table 5.1). For details in chronic stress protocols please see Table 5.1.

Golgi Staining Within the chronic stress experiment for the Cor26Nes and *CRH-R1*KO mice were sacrificed at the indicated time points (day 15, 29 and 43) with CO_2. Also the two-year old cohort of animals was sacrificed at day 15 and 29. Four brains per group were rapidly dissected and immersed in the prepared solution from the Rapid Golgi-Cox Stain Kit (FD NeuroTechnologies, MD, USA). Brains were processed according to the manufacturers guide. In brief, brains were immersed in the given mixture of solutions containing mercuric chloride, potassium dichromate and potassium chromate. After two weeks brains were transferred into a clearing solution for approximately three days until they were cut on a cryostat into 140 µm thick sections. After letting them dry, they were hydrated, stained, dehydrated and cover-slipped. Analysis of pyramidal cells of the dorsal as well as ventral CA3 region of the hippocampus was performed with the Neurolucida program (Version 6.0, MicroBrightField, Inc, Williston, USA). Only solid-coloured neurons within the middle part of the section and without breaks and ruptures were analysed.

Adrenal and Thymus weights At the same time points at which brains were collected for Golgi staining, adrenals and thymus were dissected and after preparing them free from fat and connective tissue, they were weight on a precision balance and frozen.

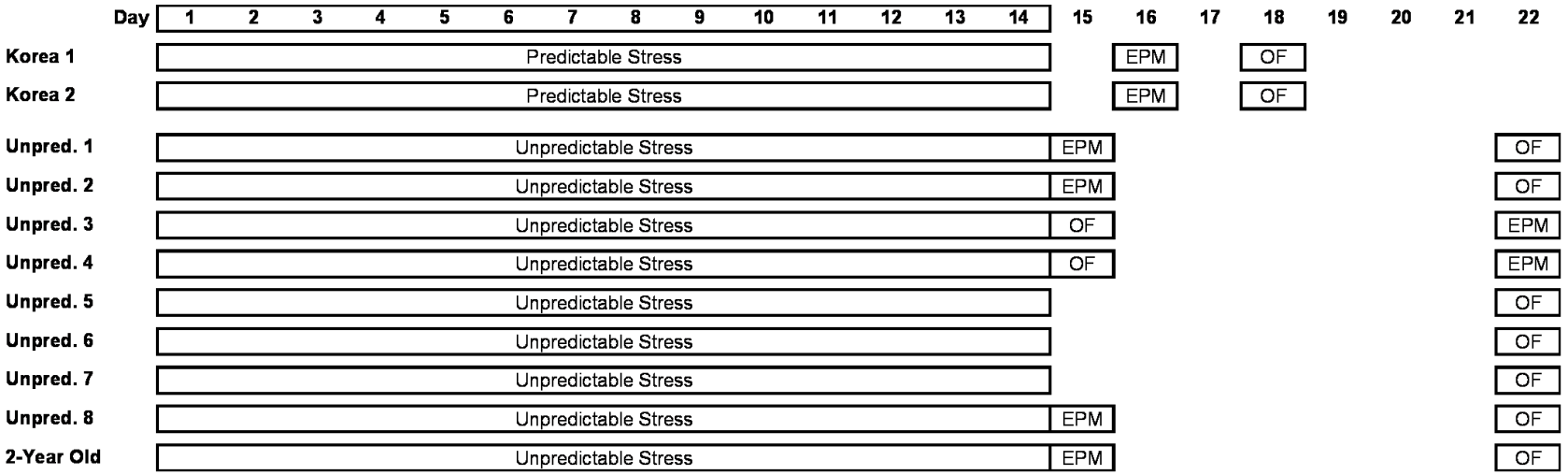

Table 5.1: Experimental design: Chronic Stress in C57BL/6 mice

EPM- Elevated Plus Maze, OF- Open Field

	Middle tube	Time	Duration	Light
Day 1	no	09:00	2h	Box
Day 2	4 cm	10:30	1h	Box
	no	13:30	3h	2800 lux
Day 3	3 cm	09:00	40min	Box
	4 cm	16:30	30min	Stroboscope
Day 4	no	07:10	30min	200 lux
Day 5	3 cm	18:00	2h	Box
Day 6				
Day 7	4 cm	07:00	4h	3000 lux
Day 8	4 cm	18:00	40min	3000 lux
Day 9	no	10:00	6h	Box
Day 10				
Day 11	no	09:00	15min	Stroboscope
Day 12	3 cm	18:00	30min	Box
Day 13	4 cm	13:30	2h	25 lux
	no	16:30	1h	Stroboscope
Day 14	4 cm	07:15	1h	Box

Figure 5.1: Chronic unpredictable restraint stress protocol
Length of the middle tube inserted, time at which restraint stress occurred, duration of restraint and light conditions under which restraint occurred

Animals of the CK-X mouse line were perfused and brains were collected for further processing. Adrenals were weighed and frozen.

5.3 Behavioural Tests

Open Field The Open Field (Actimot, TSE, Bad Homburg, Germany) is a square (45.5 x 45.5 x 39.5 cm, inner measurements) arena, illuminated with 200 lux in the centre (42 % of total arena), where the animal is traced by a system depending on infra-red light beam breaks (52 Hz). The mouse´ centre of gravity is calculated depending on the number of interrupted beams. Within a trial 34 different parameters are collected.

Each OF arena is placed in a small chamber (100 cm x 100 cm x 362 cm). Animals are put in the OF with their heads facing the middle of the back wall of the arena and the chambers door is closed during the experiment. Experiment starts with the first interruption of an infra-red light beam. After a test duration of 10 or 20 minutes the animal was collected from the arena and the OF was cleaned with disinfectant (Pursept-A, Merz Hygiene, Germany) and left to dry before starting the next animal. OF testing was conducted according to the SOP generated and standardized by the EUMORPHIA consortium (see http://www.empress.har.mrc.ac.uk)

Elevated Plus Maze The apparatus consists of four black plexiglas arms 90° apart, with two opposing arms being shielded by a 15 cm high wall (= enclosed arm) and two open arms with a small lip (0,5 cm). The length of an arm is 30 cm and the width measures 6 cm. The EPM was elevated 71 cm above the ground. The EthoVision system (Version 3.1.16, Noldus Information Technology, The Netherlands) tracks the animals via infra-red light being sent out from the bottom of the

arena. With this mice can be tested at different illumination levels and in absolute darkness. Animals were put into the closed arm facing the wall and tracked for 5 minutes. In total 28 different parameters were collected. After the end of the test the apparatus was cleaned with disinfectant (Pursept-A, Merz Hygiene, Germany) and left to dry before the next animal was started.

Forced Swim Test Mice were placed into a glass cylinder (a 10 litre beaker) filled with 25 ± 1 °C warm water to 20 cm of height, so that mice could not reach the bottom with their tails. Behaviour (Struggling: the mouse was vertical in the water, scratching vigorously the walls of the beaker; Floating: no movements of the mouse, except from minor movements to keep balance; Swimming: all other behaviour, i.e. active movement) was recorded with a hand-held computer (Psion Teklogix WorkaboutPro and The Observer, Noldus Information Technology, The Netherlands) for 6 minutes. Thereafter mice were dried with paper towels and put into a fresh cage which is placed on a heating plate or in front of an infra-red lamp. Water was replaced after each animal.

Light-Dark-Box The Light-Dark-Box test consists of two compartments placed in the Actimot frame (see OF, Actimot, TSE, Bad Homburg, Germany): the dark box (14 x 19 x 24 cm) with black plexiglas walls covered with a lid, and a light box (29 x 19 x 24 cm) with clear walls. These two compartments are connected through a small tunnel (4 x 6 x 9 cm). The light compartment is illuminated with 650 lux. The mouse was placed in the dark box facing the wall opposite the tunnel and behaviour was recorded for 5 minutes. The apparatus was cleaned with PuseptA after each mouse and left to dry before starting the next animal.

5.4 Statistics

For statistical analysis the program SigmaPlot (Version 11.0; Systat Software, Inc, Chicago, USA) was used. In cohorts with two groups a Students-t-test was applied, in cohorts with more than two groups a One Way ANOVA/ Two Way ANOVA was performed. In case the normality or equal variance test failed, a Mann-Whitney Rank Sum Test or an One Way Analysis of Variance on Ranks was applied respectively. A p-value $\leq$0,05 was considered statistically significant; a value p<0,1 was considered a trend.

For statistical analysis with more than two factors or for repeated measures, we used the SPSS program (SPSS Inc, Chicago, USA). This applied for all mutant lines, which included both males and females, for measures of body weight in the mutant mouse lines in the chronic stress test and for analysis of the morphology of hippocampal neurons.

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Appendix

Cohort name	Strain	sex	Age at 1st test [weeks]	1st acute stress duration	Interval	1st behavioural test	Naive at 1st acute stress?	Other Tests and when?
Cohort 1	C57Bl6J	m	7-9	15 minutes	20 minutes	OF	yes	LDB-EPM-FST
Cohort 2	C57Bl6J	m	9	50 minutes	20 minutes	OF	yes	LDB-EPM-FST mHB: 23d post stress
Cohort 3	C57Bl6J	m	9	50 minutes	20 minutes	OF	yes	LDB-EPM-FST
Cohort 4	C57Bl6J	m	9-10	50 minutes	20 minutes	OF	yes	LDB-EPM-FST mHB: 12d post stress old OF: 25d post stress
Cohort 5	C57Bl6J	m	9-10	2 hours	20 minutes	OF	yes	LDB-EPM-FST mHB: 7d post stress old OF: 15d post stress
Cohort 6	C57Bl6J	m	7-9	2 hours	20 minutes	OF	yes	LDB-EPM-FST
Cohort 7	C57Bl6J	m	7-8	2 hours	20 minutes	OF	yes	LDB-EPM
Cohort 8	C57Bl6N	m	8-9	2 hours	20 minutes	OF	yes	LDB-EPM-FST
Cohort 9	C57Bl6N	m	10-11	15 minutes	20 minutes	OF	yes	
Cohort 10	C57Bl6J	m	7-8	2 hours	20 minutes	EPM	yes	
Cohort 11	C57Bl6J	m	8-9	2 hours	20 minutes	EPM	yes	
Cohort 12	C57Bl6J	m	10-11	2 hours	without	EPM	yes	OF: 5h post stress repeated acute stress (see extra table)
Cohort 13	C57Bl6N	m	8-9	2 hours	10 minutes	EPM	yes	OF: 4,5h post stress
Cohort 14	C57Bl6J	m	9	2 hours	20 minutes	OF	yes	
Cohort 15	C57Bl6N	m	17	2 hours	without	OF	yes	2. Ac. Stress_EPM_10d later
Cohort 16	C57Bl6J	f	9	2 hours	20 minutes	OF	yes	2. Ac.Stress_aged: 20weeks
Cohort 17	C57Bl6N	m	27	2 hours	20 minutes	OF	yes	
Cohort 18	C57Bl6J	m	101	2 hours	20 minutes	OF	yes	Chonic Stress
Cohort 19	C57Bl6N	m	27	2 hours	20 minutes	OF	yes	2. Ac.Stress_AH_14d later
Cohort 20	C57Bl6N	m	10	2 hours	20 minutes	OF	yes	2. Ac.Stress_AH_15d later_with insert 3. Ac.Stress_AH_ 16d later_without insert
Cohort 21	C57Bl6N	m	9	2 hours	20 minutes	OF	yes	2. Ac.Stress_5d later
Cohort 22	C57Bl6N	m	12	15minutes/2 hours	20 minutes	OF	yes	
Cohort 23	C57Bl6J	m	9	15minutes/2 hours	20 minutes	OF	yes	2. Ac.Stress_2d later 3. Ac.Stress_4d later
Cohort 24	C57Bl6J	m	19	2 hours	20 minutes	OF	yes	
Cohort 25	C57Bl6J	m	13	2 hours	20 minutes	OF	yes	2. Ac.Stress_CORT_5d later

Table 5.2: Experimental details: Acute Stress in C57Bl6 cohorts

OF- Open Field; LDB-Light Dark Box; EPM- Elevated Plus Maze; FST- Forced Swim Test; mHB-modified Hole Board; AH- animal holder; CORT- corticosterone

Cohort name	Strain/ mutant line_ background strain	sex	Age at 1st test [weeks]	1st acute stress duration	Interval	1st behavioural test	Naive at 1st acute stress?	Other Tests and when?
Cohort 26	BALBc	m	9	15minutes/2 hours	20 minutes	OF	Yes	
Cohort 27	C57Bl6J, C57Bl6N, 129SvPas, BALBc	m	11	2 hours	20 minutes	OF	No	1. Ac.Stress_6d later
Cohort 28	129SvPas	m	11	2 hours	20 minutes	OF	Yes	
Cohort 29	C3H	m	12	2 hours	20 minutes	OF	Yes	
Cohort 30	AcStr01_ C57Bl6	m+f	21/24	15 minutes	20 minutes	OF	No	
Cohort 31	AcStr01_ C57Bl6	m+f	14	2 hours	20 minutes	OF	No	
Cohort 32	db/db_ C57BlKS/J	m	17	2 hours	20 minutes	OF	Yes	2. Ac.Stress_AH_4d later 3. Ac.Stress_15min_AH_13d later
Cohort 33	Emory_ CFW	m+f	8	2 hours	20 minutes	OF	No	2. Ac.Stress_~9d later 3. Ac.Stress_CORT_13d later
Cohort 34	Aphakia_ C57Bl6J	m+f	16	15 minutes	20 minutes	OF	No	
Cohort 35	Cor26Nes_ mixed (129Sv /C57Bl6)	m	24-33	15 minutes	20 minutes	OF	Yes	2. Ac. Stress_15min_14d later 3. Ac. Stress_2h_8 weeks later Chronic Stress
Cohort 36	CRHR1KO_ mixed	m	19-23	15 minutes	20 minutes	OF	Yes	2. Ac. Stress_15min_7d later 3. Ac. Stress_2h_14d later Chronic Stress
Cohort 37	HMGN1_ C57Bl6J	m+f	17	2 hours	20 minutes	OF	No	
Cohort 38	Cor26Nes_ mixed (129Sv /C57Bl6)	m	14-16	15 minutes	20 minutes	OF	No	Chronic Stress
Cohort 39	CRHR1KO_ mixed	m	12-21	2 hours	20 minutes	OF	Yes	Chronic Stress
Cohort 40	AcStr02_C57Bl6J	f	27-28	15 minutes	20 minutes	OF	Yes	2. Ac. Stress_2 weeks later
Cohort 41	HST014_C3H	m+f	42	2 hours	20 minutes	OF	Yes	2. Ac. Stress_CORT_2 weeks later
Cohort 42	Sms1_C57Bl6J	m+f	31	15 minutes	20 minutes	OF	No	2. Ac. Stress_CORT 3. Ac. Stress_2h

Table 5.3: Experimental design: Acute Stress: Different mouse lines

OF- Open Field; AH- animal holder; CORT- corticosterone

Cohort name	Strain	sex	Age at testing [weeks]	Acute stress duration	Interval [min]	Behavioural Test	Comments
Cohort 12	C57Bl6J	m	10-11	2 hours	without	EPM	OF: 5h post stress
			13-14	2 hours	20	OF	
			17-18	2 hours	20	OF	
			21-22	2 hours	20	OF	
			24-25	2 hours	20	OF	in the afternoon
			29			OF	
			34-35	2 hours	20	OF	
			37-38	2 hours	20	OF	AH
			46-47			OF	
			61	15 minutes	20	OF	
			95	2 hours	20	OF	AH
			101-102	2 hours	20	OF	AH_Actimot vs Ethovision
			127	2 hours	20	LDB	AH

Table 5.4: Experimental design for cohort 12

OF- Open Field; LDB-Light Dark Box; EPM- Elevated Plus Maze; AH- animal holder

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