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Translational assessment of cognitive impairments in depression models

PhD dissertation

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Declaration

I hereby confirm that all work carried out during this PhD was performed by me, unless otherwise stated. This work has not been submitted for any other degree or professional qualification. Declaration of co-authorship forms indicate my contribution to the respective publication and manuscripts, which are included in this thesis.

LENA-SOPHIE MARTIS

List of manuscripts

I. **The effect of rat strain and stress exposure on performance in touchscreen tasks**

Lena-Sophie Martis, Simone Krog, Thao Phuong Tran, Elena Bouzinova, Sofie Laage Christiansen, Arne Møller, Megan C. Holmes, Ove Wiborg

Published

II. **Resilient and depressive-like rats show distinct cognitive impairments in the touchscreen paired-associates learning (PAL) task**

Lena-Sophie Martis, Claudia Brision, Megan C. Holmes, Ove Wiborg

Submitted

III. **Vortioxetine recovers anhedonic-like behaviour and promotes strategic cognitive performance in a rodent touchscreen task**

Lena-Sophie Martis, Kristoffer Højgaard, Megan C. Holmes, Betina Elfving, Ove Wiborg

In preparation

IV. **BDNF$^{+/-}$ rats show depressive phenotype and altered expression of genes relevant in mood disorders**

Lena-Sophie Martis, Ove Wiborg, Megan C. Holmes, Anjanette P. Harris

Submitted

Abstract

Major depressive disorder (MDD) affects 300 million people worldwide and is a major contributor to the global burden of disease. The aetiology of depression, emerging through a gene x environment interaction, is still incompletely understood which prevents tailoring of treatment approaches. In addition to MDD core symptoms, such as anhedonia (a diminished anticipation or experience of pleasure), depressed patients suffer from a plethora of manifestations including cognitive impairments, which occur primarily in the domains of executive function, attention and memory. Patients remitted from affective symptoms of MDD often continue to display cognitive impairments. These cognitive deficits are the longest present residual symptom, predict treatment response and increase risk of relapse. Consequently, cognitive impairments need to be targeted more effectively by antidepressants for complete remission from MDD. Clinically relevant animal models are essential for developing, tailoring and testing such novel, pro-cognitive antidepressants.

This PhD project aimed to establish a preclinical screening platform for the testing of pro-cognitive antidepressants, to improve understanding of MDD risk factors and consequent symptom development, and finally, to focus on clinical relevance of the applied techniques.

The chronic mild stress (CMS) rodent model of depression was used, known for displaying the core symptom anhedonia, but also for a high construct, face and predictive validity. The environmental MDD risk factor 'stress' induces an anhedonic-like phenotype in a subgroup of exposed rats, whereas another subgroup of rats is resilient, as determined by the sucrose consumption test. The cognitive performance of different rat strains, including CMS anhedonic-like and resilient rats, was assessed employing the touchscreen operant platform, which was developed based on the Cambridge neuropsychological test automated battery (CANTAB) for assessing cognition in humans. Furthermore, a group of anhedonic-like rats was treated with the antidepressant vortioxetine, which acts as both a pro-cognitive and antidepressant treatment. Our results showed that stress exposure induced anhedonia in albino and pigmented rat strains, although stress did not affect cognitive performance of pigmented rats in a simple pairwise discrimination touchscreen task. Applying a more complex paired-associates learning touchscreen task revealed impaired cognitive performance in the CMS anhedonic-like but not in the resilient phenotype. Furthermore, vortioxetine treatment reversed anhedonia in the CMS model and altered executive functions in treated rats. The expression of genes involved in the stress response, affective disorders and neuronal plasticity was altered in the prefrontal cortex and hippocampus owned to treatment and hedonic state. Thus, we have

demonstrated that the CMS model exhibits both stress-induced cognitive alterations and depression-associated cognitive impairments in touchscreen tasks. Furthermore, touchscreen testing was sufficiently sensitive to detect alterations in cognitive performance due to pharmacological intervention. Overall, we established a potential platform for pro-cognitive antidepressant drug screening.

Furthermore, brain derived neurotrophic factor (BDNF), involved in learning and memory, was examined in the context of depression. BDNF is reduced in MDD patients as well as in preclinical models in response to stress. Although this suggests that BDNF contributes to the aetiology of depression, studies including mice heterozygous for BDNF ($BDNF^{+/-}$) have generated conflicting results. $BDNF^{+/-}$ rats may provide a more suitable model as (1) rats have a greater behavioural repertoire than mice, (2) classical behaviour tests are designed for rats, and (3) rats, like humans, produce peripheral BDNF. We found anhedonia and mild signs of anxiety in $BDNF^{+/-}$ rats, accompanied by prefrontal and hippocampal changes in expression of genes relevant in psychiatric disorders and underpinning learning. Thus, behavioural and molecular findings in $BDNF^{+/-}$ rats complement existing literature and suggest that rats are a more suitable model in BDNF research than mice.

Overall, the project uncovered environmental and genetic manifestations of risk factors in translational models and established a novel tool for translational pro-cognitive antidepressant drug screening.

Dansk resume

Depression udgør en af de største byrder i forhold til det samlede sygdomsbillede på verdensplan, hvor 300 millioner mennesker lider af denne sygdom. Selvom ætiologien bag depression er foreslået, at skyldes gen-miljø interaktion, så er det fulde sygdomsbillede stadig ukendt, hvilket forhindrer nye behandlingsmuligheder. Udover kernesymptomerne på depression, så som anhedoni (manglende evne til at føle glæde og lyst), lider depressive patienter af adskillige andre symptomer heriblandt svækket kognitive funktioner, hvor det primært er indlæring, opmærksomhed og hukommelsen, som er svækket. Patienter udviser ofte fortsat svækket kognitive funktioner selvom de er i bedring. De svækkede kognitive funktioner er de mest udbredte tilbageværende symptomer for depression efter bedring og bliver brugt til at forudsige behandlingsrespons samt forudsige en forøget risiko for recidiv. Derfor skal fremtidigt antidepressiv behandling være mere effektiv målrettet mod de kognitive funktioner for at forhindre risici for recidiv. Klinisk relevante dyremodeller er essentielle for udvikling af sådanne nye forbedrende antidepressive behandlingsmuligheder.

Formålet med dette ph.d. projekt er at undersøge en præklinisk screeningsplatform til evaluering af kognitive antidepressive midler, at øge forståelsen for risikofaktorer for depression og udvikling af de efterfølgende symptomer, samt at vurdere den klinisk relevans af de anvendte teknikker.

Kronisk mild stress (forkortet CMS for Chronic Mild Stress), som er en rottemodel for depression, bliver brugt i dette projekt, da den er velkendt for at inducere kernesymptomet for depression, anhedoni, men også for at have højt validitet. Stress, som er en miljømæssige risikofaktor for depression, inducerer et anhedonisk-lignende fænotype en i undergruppe af de stress-eksponerede rotter, mens en anden undergruppe forbliver upåvirket, og kaldes resiliente. Disse grupper bestemmes ud fra sukrose-indtaget fra en sukrose indtagelsestest (sucrose consumption test). De kognitive funktioner af forskellige rotteracer, herunder de anhedonisk-lignende rotter og de resiliente rotter, blev vurderet ved brug af en touch screen platform, som er baseret på Cambridge neuropsykologisk test (forkortet CANTAB for Cambridge Neuropsychological Test Automated Battery), der bruges til at teste kognitive funktioner i mennesker. Ligeledes blev en gruppe af de anhedonisk-lignende rotter behandlet med det unikke antidepressive middel, vortioxetin, som både virker på de kognitive funktioner og har en antidepressiv virkning. Vores resultater viser, at stress-eksponering inducerer anhedoni i albino og pigmenterede rottestammer, mens stress ikke påvirker de kognitive funktioner i den pigmenterede rottestamme ved en simpel touch screen diversitetstest. Ved at bruge en mere

kompleks touch screen indlæringsassociation test har vi vist, at de anhedonisk-lignende rotter har nedsat kognitive funktioner i forhold til den resiliente fænotype. Ligeledes har vi vist, at behandling med vortioxetin modvirker den anhedonisk fænotype i CMS modellen samt ændrer udførelsen af opgaver i touch screen testen. Ekspressionen af gener, som er involveret i stressrespons, affektive lidelser og neuronal plasticitet, ses ligeledes ændret i præfrontal cortex og hippocampus i forbindelse med vortioxetine behandling. Således har vi vist ved brug af touch screen platformen, at CMS modellen forårsager stress-relateret kognitive ændringer og forringet kognitiv funktion som også ses ved depression. Derfor er touch screen platformen tilstrækkelig sensitiv til at måle ændringer i de kognitive funktioner, som både skyldes stress og farmakologisk behandling. Overordnet har vi etableret en potentielt platform, som kan bruges til screening af antidepressive behandlinger med fokus på de kognitive funktioner.

Brain Derived Neurotropic Factor (BDNF), som er involveret i indlæring og hukommelse, har tidligere været undersøgt i forbindelse med depression. Tidligere studier har vist, at BDNF er reduceret i depressive patienter og i prækliniske dyremodeller, som et respons på stress. Selvom dette antyder, at BDNF er involveret i ætiologien bag depression, giver studier med $BDNF^{+/-}$ mus modstridende resultater. Derfor ville $BDNF^{+/-}$ rotter muligvis være en mere passende model fordi (1) rotter har et større adfærdsrepertoire end mus, (2) klassiske adfærdstests er designet til rotter, og (3) rotter producerer perifer BDNF ligesom mennesker. Vores resultater viser, at $BDNF^{+/-}$ rotter udviser både anhedoni og milde tegn på angst, samt ændringer i ekspressionen af præfrontale og hippocampale gener, som er relevante for psykiatriske lidelser og særlig indlæring. Derfor passer adfærden og de molekylære resultater fra $BDNF^{+/-}$ rotterne til den allerede eksisterende litteratur, hvilket indikerer, at rotter er mere brugbare til undersøgelse af BDNF ekspressionen end mus.

Overordnet har dette projekt demonstreret miljømæssige og genetiske risikofaktorer for depression i translationelle modeller og etableret et nyt translationel værktøj til screening af antidepressiv behandling, hvor de kognitive funktioner bliver undersøgt.

Lay Abstract

Depression is a severely disabling disease affecting 300 million people worldwide and their social surrounding including family, colleagues and friends. The main symptoms of depression are being in a sad mood and showing a decreased interest or experience of pleasure (which is called being anhedonic). Depressed patients may also show a variety of other symptoms; amongst them are cognitive impairments, such as difficulties in decision-making, paying attention, learning or memory. After remission from depression, and normalisation of the mood-related symptoms, these cognitive impairments can still persist and continue impact day-to-day life. Everyday tasks may be perceived as more difficult and the risk of having more depressive episodes is increased. Therefore, novel antidepressant treatment that helps to relieve the cognitive impairments as well as the depression is needed. Furthermore, animal models to test the novel treatments on are indispensable. Usually, such treatment effects are assessed in animal tests that are specific to the animals' nature. However, such studies may not be clinically relevant nor help to draw conclusions from animal to men.

Hence, we applied the "touchscreen operant platform", a touchscreen setup developed on a touchscreen setup used in humans (e.g. iPad tests). Rats need to learn that some symbols on the touchscreen will be rewarded when chosen, which is similar to the test where humans should only touch the correct symbol on their touchscreen. There are different tasks available for testing different forms of cognition (e.g. memory, attention). The animal model of depression that was used is called the chronic mild stress (CMS) model because it uses mild stressors over weeks. This mimics daily stressors in humans that can lead to depression. However, not all humans, as not all rats, develop depression; some are resilient to it, which makes a good analogy between men and animals.

We found that different rat strains can lead to different results in the touchscreen learning performance. Stressed rats seem to be more impulsive, meaning they touch the screen although no symbols are displayed or they do not take as much time as non-stressed rats to make their choice on the touchscreen. Surprisingly, these impulsive behaviours were also seen in rats that were treated with antidepressants. Very importantly, we found that stressed rats that develop depressive-like behaviour, need longer to learn the touchscreen task, whereas rats that were stressed but stayed resilient did not take longer than the non-stressed rats to acquire the task. This shows us that some cognitive impairments are specific to depression and not to a general stress exposure. The antidepressant treatment used in this test did not improve learning.

Next, we looked at brain-derived neurotrophic factor (BDNF), a protein that is known to be expressed at lower levels in the brain of animals exposed to stress and similarly in patients with depression. Hence, we were interested if rats designed to have around 50% of the normal BDNF levels show depressive-like behaviour. We found that these rats have changes in expression levels of genes involved in depression or psychiatric diseases, such as Disc1, Fkbp5, GR and Nrg1. Furthermore, the lower-level BDNF rats displayed anxiety-related behaviour in some tests and anhedonic-like behaviour. These are important findings since anxiety often co-occurs with depression. Hence, we could show how BDNF may be involved in altered behaviour and expression levels of other gene; and further untangle the complex gene-environment interactions that lead to depression in humans. It is important to understand these interactions in order to tailor antidepressant treatment for depressed patients with showing different sets of symptoms. Furthermore, using stress exposed rats in touchscreen tasks we are able to assess the effects of such novel treatments in a clinically relevant model allowing conclusions to be drawn from animal to humans.

Table of content

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HTTLPR	Serotonin-transporter-linked polymorphic region
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
$BDNF^{+/-}$	Heterozygous for the BDNF gene
BOLD	Blood oxygen-level dependent
cAMP	Cyclic adenosine monophosphate
CANTAB	Cambridge Neuropsychological Test Automated Battery
cDNA	Complementary deoxyribonucleic acid
CMS	Chronic mild stress
CUMS	Chronic unpredictable mild stress
CRH	Corticotropin-releasing hormone
C_t	Cycle threshold
CVLT-II	California Verbal Learning Test
DG	Dentate gyrus
DISC1	Disrupted in Schizophrenia 1
dPAL	Different paired-associates learning
ELS	Early life stress
EPM	Elevated plus-maze
FKBP5	FK506 binding protein 5
fMRI	Functional magnetic resonance imaging
FSE	Fast spin echo
FST	Forced swim test
FWHM	Full width half maximum
GR	Glucocorticoid receptor
GSK3B	Glycogen synthase kinase 3 beta
GWAS	Genome-wide association study
h	Hour
HPA	Hypothalamic-pituitary-adrenal
HPC	Hippocampus
ICSS	Intracranial self-stimulation
KD	Knock-down
LE	Long Evans
LH	Learned helplessness
MDD	Major depressive disorder
min	Minute
MR	Mineralocorticoid receptors
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NIH	Novelty induced hypophagia
NRG1	Neuroregulin 1
NRI	Norepinephrine reuptake inhibitors
OF	Open field
pCREB	Phosphorylation of cAMP-response element binding protein
PAL	Paired-associates learning
PD	Pairwise discrimination
PET	Positron emission tomography
PFC	Prefrontal cortex
RT-qPCR	Real-time quantitative polymerase chain reaction
PVN	Paraventricular nucleus
SAB	Spontaneous alternation behaviour
SCT	Sucrose consumption test
SD	Standard deviation
SEM	Standard error of the mean
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
SNRI	Serotonin-norepinephrine reuptake inhibitor
sPAL	Same paired-associates learning
SPM	Statistical parametric map
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
STFP	Social transmission of food preference
WT	Wild type

1 INTRODUCTION

"There is an ancient Indian parable of six blind men attempting to describe an elephant. Unable to see the giant animal as a whole, each describes the elephant according to what he feels with his hands, as either a tree trunk, a wall, and so on. In a sense, MDD, and perhaps every psychiatric disorder, is like the proverbial elephant. If we make the mistake of equating one small part of a larger process with the disease itself, we run the risk of missing the big picture, which is that most psychiatric disorders probably represent an interactional matrix of many factors, and cannot be reduced to those factors alone."

Dean & Keshavan, 2017

1.1 Major depressive disorder

Major depressive disorder (MDD) is the leading cause for disability worldwide. Around the globe, there are more than 300 million people that suffer from depression[1]. In the adult population, an estimate of 6% show a 12-months prevalence for MDD[2], whereas the lifetime prevalence is approximately around 20%[3]. Approximately, two times as many women suffer from MDD as men[2,3]. In high-income countries, the life time prevalence is significantly greater (14.6%) than in middle- or low-income countries (11.1%)[2]. MDD explains 8.2% of the global years lived with disability and thus occupied the second leading cause for disability in 2010[4]. The years lived with disability are still on the rise due to a growing and aging population, which was demonstrated by an increase of years lived with disability by 37.5% from 1990 and 2010[4]. Median disease onset is at 25 years of age, and the high-risk period ranges from mid-late adolescence until early forties[2]. However, earlier onset of MDD is associated with increased disease severity and recurrence[5,6]. Furthermore, depression is an episodic disease and the poor response of only 50% of patients to a two-step pharmacological treatment regimen, as well as persistence of residual symptoms even after remission from MDD, increase the risk for relapse and chronicity of the disease[7]. Overall, MDD is a highly prevalent disease and its inefficient cure is of concern.

MDD patients display a heterogeneity of symptoms. Core symptoms of MDD are a depressed mood and anhedonia (a diminished anticipation or experience of pleasure). Additionally, patients can suffer from cognitive impairments primarily in attention, executive function and memory. A complete list of symptoms used for diagnosis is presented in Table 1.

Table 1. Diagnostic criteria for major depressive episode according to DSM-5, the Diagnostic and Statistical Manual of Mental Disorders.[67] At least five of these symptoms including at least one of the core symptoms have to present nearly every day during a two-week period.

Core symptoms	Depressed mood Loss of interest or pleasure (Anhedonia)
Additional symptoms	Body weight changes Insomnia/ hypersomnia Psychomotor agitation/ retardation Fatigue/ Loss of energy Feelings of worthlessness and inappropriate/ excessive guilt Cognitive impairments Suicidal thoughts

The suicide rate is twenty times higher in MDD patients compared to the general population[8]. Furthermore, MDD patients have an increased risk of developing comorbidities, such as heart diseases, diabetes mellitus or stroke[9,10]. The high suicide rate as well as occurrence of other comorbidities shorten the life span of MDD patients, which further underlines the need for countering depression. MDD treatment is additionally complicated by psychiatric comorbidities, for example anxiety disorders, which occur in 60–70% of MDD patients during their lifetime[11,12]. In conclusion, MDD affects many people, reducing their quality of life and potentially resulting in a premature death.

1.1.1 Risk factors: gene x environment interaction

Although depression research has received much attention and resources, the neurobiological mechanisms of MDD are not well understood[13]. Lack of knowledge is due to the complex gene x environment interaction eliciting a heterogeneity of symptoms across patients[14,15]. Potential genetic as well as environmental risk factors are identified; but their interactions resulting in a patient-specific MDD pathology remain unknown[16] and prevent optimal treatment strategies.

1.1.1.1 Genetic predisposition

The heritability of MDD is estimated to be 30–40%[16,17] whereas heritability appears to be higher in women (40%) than in men (30%)[18,19] and is increased two- to threefold in first-degree family members[20]. A family history of depression introduces differences in MDD pathology compared to MDD patients without such family history. For example, individuals with a family history show an earlier onset of MDD, increased severity of symptoms and higher relapse rates[20–23]. Although such observations indicate a genetic component in MDD development, genome-wide association studies (GWAS) keep failing to identify candidate genes associated with MDD[11,24]. The world's second biggest GWAS in psychiatry was conducted in depression research and included over 18 000 people (half of them with MDD) in the discovery phase and analysed over 1.2 million single-nucleotide polymorphisms (SNPs).

Still, not one SNP reached significance[25]. The same result was ascertained in the replication phase of the study including close to 7 000 MDD patients and over 50 000 controls targeting 554 SNPs. This GWAS mega analysis in the European population embodies the biggest MDD GWAS study, yet the authors suggest insufficient statistical power as reason for the lack of significant findings. They state that the high prevalence of MDD in the population implies only moderate genetic changes in MDD patients compared to controls and, thus, demands for an even greater sample size[25]. In the review from Flint and Kendler[11], the lack of significant findings of candidate genes related to MDD pathogenesis are explained either by overestimation of genetic effects in depression or by depression being falsely categorized as one disease. MDD might be reflecting a heterogeneity of diseases, which result in similar symptoms but are caused by alterations in different pathways. An alternative explanation could be that, gene x gene interactions, involving many genes with small effects on their own, contribute to the genetic side of MDD[26].

Nonetheless, a few studies observed associations of a genetic predisposition with MDD pathology and treatment response. For example, Binder *et al.*[27] discovered that SNPs in FK506 binding protein 5 (FKBP5) are associated with treatment response. A specific polymorphism (TT genotype at rs1360780) was linked to individuals with a high number of depressive episodes but also to a faster response to antidepressants. Polymorphisms in Fkbp5 can increase its gene expression. Incremented levels of FKBP5 desensitize the hypothalamus-pituitary-adrenal (HPA) axis' negative feedback loop via competing with free glucocorticoids for binding to the glucocorticoid receptor (GR). Furthermore, the GR polymorphisms *Bcl*I and ER22/23EK were found to increase risk for MDD in homozygous carriers[28]. Thus, genetic predispositions involved in HPA axis regulation appear to be involved in MDD and treatment response.

Another polymorphism that was associated with MDD pathology is an amino acid substitution of valine with methionine at codon 66 (Val66Met) in the brain-derived neurotrophic factor (*BDNF*) gene. Val66Met was increasingly found in MDD patients and resulted in an attenuated secretion of the activity-dependent form of BDNF[29]. Val66Met was linked to neuroticism, an affective-state dependent personality trait[30], which is associated with depression[31]. Furthermore, Val66Met appears to modulate the impact of stressful life events and, to a moderate degree, the impact of childhood adversity on MDD pathogenesis[32]. Thus, polymorphisms in the BDNF gene might create a vulnerability in individuals for developing MDD[33–35].

Furthermore, a polymorphism in the promotor region of the serotonin transporter gene (*5-HTTLPR*) is suggested to be involved in MDD pathogenesis. Originally, the polymorphism

in *5-HTTLPR* was directly associated with depression. This theory was then modified and it was shown that carriers of the polymorphism had an increased risk of developing MDD upon exposure to stressful life events[36]. However, this association seems dependent on the timing of the stress experience, the type of stressor and interactions with other genetic polymorphisms[17,36]. Furthermore, carriers show an increased risk to experience multiple-episodes of MDD, but the risk was not increased for a single-episode[37]. Overall, the examples of polymorphisms in *FKBP5*, *BDNF* and *5-HTTPLR* illustrate the difficulty when attempting to entangle the causal relationship of genetic risk factors and the emergence of MDD symptoms due to the environmental interference.

1.1.1.2 Environmental risk factor -Stress

Stress is a major environmental risk factor for developing depression[14,38]. "Stress" can be ascribed as the non-specific response of the body to any demand for change and, thus, as a threat to the maintenance of the body's homeostasis[33,39,40].

The brain plays a central role in the stress response: Stressors can be real (e.g. predator) or perceived (e.g. phobia). The brain is instrumental in defining a threat, thus a stressor, and initiates an appropriate physiological or behavioural response[41]. Various systems are involved in the succeeding stress response. Intensively studied is the hormonal stress response regulated by the HPA axis (Figure 1). Sensory input to the hypothalamus leads to secretion of corticotropin releasing hormone (CRH) from the paraventricular nucleus. Thereupon, the anterior pituitary releases adrenocorticotropic hormone (ACTH), which stimulates the adrenal glands to secrete glucocorticoids[42,43]. GRs are transcription factors, i.e. they regulate the transcription of other genes and, thus, are vital for cellular processes. Glucocorticoids are involved in important downstream processes for the 'fight or flight' response, including a rapid energy availability, down-regulation of the immune system and enhanced cognition (e.g. increased vigilance)[43]. A rapid activation of the HPA axis is beneficial to cope with an acute stressor and to restore homeostasis. For a healthy stress response, the increased HPA axis activation is short-term and quickly terminated in the absence of a stressor. Other brain regions are also involved in the regulation of the HPA axis, for example, the hippocampus (HPC). Increased levels of glucocorticoids secreted by the HPA axis reinforces the inhibitory control of the HPC on the HPA axis and resembles a negative

feedback loop of the HPA axis. However, prolonged high levels of stress result in long-term hyperactivation of the HPA axis, which is associated with pathogenesis of neuropsychiatric disorders[44–46].

The time point of stress exposure is also important. The brain undergoes different stages of plasticity throughout life depicting distinct phases of vulnerability. Stress programming is categorized accordingly, such as prenatal, early life (neonatal or juvenile) or adulthood stress[42,47]. It was shown that early life stress (ELS), such as experiencing abuse and witnessing violence, increases risk for depression later in life[17]. Thereby, the severity of the adverse event during childhood correlates with the emergence of MDD pathology[48]. Thus, stress increases the individual's vulnerability to develop affective disorders later in life. Markers of such a vulnerable phenotype are glucocorticoid resistance, increased inflammation, central CRH secretion and decreased activity of oxytocin[49–52]. Structural and functional changes occur in brain regions in response to ELS, including a smaller HPC volume[53] and an increased amygdala activity[54]. Symptoms and treatment response observed in depressed individuals with a background of ELS appear distinct from MDD patients without ELS, suggesting MDD in association with ELS as a subtype of depression[48,55].

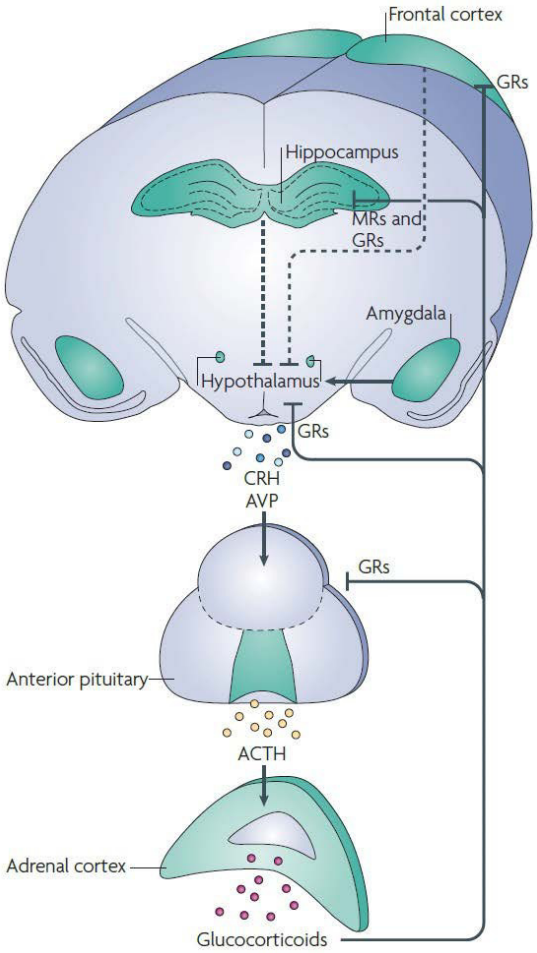

Figure 1. Hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, increased levels of arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) are released from the hypothalamus. This stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and consequently, elevated levels of glucocorticoids are released into the blood stream. The free glucocorticoids augment the inhibitory control, e.g. of the hippocampus on the HPA axis, resembling a negative feedback loop. The negative feedback is initiated by glucocorticoid (GR) and mineralocorticoid receptors (MR).[42]

In adult life, long periods of stressful experience, such as unemployment, poverty and family conflicts can induce a depressive episode[56]. Moreover, chronic stress can impair

cognitive function in e.g. HPC-dependent tasks, cognitive flexibility and decision-making[57–59]. Exposure to stress can lead to a shift from effortful, resource-demanding behaviour to less effortful, habitual cognitive strategies[60]. Such a shift is beneficial in times of high stress, but a failure to adapt in the long-term results in impairment of choosing the most appropriate cognitive strategy. Furthermore chronic stress causes dendrites to experience shrinkage in the HPC and prefrontal cortex (PFC), while they expand in the amygdala[56,59,60]. Most likely, dendritic changes are modulated by enhanced glucocorticoid secretion by an over-active HPA axis[61]. Furthermore, stress can also decrease neurogenesis in the HPC, a brain region involved in learning and memory[59]. This effect might be linked to BDNF, a growth factor modulating neuronal plasticity and its expression is diminished in response to stress[62]. Thus, the neurotrophic hypothesis of depression unifies the gene x environment interaction of MDD pathogenesis[63]. Therefore, stress might be directly responsible for the depression-associated cognitive impairments.

However, it should be clarified that not every individual experiencing prolonged periods of stress necessarily develops depression. Genetic predisposition but also environmental factors, such as social support, can prevent disease development[64]. Especially the individual's perception of control – or loss of control over the stressful situation impacts the vulnerability to MDD[65]. Resilience to stress is associated with the ability of an individual to consider challenges as opportunities in which one can actively manoeuvre instead of an externally imposed burden[66].

1.1.2 Symptoms associated with depression

Many symptoms are associated with depression. These can emerge in numerous combinations in patients enduring depression. The following sections focus on two symptoms: anhedonia and cognitive impairments.

1.1.2.1 Anhedonia

Anhedonia is one of the two core symptoms of MDD according to DSM-5 and contributes to the affective symptoms of depression. Anhedonia is described as a diminished anticipation or experience of pleasure in activities found rewarding prior to disease onset[67]. Brain regions involved in anhedonia are the PFC, dorsal striatum, amygdala and nucleus accumbens[68]. Anhedonia is the only negative predictor of time to remission and number of depression free days[69]. In depression research, anhedonia is often assessed by providing a reward-inducing experience and testing the subject's response to it. The rewards can be categorized as primary or inherent (such as food or sex), or as secondary rewards (such as money). In clinical studies,

mostly secondary rewards are used, whereas in preclinical research mostly primary rewards are applied. Together with the very general but also varying definition of anhedonia[68], comparisons between studies, performance of meta-analyses and translation between clinical and preclinical research is intricate.

Four clinical self-rating scales dominate assessment of anhedonia: Chapman Social Anhedonia Scale, Revised Chapman Physical Anhedonia Scale, Fawcett–Clark Pleasure Capacity Scale and Snaith–Hamilton Pleasure Scale. However, the criteria of anhedonia is inconsistently fulfilled across the four scales (reviewed in Rizvi *et al.*[68]). Thus, more recent scales were developed (Temporal Experience of Pleasure Scale, Motivation and Pleasure Scale-Self, Report, Specific Loss of Interest Scale, Anticipatory and Consummatory Interpersonal Pleasure Scale, Dimensional Anhedonia Rating Scale). However, these also encompass different foci and, therefore, the Snaith–Hamilton Pleasure Scale remains the most frequently used scale in assessing anhedonia although it mainly determines consummatory pleasure[68].

Preclinical models utilize the sucrose consumption test (SCT), the sucrose preference test (SPT), the place preference test or intracranial self-stimulation (ICSS) to evaluate the hedonic state of animals. Assessment of sucrose intake as reflection of the hedonic state was carefully evaluated by Willner and colleagues in connection with the chronic mild stress (CMS) model[70–73]. It can be disputed if the preclinical SPT or SCT reflects the subjective character comprising anhedonia in humans or if these tests can be reduced to alterations in energy seeking or changes in fluid consumption in animals. However, Muscat and Willner[70,73] have extensively evaluated these tests in the context of the CMS model and found the SPT and SCT to be a valid tool for assessing anhedonia in animals[71].

In the place preference test, control animals prefer the compartment of a box in which something rewarding, e.g. food, was presented on the previous days. In contrast, stressed animals do not show a preference for either box compartment. Nevertheless, it is questionable if the place preference test is a readout of anhedonia or rather reward-location association learning[74].

ICSS resembles the most elegant method for assessing anhedonia. The animal can induce activation of a brain region by pressing a lever, which elicits a reward sensation in the animal. The degree of effort an animal is willing to perform to receive a reward can be determined by modifying the number of lever presses necessary to elicit a reward sensation. This makes ICSS the gold standard for testing the abusive risk of drugs. However, ICSS is invasive and is inconvenient for models such as the CMS, which employ large numbers of

animals. Thus, the SCT or SPT are a valid middle-ground for assessing anhedonia in a reliable and straightforward practical manner.

1.1.2.2 Cognitive impairments in MDD

Cognition can be depicted as "the mental action or process of acquiring knowledge and understanding through thought, experience, and the senses"[75]. Cognitive processes underlie our everyday actions. We intentionally use cognitive functions, like learning and memorising, in school or at work. Often, however, cognitive processes are unconscious, such as speech production, decision-making or retrieval of short-term or long-term everyday knowledge or events. Cognition is a major entity of our life and essential for normal, daily functioning.

Cognitive function can be severely disturbed in patients with neuropsychiatric disorders, such as schizophrenia or depression. In depression, cognitive impairments are not listed as a core symptom of MDD[67]. However, cognitive impairments are a major contributor to the deficits in daily functioning in subjects with MDD[76], resulting in insufficient work performance, household maintenance or upholding of social relationships[77]. Furthermore, cognitive impairments are a prominent residual symptom following remission from depression and their persistence prevents success of antidepressant therapy[78,79]. These findings oppose the idea of cognitive impairments being a sole epiphenomenon of depression[79–81].

The cognitive domains that appear most impaired are those that require effortful processes[82]. Primarily, cognition is affected in the domains of attention, executive function and memory[77,80–83].

Various studies have shown that executive functions are impaired in MDD patients. For example, MDD patients displayed deficits in the intradimensional/extradimensional set shifting task requiring cognitive flexibility. This deficit was especially evident during the extradimensional set shift suggesting impairments in the lateral PFC[84]. A study introduced the concept of "depression-executive dysfunction syndrome", which refers to the symptoms of reduced fluency, impaired visual naming, paranoia, psychomotor retardation and loss of interest in activities, based on their research in elderly patients[85]. The depressive patients with executive dysfunctions in the study displayed increased functional disability compared to depressed patients with normal executive functions. The authors suggest that executive dysfunctions are a substantial part of geriatric depression, likely provoked by deficits in the medial frontal lobe[85]. This emphasises the disabling impact of cognitive impairments on life functioning. Furthermore, patients with depression-executive dysfunction syndrome exhibit clinically increased risk for relapse and poor treatment response[85,86]. Moreover, elderly MDD

patients demonstrate impairments in executive functions in the trail making test, verbal fluency test and go/no-go task. Thus patients showed deficits in semantic fluency, inhibition of automated motor reactions, cognitive flexibility and augmented psychomotor slowing[79]. The severity of depression correlated with lower performance in the go/no-go task, assessing response inhibition[79]. Similarly, another study found that adult, but non-elderly, depressed patients performed worse in the Stroop (executive attention), visual orienting (attentional shift), and continuous concentration (sustained attention) tasks. Performances were poorer in severely depressed patients (three or more depressive episodes) compared to patients with one or two depressive episodes[87]. Moreover, other depression-associated processes, such as rumination, appear to interfere with the inhibitory executive control, as shown with the random number generation task[88]. Hence, depressed patients fail to execute an inhibitory control over the ruminating thoughts and therefore forfeit in task performance. Clearly, executive functions are impaired in depressed patients and suggest alterations in the PFC.

"Attention" is another cognitive domain that received awareness in depression research. Paelecke-Habermann and colleagues[87] suggest that normal attentional performance is a prerequisite for executive functions. Hence, deficits in executive function might be interconnected with impairments in attention. Investigation of the three attentional network functions (Figure 2) in acute and remitted depressive patients suggests attentional deficits as trait- rather than state-dependent characteristic of MDD[87]. However, the same study suggests independence of deficits in attention and executive function in depression[87]. Furthermore, the concept of a negativity bias is well known in depressed patients. This mechanism was proclaimed mainly for memory[89], but is already active in attentional processes as shown with the dot probe task[90]. The attentional bias was also prominent for faces[91]. Depressed patients focused more on sad faces and healthy controls more on happy faces. Such a bias in depressed individuals might negatively influence interpersonal perception and social functioning. The negative bias towards sad faces was still present in patients remitted from depression[91].

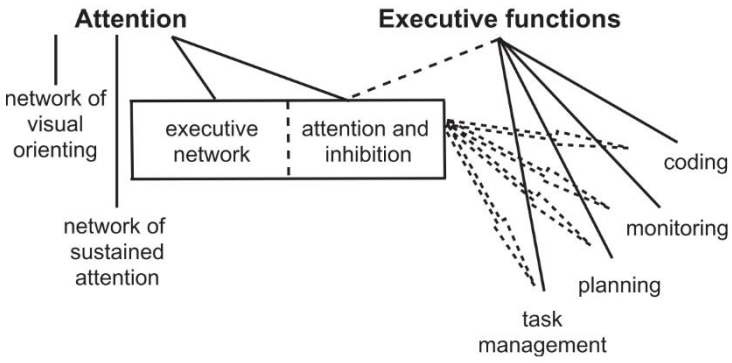

Figure 2. Interplay of the cognitive domains of attention and executive functions. Attention is a term that spans over three, independent networks, which are the basis for the five components of executive functions. These executive components are not independent from each other and deficits in one impedes the other components as well.[87]

Similarly, reading words revealed a negativity bias in depressed patients by being selective attentive to socially threatening, but not physically menacing words[92]. Thus, attentional processes can be impaired in depressed patients and, moreover, they redirected the patient's focus towards negative information, which possibly augments the depressive mood of the patient.

Another cognitive domain impaired in association with depression is "memory". Patients with depression performed poorer in a verbal learning test (CVLT-II) during both, the learning and recall phase[93]. Thus, declarative memory was impaired in depressed individuals compared to healthy controls. These impairments could neither be explained by verbal nor non-verbal IQ scores nor with secondary, strategy-related processes. Impairments in encoding and retrieval of verbal memory suggest altered function in medial temporal lobe regions[93]. In non-dementia late-onset MDD patients, memory deficits were clearly observed during the acute phase of depression independent of "general" cognitive performance or motivation[94]. Interestingly, only autobiographic memory but not memory for public events was affected in these patients. Generally, MDD patients appear particularly affected in explicit compared to implicit memory[80,81,95]. Another study applying the Cambridge Neuropsychological Test Automated Battery (CANTAB) revealed that MDD patients performed poor in tasks depending on episodic memory, recognition memory, problem solving, spatial recognition memory, and attentional set shifting from the CANTAB test battery[96–98]. Conclusively, memory deficits are present in MDD patients, which might be a result of augmented HPA axis activity and the consequently prolonged release of corticoids affecting the HPC[98,99].

The severity of cognitive impairments in depression appears to be augmented by the severity of depression experienced. Cognitive impairments are impacted by multiple episodes versus a single episode, number of recurrent episodes, history of psychotic depression, poor response to pharmacotherapy, increased residual symptom severity, younger age at MDD onset, and by older age (implying a higher amount of prior depressive episodes or increased vulnerability of the brain due to aging processes)[98,100]. Cognitive impairments are robustly found especially in elderly MDD patients[98,101,102]. However, it remains unclear how depression-associated cognitive impairments emerge. They could be a consequence of the neuroanatomical changes observed in MDD (reviewed in 1.1.3) or a result of the stress-induced hypercortisolism[77].

Several studies and meta-analyses investigated the alleviation of cognitive impairments after remission from the affective symptoms of MDD. Bhalla and colleagues[103] found that in late-life depression, 45% of patients display cognitive impairments one year after remission from

the affective symptoms. In fact, 94% of patients with persistent cognitive impairments during depression remained affected by these impairments one year following remission[103]. Additionally, 23% of patients newly developed cognitive deficits after remission from depression[103]. Reppermund *et al.*[80] observed cognitive impairments in executive function, attention and memory. These cognitive impairments were still present in 57% of patients after remission. Furthermore, cognitive performance was not statistically significant different between remitted and non-remitted MDD patients[80]. A study by Jaeger *et al.*[77] found that 6 months after hospitalization for MDD, 60% of patients exhibited significant neurocognitive deficits, which were also a predictor of life functioning disability. A meta-analysis including only studies that used the CANTAB test battery for assessing cognitive performance discovered that deficits in executive functions and attention as well as small deficits in memory persisted in patients remitted from the affective symptoms of MDD[81]. Furthermore, remission from affective symptoms does not result in normal daily functioning, which indicates cognitive impairments as a disability factor in MDD pathology[77,83,104]. Hence, even when remitted from depression, patients are still impaired in their daily functioning. This possibly induces lower self-esteem and feelings of worthlessness in such individuals, which in turn might trigger another depressive episode.

During a three-year period, cognitive symptoms were the most dominant residual symptom of MDD. In patients experiencing a depressive episode, cognitive impairments were present 94–100% of the time, and during a non-depressive period 35–44% of the time[105] (Figure 3). This long-term study emphasises the predominant appearance of cognitive impairments in the pathology of MDD. Furthermore, cognitive impairments appear to be greater with every

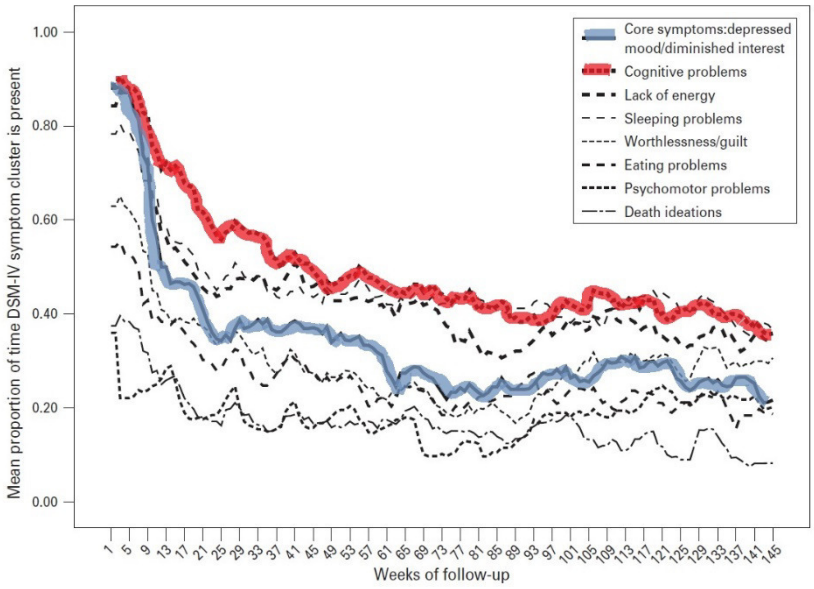

Figure 3. The presence of depression-related residual symptoms during a 3-year follow up. Cognitive deficits are a predominant residual symptom (red), whereas the core symptoms of depression are less present (blue).[105]

episode of MDD and in-between episodes, cognitive symptoms recover less and less[6,87] (Figure 4). In young and elderly MDD patients, cognitive symptoms predict treatment outcome[106,107]. Furthermore, treated patients perform better in cognitive tasks than unmedicated patients, however, medicated patients still perform worse compared to healthy controls[108], emphasizing the demand for pro-cognitive antidepressants. If cognitive symptoms can be mitigated, the risk of relapse can be reduced[109,110]. So far, treatment with serotonin-norepinephrine reuptake inhibitors (SNRIs) appears to be superior to selective serotonin reuptake inhibitors (SSRIs) in regard to alleviating cognitive deficits, such as episodic and working memory[111]. Both treatments improved attention and executive functions alike, but failed to normalize cognitive performance to the level of healthy controls[112]. These findings demonstrate that antidepressants still lack to efficiently target cognitive impairments but mainly focused on alleviating the affective symptoms of depression only. In the long-term, it is estimated that only 20% of MDD patients fully recover and regain their daily functioning to a level prior to depression onset[113,114]. Thus, normalization of cognitive performance in depression is required for complete remission of depression.

1.1.3 The brain in MDD

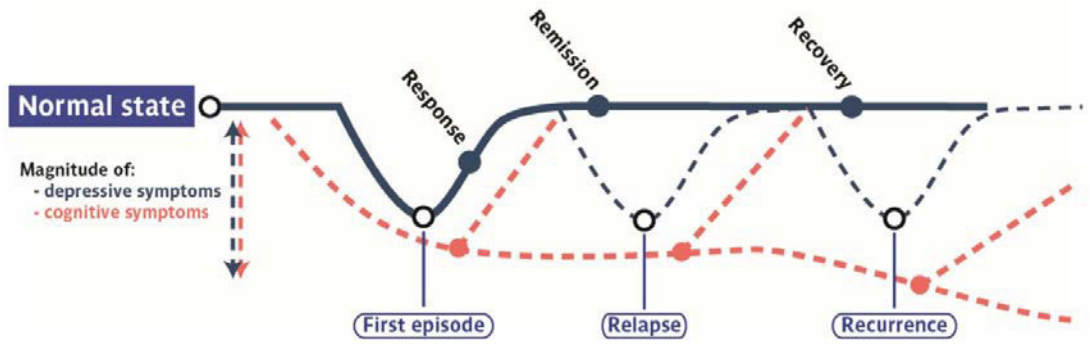

Figure 4. Scheme of major depressive episodes. Illustrated in blue is the episodic nature of the affective symptoms. Depression-associated cognitive impairments are displayed in red. Cognitive abilities may recover between depressive episodes or increasingly deteriorate with every relapse.[6]

In MDD, brain regions with functional changes are predominantly the PFC, the HPC, the amygdala and the nucleus accumbens[115].

The PFC is associated with complex cognitive processes and involved in executive functions. These include regulating attention, cognitive flexibility, response inhibition, working memory, verbal fluency, decision-making and problem solving amongst others. In depressed patients, the PFC shows decreased activity and a reduction in volume[116,117]. Diminished PFC activity was associated with MDD severity[117].

Alterations in the HPC are a robust finding in MDD pathology. The HPC is associated with formation of long-term and spatial memory. The HPC is linked directly to the hypothesis of stress as a risk factor of depression as it is an inhibitory regulator of the HPA axis activity mediated by its glucocorticoid (GR) and mineralocorticoid receptor (MR) actions. In depressed patients, reduced volume of the HPC was robustly found in several studies[118–120] and may be explained by dendritic retraction, cellular shrinkage, apoptosis, reduced number of glial cells, lower levels of extracellular fluid content or lower neurogenesis or gliogenesis rates[61]. Volume reduction appears to correlate with the duration of the depressive episodes[119–121]. Furthermore, HPC neurogenesis was shown to be decreased in response to stress, increased after antidepressant treatment[122] and required for antidepressant action[123]. These findings, in both pathology and treatment substantiate a central role of the HPC in MDD.

Changes in reward sensitivity and anhedonia are suggested to be a consequence of alterations in the nucleus accumbens and the ventral tegmental area[124]. Both areas are involved in reward and motivation and it was shown that, e.g. the nucleus accumbens is involved in maintaining resilience and response to antidepressants after stress exposure[125].

Generally, it is more likely that MDD pathology is introduced by a network dysfunction mode than by single brain lesions[126]. Imaging studies found that the connectivity of brain regions is altered in MDD patients, such as higher activity in the subgenual cingulate and thalamus, investigating the default mode network with magnetic resonance imaging (MRI)[127]. Additionally, positron emission tomography (PET) reveals that depressed patients' metabolism is reduced in the lateral PFC, but increased in medial PFC and subgenual cingulate[128,129]. Even in patients remitted from depression, the brain metabolism was altered involving an increase in activity in the anterior cingulate, orbitofrontal cortex and medial thalamus[130]. These findings suggest that brain changes do not simply recover after a depressive episode and more likely increase vulnerability to experience another subsequent episode.

1.1.4 Antidepressant treatment

Antidepressant medication was discovered by serendipity in the 1950s. Patients treated for tuberculosis showed signs of elevated mood following treatment with iproniazid[131]. The first antidepressants included monoamine oxidase inhibitors and tricyclic antidepressants, which prevent the metabolism or reuptake of monoamines and therefore increase their availability. These broad-acting antidepressants were mostly replaced by SSRIs, noradrenaline reuptake inhibitors (NRIs) and SNRIs in the 1980s[131,132] and are still the most common first-line treatment at present. A major detriment of currently used antidepressants is the long period of 2-4 weeks until therapeutic onset[132]. Furthermore, with a remission rate as low as 50% in a

two-steps treatment regimen, valuable time is lost to find a potent drug for individual patients[7]. Novel antidepressants, such as ketamine, are being constantly investigated, but there has been no major breakthrough in antidepressant drug research for nearly 40 years[132]. Current first-line antidepressants insufficiently target all of the potential biological underpinnings of MDD pathogenesis, such as dysregulation of the HPA axis, inflammation, or the involvement of other neurotransmitter systems, such as glutamate or γ-aminobutyric acid (GABA) but primarily focus on symptom relief[11,132]. As previously mentioned, cognitive impairments were identified as a major contributor to the functional disability of MDD patients in daily life and remain a main residual symptom of MDD. However, antidepressant treatment with pro-cognitive characteristics is rare. A candidate drug for targeting affective symptoms as well as cognitive impairments in MDD is vortioxetine.

1.1.4.1 Vortioxetine

Vortioxetine ((1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine, Lu AA21004) is an antidepressant that was approved by the FDA and European Medicines Agency for treatment of MDD in 2013[133]. Vortioxetine has a multimodal mechanism of action by being a serotonin transporter (SERT) inhibitor; serotonin $(5\text{-HT})_3$, 5-HT_7 and 5-HT_{1D} receptor antagonist; 5-HT_{1B} receptor partial agonist; and 5-HT_{1A} receptor agonist[133]. The pro-cognitive effects of vortioxetine are attributed to its actions on the 5-HT_{1A}, 5-HT_{1B}, 5-HT_3, 5-HT_7 receptors; additionally to relieving the affective symptoms of depression[133,134].

In fact, seven out of ten studies in humans show that vortioxetine is superior to placebo in alleviating the affective symptoms of MDD and superior to agomelatine, an atypical antidepressant[133,135,136]. Furthermore, vortioxetine improved performance in the cognitive domains of executive function, attention, memory, processing speed, and verbal learning compared to placebo treatment in elderly MDD patients[137]. Executive function, attention, processing speed, learning and memory were also improved by vortioxetine compared to placebo treatment in non-elderly MDD patients[138]. Thus, these results suggest that vortioxetine has a unique treatment profile in targeting both, the affective and cognitive symptoms associated with MDD. Moreover, improvement on the cognitive symptoms appears to be a direct effect of treatment and not a by-product of alleviated mood symptoms[133].

Preclinically, the antidepressant effect of vortioxetine was demonstrated in several behavioural rodent studies. Vortioxetine has an antidepressant effect in the forced-swim test (FST), social interaction, fear-induced vocalization, novelty suppressed feeding and open field (OF) test. These effects were observed after acute treatment with vortioxetine; and in the FST, OF and novelty suppressed feeding test also after chronic administration[139,140]. In the clinic,

classical antidepressants need to be given chronically to elicit an effect, thus, there is some divergence between preclinical and clinical studies. Pro-cognitive effects of vortioxetine treatment were also observed in behaviour studies. Phencyclidine-induced deficits in the attentional set-shifting test were reversed by vortioxetine [141,142]. Similarly, vortioxetine prevented chronic cold intermittent stress-induced cognitive impairments in the reversal learning task for cognitive flexibility[143]. Furthermore, vortioxetine treatment prevented age-related deficits in the object placement test assessing spatial memory[144]. Novel object recognition and spontaneous alternation behaviour (SAB) was rescued by acute vortioxetine treatment in 5-HT depleted rats[134,145]. Treated, but otherwise naïve rats showed memory improvements in the contextual fear conditioning and the novel object recognition test due to acute vortioxetine treatment[146]. Thus, vortioxetine appears to have a pro-cognitive effect on memory and executive functions.

The pro-cognitive effects of vortioxetine are supported by electrophysiological studies and may unravel the biological mechanisms involved. Dale ***et al.***[147] show that vortioxetine amplifies theta-burst long-term potentiation (LTP) in HPC slices determined by patch-clamp recordings and increased theta power in the frontal cortex of awake rats assessed by electroencephalography. Interestingly, acute administration of vortioxetine reduced LTP provoked by high frequency stimulation in anaesthetised, naïve rats but prevented stress-induced derogation of LTP in pre-treated rats[148]. Additionally, vortioxetine stimulates neurogenesis and neuron maturation in mice and rats more rapidly than with other antidepressants, such as the SSRI Fluoxetine, or compared to vehicle controls[139,148]. Thus, on a molecular level, vortioxetine improves functional processes commonly associated with cognitive performance.

1.2 Animal models of depression

Animal models are a corner stone for clinical research. Compared to clinical research, animal models allow for better control of the environment the organism is exposed to, for manipulation of fundamental parts of the organism (e.g. knock-out of a gene), greater homogeneity of experimental groups, the study of long-term effects and generational effects in a shorter time-period, first stage drug testing, and the use of invasive techniques. Therefore, animal models provide insight to the causal relationships in disease pathogenesis.

Preclinical models try to mimic a specific human condition. However, it is often difficult to fully examine and model all modalities of psychiatric diseases in animals. Thus, the concept of "validities" was introduced to elucidate which criteria of the human condition are fulfilled. Ideally, an animal model should encompass predictive, face, and construct

validity[149]. Construct validity requires that similar neurobiological underpinnings result in similar symptoms clinically and preclinically. It is often supplemented by aetiological validity, meaning the aetiological origin of the condition is the same in the preclinical and clinical condition. Face validity entails the bridging of clinical symptoms with the animal's behavioural phenotype. Finally, predictive validity demands that treatment, which is non-/effective in the clinic, provokes the same action in the preclinical model[132,149,150]. These primary validities can be extended to introduce relevant criteria for specific animal models. For example, "population validity", which describes that only a subpopulation of animals should exhibit a vulnerable phenotype in response to an external stimuli, i.e. that only some animals should display a depressive-like phenotype due to exposure to environmental risk factors to match the situation in humans[151].

1.2.1 Difficulties in modelling depression

Some difficulties arise when modelling depression in animals. MDD symptoms, such as weight changes or altered sleep architecture can be readily assessed in animals. However, "internal" symptoms including feelings of worthlessness, inappropriate guilt or suicidal thoughts, which are typically assessed by questionnaire or oral communication in humans, are challenging to model and evaluate in animals. Additionally, the inter-patient heterogeneity of symptoms further exacerbates preclinical MDD modelling. Still, various depression models have been established exhibiting different aspects of clinical depression. In the following, preclinical models are presented, subdivided into environmental and genetic paradigms.

1.2.2 Genetic models

Genetic models can be created by targeted gene manipulation or by selective breeding of animals with a relevant trait. Genetic models include knock-down, knock-out or overexpression of a candidate gene. These gene manipulations can be genome-wide or conditionally expressed in a specific tissue or cell type. Single-gene modifications allow for investigating the causal relationship of a gene in disease symptomatology or in treatment efficacy. An example model is the genetic modification of the *Bdnf* gene in mice and rats.

The *BDNF* gene is of particular interest in MDD. For example, the BDNF polymorphism Val66Met, which results in attenuated BDNF activity, was linked to MDD pathology[29]. Furthermore, BDNF levels were found reduced in the PFC and HPC of MDD patients' *post-mortem* tissue[152]. Accordingly, BDNF was proposed as a candidate gene of MDD, which ensued in a plethora of clinical and preclinical studies.

A full knock-out of the *Bdnf* gene in mice was attempted in 1994, but was developmentally lethal[153], thus, heterozygote knock-out mice were developed ($BDNF^{+/-}$). These mice were behaviourally indifferent from wild types (WTs) regarding behavioural despair in the FST, anxiety in elevated plus-maze (EPM), elevated zero-maze, light-dark box and novel cage test or anhedonia in the SPT[154,155]. $BDNF^{+/-}$ mice were slower to escape in the learned helplessness paradigm, but this finding might be ascribed to a lower pain sensitivity demonstrated with the hot plate test[155]. In a different study, however, $BDNF^{+/-}$ mice showed anxious-like traits in the novel object test in the OF and displayed increased anxiety in the light-dark box[156]. Overall, studies using $BDNF^{+/-}$ mice provided conflicting results and therefore findings should be interpreted cautiously. Since behavioural tests for rodents were originally designed for rats but not mice[132], behavioural investigation of $BDNF^{+/-}$ rats might be more appropriate. This has been achieved recently and a temporal and region-specific knock-down (KD) of BDNF in Sprague Dawley rats led to depression-associated behavioural changes. BDNF KD in the dorsal dentate gyrus (DG) elicited anhedonia in the SPT, behavioural despair in the FST, cognitive impairments in the Morris water maze (MWM) and decreased locomotor activity[157]. Whereas BDNF KD in the ventral subiculum only induced anhedonia and BDNF KD in the HPC CA3 region did not alter any of these readouts[157]. Naïve congenital $BDNF^{+/-}$ rats displayed no behavioural alterations in the EPM or FST, but showed anxiety-related behaviour by spending less time in the centre of the OF. Chronic corticosterone treatment, simulating stress in these $BDNF^{+/-}$ rats resulted in an anxiolytic effect in the EPM. In the OF, $BDNF^{+/-}$ rats displayed decreased locomotor activity, which was further diminished after corticosterone treatment. Therefore, this study suggests an involvement of BDNF and its interaction with stress in an anxiety-related phenotype[158]. These findings were complemented by another study applying a Pavlovian fear conditioning paradigm. $BDNF^{+/-}$ rats displayed a weaker association of a foot shock with a neutral light stimulus. Furthermore, retrieval of the association in an MRI scanner revealed decreased activation of the amygdala in $BDNF^{+/-}$ rats[159], thus suggesting altered emotional fear processing. Moreover, cognition was impaired in $BDNF^{+/-}$ rats as demonstrated in the Y-maze for short-term spatial memory and in the acoustic startle prepulse response inhibition associated with disrupted sensorimotor gating, a symptom in many neuropsychiatric disorders. However, novel object recognition assessing memory was not impaired[160]. Overall, findings in $BDNF^{+/-}$ rats appear more consistent than in mice, but still no evident depressive-like phenotype could be associated with these rats.

By contrast, findings about the interaction of antidepressant treatment and BDNF levels are robust[63]. BDNF$^{+/-}$ mice as well as mice with an induced BDNF knock-out showed resistance to antidepressant treatment[161–163] suggesting sufficiently high BDNF levels are requirement for antidepressant drug efficacy. In contrast, overexpression of the BDNF gene in mice resulted in antidepressant-like behaviour in the FST[164]. Furthermore, infusion of BDNF in the midbrain of rats resulted in an antidepressant-like effect in the learned helplessness paradigm and the FST[165]. In conclusion, genetic modification of BDNF suggests a negative relationship of BDNF levels and MDD pathogenesis as illustrated in Figure 5.

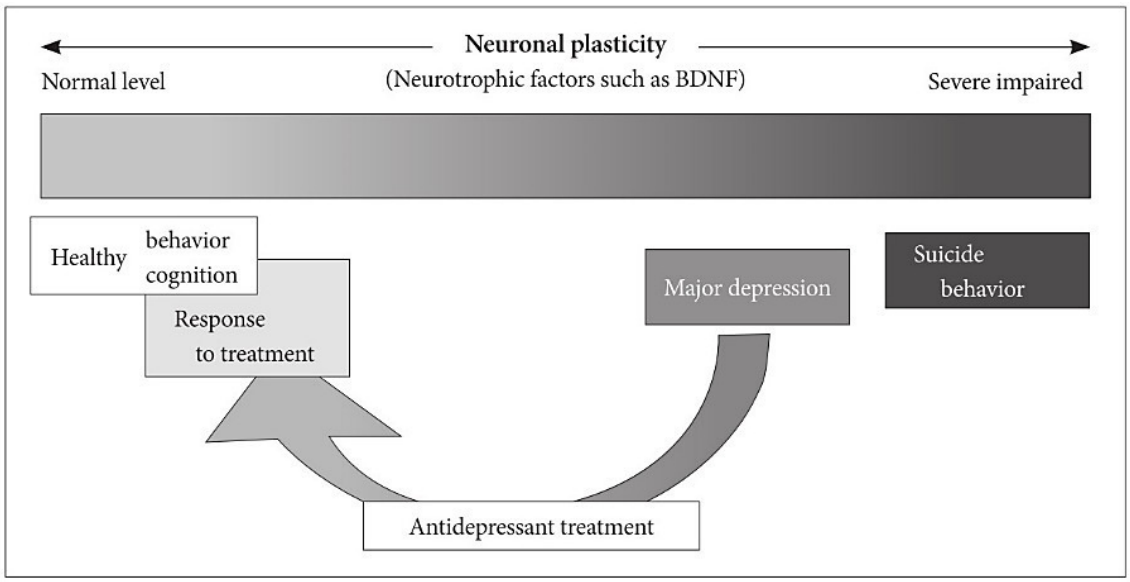

Figure 5. The relationship of brain-derived neurotrophic factor (BDNF) levels and depression pathology. Reduced cerebral BDNF levels are found in depressed suicide victims, whereas antidepressant treatment requires and increases BDNF levels.[316]

Generally, a caveat of genetic models, which innately display disease symptoms, is that the literature evidently suggests a gene x environment interaction causes MDD. However, generating genetic modifications might be helpful to uncover predispositions in animals, which lead to disease pathogenesis in the event of an environmental trigger[132]. Therefore, the following section addresses preclinical depression models constructed by manipulating the animal's environment.

1.2.3 Environmental models

Stress is a main environmental risk factor for inducing depression in humans[166,167]. Thus, stress has been extensively used to induce a depressive-like phenotype in animals. However, the term "stress" covers a plethora of different micro-stressors and protocols, which are often lab-specifically modified. Hence, different stress protocols result in a variety of depression-related

phenotypes. On the one hand, such a variety possibly covers divergent subtypes of MDD, but can also exacerbate comparisons of study results and meta-analyses. Well-known stress models in preclinical depression research include the CMS[168,169], chronic unpredictable mild stress model (CUMS), learned helplessness (LH), social defeat (such as the resident-intruder paradigm) and early life stress (ELS, such as maternal separation)[132,170,171]. Paradigms that are based on or include social stressors in their protocol appear to mimic the human condition more closely than non-social stress protocols[172]. In the following, different stress models are presented.

1.2.3.1 Early life stress

ELS attempts to model adverse childhood events in humans, such as abuse, neglect or parental loss. In humans as well as in rodents, such events can induce a vulnerable predisposition for developing neuropsychiatric diseases, including depression and anxiety, later in life[17,132].

ELS encompasses a plethora of models across the early life span including prenatal, early postnatal or juvenile stress paradigms[173]. A frequently applied paradigm of ELS is maternal separation, in which the mother is separated from its pups for a limited time inducing psychological and physiological stress to the pups[174]. Endocrinological and behavioural changes are observed immediately after separation and into adult life. Separation results in an altered HPA axis activity (a prolonged, hypo- or hyperactive response to acute stress and novelty) and disturbs the dopaminergic, noradrenergic and mesolimbic system.[175–178]. Behaviourally, pups display an altered sleep architecture, weight changes, altered avoidance learning and increased anxiety[179–181].

However, divergences in the resulting pup phenotype are reported, such as HPA axis hypo- or hyperactivity. These divergences result from differences in the ELS protocol including duration of maternal separation, a single versus multiple separation sessions, pup age during separation and age of endocrinological assessment, pup gender but also the choice of the control group (undisturbed, handled or litter-mates) to which the experimental group is compared to[175]. Furthermore, maternal behaviour, i.e. pup care, is influenced by litter size[182] and litter gender composition[183,184]. Finally, a caveat of the ELS model is that brain maturation is less developed in rodents than in primates after birth complicating translation between species[42]. Consequently, ELS models appear prone to high phenotype variability due to unstandardized protocols. Besides this caveat, ELS is the best approach to model depression with childhood trauma, a subtype of MDD[17].

1.2.3.2 Learned helplessness

The LH model is thought to model a unique cognitive aspect of depression –the perceived loss of control over an aversive situation[185]. In the LH paradigm, animals receive electric shocks without being able to escape the situation. However, once the option for escape exists, i.e. cage door open, animals still do not attempt to escape due to their previous inevitable experience[186]. This led to the term "learned helplessness". It was shown that it is the uncontrollable aspect and not the foot shock *per se* that defines the depressive-like phenotype[187]. Loss of control is likely processed in the ventromedial PFC[188]. Consequently, sensation of an uncontrollable stressor desensitizes the 5-HT_{1A} receptor in the dorsal raphe nucleus resulting in an altered behavioural phenotype[189]. LH animals display anhedonia, agitation, sleep disturbances, reduced sex drive and weight loss. Antidepressants can reverse LH behaviour[190]. However, it was reported that these behavioural deficits only persist for few days[191] preventing testing of chronic treatment.

1.2.3.3 Chronic social defeat

Generally, social defeat models induce social avoidance behaviour by exposing a subordinate animal to a dominant animal. Different social defeat models exist involving one or more subordinate animals and can be used to apply acute or chronic stress[132,192,193]. Social defeat paradigms provoke social withdrawal, a symptom known to accompany MDD pathology. This symptom is reversed by chronic but not acute antidepressant treatment[194]. Furthermore, the chronic social defeat model provokes susceptible as well as resilient phenotypes differing in social interaction and anhedonic-like behaviour, increased immobility in the tail suspension and FST and increased anxiety in the OF test, sleep architecture, lower body weight, increased CRH levels in the paraventricular nucleus and decreased MR levels in the HPC[193,195]. Anhedonic-like behaviour and behavioural despair were renormalized with chronic citalopram (SSRI) treatment[196]. Depending on the study, some of the symptoms, such as anxiety, stress reactivity, behavioural despair or body weight are indifferent between both social defeat groups possibly due to differently defining susceptibility and resilience[193,195]. Not all studies separated the social defeat group[192,196–198], and of those only some observe a depressive-like phenotype[196,197] . A critical view of this model suggests the social defeat model as model of stress rather than a model of depression since animals display an increased startle amplitude, which is characteristic of the post-traumatic stress disorder and thus non-specific to depression[199].

1.2.3.4 Chronic mild stress model

The CMS paradigm has been extensively studied and is well known for exhibiting the MDD core symptom anhedonia in rodents. The model was first introduced by Katz and Hersh in 1981[200]. They applied stressors that are considered to be comparatively severe. Willner[71,73] modified the protocol by abandoning the severe stressors for milder ones and, thus, the paradigm mimics more closely everyday stressful situations in humans. Micro-stressors in recent CMS protocols include, dampened bedding, changes in illumination (e.g. lights on during the dark phase), stroboscopic light, food and water deprivation, grouping of two CMS rats (resident-intruder) and cage tilting[132,201]. The micro-stressors are applied in an unpredictable order and time point of the day, introducing the element of uncertainty, which in itself is a core element on the asperity of stress perception[65]. Furthermore, the episodic nature of depression can be modelled by cessation and reintroduction of stress exposure. CMS exposed, depressive-like animals respond to chronic antidepressant treatment but not acute administration of antidepressants, demonstrating good predictive validity[201].

Typically, the CMS protocol is applied over several weeks and the animals' reward sensitivity is monitored with the SPT or SCT. Thereby, alterations in reward sensitivity induced by stress exposure can be traced within each rat. Individual rats respond differently to the stress exposure and a spectrum of phenotypes develops. The strongest phenotypes display either the MDD core symptom anhedonia, shown by reduced reward sensitivity (~40% of rats), or are resilient and maintain their reward sensitivity as prior to stress onset (~20% of rats)[201]. Resilience is another feature of the CMS paradigm that demonstrates the clinical pertinence of the model; likewise, only a subset of humans develops MDD associated with stress challenges. Furthermore, rats that are stress exposed but resilient in their hedonic state allow for the distinction of stress- and depression-specific alterations. This distinction is not addressed in every preclinical model and often the stress-exposed group is taken synonymously as depressive-like. In the CMS paradigm, usually only the strongest phenotypes, i.e. the rodents that show a robust resilient or anhedonic-like phenotype, are used in subsequent experiments.

CMS exposed rodents also display other behavioural abnormalities. Exploration, sexual behaviour, locomotion and aggression are reduced, and sleep architecture is altered in CMS susceptible rodents[168,202,203], reminiscent of MDD symptomatology. Interestingly, CMS exposure promoted anxiolytic-like behaviour in the EPM test[202]. Social interaction is not altered in rats subjected to the CMS paradigm[202], although patients with MDD can express social withdrawal[67]. After cessation of CMS for 4–5 weeks rats show spontaneous recovery from the stress exposure and depressive-like state[201].

Various physiological changes can be observed in rats subjected to the CMS paradigm. Augmented stress exposure activates the HPA axis, which consequently increases release of glucocorticoids attempting to restore homeostasis. However, prolonged hyperactivity of the HPA axis can result in adverse alterations, e.g. cerebral atrophy, and transition to disease state, such as depression[44]. Such changes were also observed in the CMS model. CMS susceptible, anhedonic-like rats showed increased corticosterone levels for four weeks during stress exposure. CMS resilient rats only displayed an initial increase in corticosterone at the beginning of the CMS paradigm, but glucocorticoid secretion quickly returned to baseline level[201,204]. CMS exposed rats also display weight changes, weighing less than same-aged non-stressed controls[201]. In classical behavioural paradigms assessing cognition, CMS rats (resilient and anhedonic-like) were impaired in the SAB task assessing working memory[205]; displayed a negativity bias in a lever pressing test using an ambiguous tone[206]; but only anhedonic-like rats increased in contextual fear conditioning determining long-term memory of aversive stimuli[205]. Stress exposure did not affect performance in the step-down, passive avoidance or cued fear conditioning test[205]. Thus, CMS rats display cognitive alterations in behavioural paradigms involving negativity bias, long-term and working memory.

On a cellular level, CMS anhedonic-like rats showed decreased levels of neurogenesis in the ventral HPC compared to non-stress controls[122]. Neurogenesis was restored in animals that responded to antidepressant treatment with escitalopram. Non-responders, determined by their persistent anhedonic-like state, continued to show decreased neurogenesis[122]. Although neurogenesis was restored in response to treatment in CMS animals, the number of granule cells in the HPC was not[207]. Moreover, the neuronal growth factor BDNF was decreased (protein and gene expression) in CMS exposed animals[208]. Thus, rectified neurogenesis in response to antidepressant treatment in CMS animals complements the bidirectional relationship of increased BDNF levels, and thus elevated neuronal plasticity, and antidepressant efficacy[35]. Similar to studies in $BDNF^{+/-}$ animals, molecular findings are robust regarding the antidepressant treatment effects but less clear when it comes to the depressive-like phenotype itself. For example, decreased cell proliferation in the HPC was found in both, anhedonic-like and resilient rats and therefore this effect was not specific to the depressive-like phenotype but a general effect of stress exposure[207]. Furthermore, pharmacological blocking of neurogenesis did not result in a depressive-like phenotype[209]. Consequently, neurogenesis appears implicated in the depressive-like phenotype and involved in treatment response although on its own, restricted neurogenesis does not induce depression.

Brains of CMS anhedonic-like, resilient and non-stressed controls were analysed with magnetic resonance imaging. Anhedonic-like animals displayed altered metabolite levels, resilient rats exhibited changes in ventral HPC shape, and both CMS groups possessed subtle substructural changes revealed by diffusion kurtosis imaging of the HPC[210]. Moreover, diffusion characteristics were distinct between the anhedonic-like and resilient phenotype in the caudate putamen, and increased in CMS rats for axial as well as radial diffusion in the caudate putamen and amygdala compared to controls. The volume of the caudate putamen was increased in the anhedonic-like compared to the control group[211]. Thus, the HPC appears to be a key region in depression pathology revealing physiological alterations due to stress in general as well as to the depression state.

Limitations of the CMS model include the initial amount of animals needed to obtain a sufficient group size of anhedonic-like animals. This is especially the case when multiple anhedonic-like groups are required to study, for instance, the efficacy of antidepressant treatment. Furthermore, the CMS paradigm is labour-intensive due to the amount of animals and the daily application of micro-stressors over weeks. Still, the CMS model has good face, predictive, construct and aetiological validity and is thus a unique model for preclinical depression research[132].

1.3 Translational testing

1.3.1 The touchscreen operant platform

The importance of translation from preclinical research to clinical is being increasingly recognised. Initiatives, such as CNTRCIS[*], have been established aiming to facilitate translational crosstalk. In this context, standardization of tests is central as it facilitates the replication and comparison of studies across institutions and uniforms data acquisition for meta-analysis. The CANTAB test battery is such a standardized, clinical tool and the most frequently applied battery for assessing cognition in MDD research[6]. Various touchscreen tasks allow evaluation of abilities from different cognitive domains.

Based on CANTAB, the preclinical touchscreen operant platform was developed for non-human primates and rodents. The touchscreen operant platform uses the same tests as CANTAB adapted for testing in animals (face validity) and, as such, represents one of the

[*] Cognitive Neuroscience Treatment Research to Improve Cognition in Schizophrenia

most translational tools in preclinical research. Additional advantages to being clinically pertinent are objective readouts and minimization of experimenter's bias. Moreover, exactly the same tests can be applied across institutions while visual stimuli can be modified and new stimuli or tests can be shared between groups, ensuring standardization. Furthermore, the operant conditioning for touchscreen testing is based on appetitive learning instead of adverse conditioning, which is frequently used in classical behavioural paradigms. Thus, the operant conditioning for touchscreen testing is preferable when working with stress-induced models and non-stressed controls due to the use of non-aversive testing. Although the touchscreen operant platform was developed in the 1990s, it was not extensively used[6,212]. However, the young field of preclinical touchscreen testing is growing and aims for *par for par* comparisons with clinical research as shown by an elegant study of Nithianantharajah and colleagues[213]. In that study, a modification on the *Dlg2* gene was introduced in mice as it is naturally occurring in some patients with bipolar disorder, schizophrenia, autism and intellectual disability, and the two species were tested in the paired-associates learning (PAL) task. Individuals, human and mice, carrying the genetic mutation showed strong cognitive impairments, whereas healthy controls were able to learn the PAL task. Thus, results were replicated across species applying a touchscreen paradigm. Hence, translational touchscreen testing is at the forefront of bridging preclinical and clinical research.

1.3.1.1 Pairwise discrimination and reversal learning

The pairwise discrimination (PD) and reversal task requires the association of one of two symbols with a reward (S+), whereas the other symbol is non-rewarded (S-). After task acquisition, the reward is switched to the previously non-rewarding symbol (S- becomes S+ and *vice versa*)[214]. Thus, visual discrimination, stimulus-reward association and response inhibition during reversal learning is evaluated with this task, as well as perseverative behaviour is tested.

Reversal learning is an interesting component of this task and is composed of two different phases: first, perseverative behaviour is tested and, second, evaluation of the new stimulus-reward association learning. Animals with orbitofrontal cortex lesions were impaired in the first phase (perseveration)[215,216] and prelimibic subregions are required for the strategy shift in reversal learning[216]. Likewise, the medial PFC may too be vital for the second phase (new stimulus-response association)[214,217] and the dorsomedial striatum for the maintenance of the new associations[216]. Hence, although the PD and reversal task is a rather simple test, the reversal learning element evaluates domains, such as cognitive flexibility, that are often impaired in stress-related disorders.

Unmedicated MDD patients displayed no impairments regarding task accuracy but showed psychomotor slowing during PD and reversal learning, which correlated with disease severity. Furthermore, hyperactivity of the anterior insula and putamen and hypoactivity in the PFC, anterior cingulate cortex, thalamus, and inferior parietal cortex were found in MDD patients during the PD and reversal task. Therefore, this study indicates functional alterations in frontal-striatal and limbic brain areas during PD and reversal learning[218].

Preclinically, one study has indicated that the PD and reversal task might be sensitive to affective state-dependent cognitive impairments in the CMS model[219]. However, many animals did not even acquire the first PD learning task limiting the interpretation of the more interesting reversal learning step. The high drop-out rate might be explained by the choice of rat strain since the included albino Wistar rats might be limited in their visual ability[220]. Another study observed that stress exposure facilitated reversal learning likely by inhibiting ventromedial PFC function[221] proposing the PD and reversal task as good starting point for assessing the effects of CMS in rats.

1.3.1.2 Paired-associates learning task

The PAL task for assessing cognition in humans is part of the CANTAB test battery[222]. On a computer screen, various visually equal boxes are displayed. One at a time, the boxes become temporarily invisible to reveal a unique pattern underneath the box. After the presentation phase, one pattern is displayed in the centre of the computer screen and the participant has to indicate underneath which box the equivalent pattern is hidden (Figure 6). The task evaluates spatial memory associative learning and was shown to effectively detect cognitive impairments in neuropsychiatric disorders, such as schizophrenia, autism or Alzheimer's disease[82,223]. Cognitive impairments in the PAL task appear to positively correlate with restricted daily functioning in schizophrenic patients[223]. Furthermore, discrimination between Alzheimer's disease, mild cognitive impairment and healthy controls was possible via the PAL task, whereas MDD patients and controls appeared similar in their performance in this study[224]. Similarly, unmedicated MDD patients and controls completed a comparable number of levels in the PAL task, but depressed patients completed more trials unsuccessfully on the

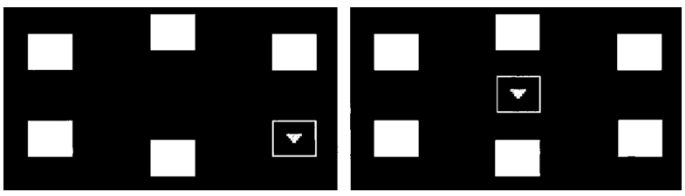

Figure 6. Paired-associates learning (PAL) task for humans. First, the different patterns are sequentially presented to the participant (left screen); then the box matching the pattern underneath with the one in the centre needs to be indicated (right screen).[82]

first presentation[225]. In elderly MDD patients, performance in the PAL task was impaired in a stimulus set size of six and higher compared to age-matched controls. PAL performance was not statistically different to controls in patients recovered from MDD (within-design)[96]. Regarding brain activity, Altered HPC activation was observed in mild cognitive impaired patients compared to healthy controls when executing the PAL task in an MRI scanner. Structural changes in the HPC, i.e. a decrease in grey matter, might be responsible for the functional deficits in this region and inferior cognitive performance[226]. Furthermore, schizophrenic patients' PAL performance correlated negatively with HPC volume loss[227]. Patients with frontal lobe and HPC lesions showed impaired performance in the PAL task indicating the functional relevance of these brain regions for the task[228].

In the PAL task for rodents, the touchscreen is covered by a mask leaving only three windows to touch the screen. The animals need to associate three symbols to three touchscreen locations, respectively (Figure 7A). During a trial, two of the three symbols are presented; one in its correct location and one in an incorrect location. The animal is required to choose the correct pairing to receive a consumable reward. Two versions of the PAL task exist for rodents. In the same PAL (sPAL) task, two identical symbols are presented in two of the three windows; whereas in the more difficult different PAL (dPAL) task, two out of the three possible symbols are shown (Figure 7B). The dPAL task is thought to be HPC-dependent as it requires association of a stimulus with a specific location on the touchscreen (similar to the object-in-place task); whereas the sPAL task is thought to be cue-driven[229]. A study by Delotterie *et al.*[230] observed that a lesion in the dorsal striatum but not in the HPC attenuated acquisition of the dPAL task, whereas only post-acquisition lesioning of the HPC debilitated cognitive performance in dPAL. They understand this as a different to the human PAL task, which requires the HPC also for task acquisition[82,226]. This is an interesting finding because classical object-location or odour-location tasks require the HPC in rodents[231]. Another study using mice showed that post-acquisition lesioning of the HPC impairs dPAL performance, but so did pre-acquisition lesioning, although to a smaller, but still significant, extent[232]. This suggests that alternative brain structures may take over during learning of object-location association in the dPAL task if the HPC is impaired. *C-fos* staining revealed increased activation of the orbitofrontal cortex, the

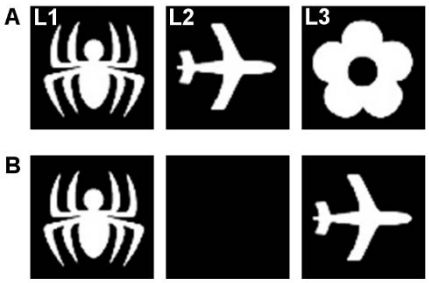

Figure 7. Paired-associates task for rodents. (A) The three symbols are displayed in their correct location (L): Spider-L1, Plane-L2, Flower-L3. (B) An example trial of the dPAL task with the spider in the correct and the plane in its incorrect location.

retrosplenial cortex and the HPC during PAL acquisition[229,233,234]. Other potential brain areas that are implicated in the dPAL task are the entorhinal cortex for encoding the spatial component and the perirhinal cortex for object recognition[235–237]. Furthermore, HPC NMDA and AMPA receptor activation is required for dPAL performance[229]. These receptors as well as PFC and HPC functioning are central in cognition, such as executive functions, learning and memory. Furthermore, PFC and HPC function and structure is altered in MDD patients as well as in the CMS model[122,210,211,238]. Accordingly, the dPAL task might be sensitive for detecting cognitive alterations in the CMS model similarly to depression-associated cognitive impairments in memory, attention and executive function[81].

1.3.2 The model organism

The term "preclinical research" encompasses a variety of model organisms, such as cells and animals. The research question and the experimental technique determine the animal model of choice. Therefore, animal models range from relatively simple organisms such as the fruit fly to more complex organisms, like non-human primates. In preclinical research, rodent models (in particular mice and rats) are frequently applied balancing translational value with time and expenditure.

Commonly, mice and rats are regarded as interchangeable animals only differing in size. However, their common ancestor existed approximately at the same time as the ancestor of macaque monkeys and great apes, which includes *homo sapiens*[239]. Therefore, genetic disparity has evolved between the two rodent species. For example, 44% of genes are differentially expressed in HPC dendrites of mouse and rat[240]. Consequently, it is unsurprising that mice and rats differ on a cellular level. For instance, rats and humans, unlike mice, highly express the 5-HT_6 receptor in the basal ganglia additionally to a much higher ligand binding affinity of the receptor[241]. The 5-HT_6 receptor appears to be involved in higher cognitive processes, like learning and memory, and the modulation of several neurotransmitter systems including glutamate, acetylcholine, dopamine, GABA, epinephrine and norepinephrine secretion. Furthermore, the 5-HT_6 receptor might affect anxiety- and depression-like behaviours[242]. Moreover, plasma of rats and humans but not mice contains BDNF[243]. Peripheral BDNF was shown to affect central cellular signalling as well as depression-related affective behaviours[244]. In summary, the closer physiological similarity of rat to human than mouse to human suggests the rat as the more translational model in cognition and depression research[239].

Rats and mice also differ in their natural behaviour. In nature, both species live in groups. However, rats are less aggressive, less territorial and male hierarchies are less absolute

compared to mice. Moreover, female group rearing in mice resulted in increased anxiety in the adult offspring, whereas anxiety was reduced in the rat setting[239,245,246]. Therefore, the rat appears to have greater social cognition than the mouse, and is therefore suggested to better model the complex social behaviour or social deficits observed in humans[239].

Finally, rats and mice appear to differ in their cognitive performance. In the Morris water maze, rats have lower intra-trial variance possibly due to applying spatial learning strategies for task acquisition, whereas mice rely partly on swimming around the outside pool area[247]. Thus, rats' representation of space appears more stable and their learning strategy of spatial tasks appears different to mice[248,249]. Generally, rats seem to acquire learning tasks faster than mice[250,251] suggesting the rat's learning ability to being more sophisticated and therefore closer to the human cognition[239]. Conclusively, the rat compared to the mouse appears to be a more relevant model for MDD-related and cognitive research.

2 AIMS

Many people suffer from MDD worldwide. However, antidepressant treatment is suboptimal, with therapeutic relief commencing only after 2–4 weeks of treatment and only 50% of patients responding to a two-step treatment regimen. Challenges in optimizing antidepressant medication result from the lack of understanding of MDD pathogenesis caused by a complex gene x environment interaction and emerging in a heterogeneity of symptoms between patients. Finally, antidepressants primarily target the affective symptoms of depression, while more and more studies demonstrate that depression-associated cognitive impairments persist after remission and decrease daily functioning as well as increase risk of relapse. Thus, the demand is high for pro-cognitive antidepressants to approach a complete remission from MDD. Essential for the development and testing of such pro-cognitive antidepressants are a better understanding of symptom aetiology and valid preclinical models for drug screening.

Therefore, this PhD project aimed to further investigate the relationship of risk factors and symptom development by applying environmental as well as genetic risk factors in MDD pathogenesis. Furthermore, we aimed to design translational studies with clinical relevance. First, this was implemented by employing a rat instead of a mouse model for preclinical BDNF research and, second, by applying the touchscreen operant platform, which is based on the CANTAB test battery for assessing cognition in humans. Finally, the aim has been to establish a preclinical platform for pro-cognitive drug screening.

Specifically, the conducted studies aimed to answer the following questions:

Study I

The preclinical touchscreen platform for testing cognitive performance is proposed to be more translational to human testing, more standardized, objective in its readout and better transferable across institutes than classical tests[214]. At present, preclinical touchscreen testing has been scarcely applied in depression- and anxiety research[6]. A caveat of the vision-based touchscreen testing is the requirement of sufficient visual accuracy, which is known to be poorer in albino strains. Furthermore, the touchscreen platform might not be sensitive enough for detecting mild cognitive impairments as consequence of stress exposure, such as deficits in the PFC[252]. We therefore compared naïve albino rats with pigmented rats in a simple pairwise discrimination and reversal task. Furthermore, pigmented rats were CMS exposed

and cognitively examined to investigate the sensitivity of the touchscreen pairwise discrimination task. We anticipated that albino rats perform poorer than pigmented rats due to impaired vision. Furthermore, it was hypothesized that stress exposure would decrease cognitive performance suggested by the detrimental effects of stress on brain structure and function[45,60,61].

a) Are albino rats (e.g. Wistar) suitable for testing cognitive performance in the vision-based touchscreen platform compared to pigmented rats (e.g. Long Evans), since albinism is associated with poorer vision?
b) Can the CMS paradigm be transferred from the established Wistar strain to the Long Evans rat strain and elicit a comparable, depressive-like phenotype?
c) Do CMS exposed rats show cognitive impairments in the touchscreen task?

Study II

In preclinical models, chronic mild stress was shown to induce anhedonia as well as cognitive impairments. However, the stress exposed group is taken synonymously as depressive-like group although it was demonstrated that a homogenous response to stress cannot be assumed[253]. Therefore, dissociation of cognitive impairments being a sign of the depressive-like state or a general consequence of stress exposure is impossible. Stress was shown to induce atrophy in the brain including cellular shrinkage, apoptosis, dendritic retraction and decreased neurogenesis, which could be the cause of depression-associated cognitive impairments. However, synaptic connectivity in the medial PFC appears to be dependent on stress responsiveness in the learned helplessness paradigm, which might suggest the linkage of cognitive impairments specifically to the depressive-like state[254]. These hypotheses are assessed by including stress exposed resilient and susceptible, i.e. anhedonic-like rats as well as non-stressed controls in the study allowing to associate cognitive impairments to stress exposure (both CMS groups) or to the depressive-like state (CMS anhedonic-like group only). Based on the literature, we predicted that both CMS groups would show cognitive alterations with the CMS anhedonic-like, i.e. stress susceptible group being more severely impaired.

a) Are cognitive impairments a consequence of general exposure to CMS or is this effect depression-specific?
b) Which cognitive domains are impaired in CMS anhedonic-like or resilient rats?

Study III

The CMS model is well evaluated and comprises good construct, face and predictive validity. However, the recently introduced pro-cognitive antidepressant vortioxetine appears ineffective in restoring the hedonic state in CMS exposed rats[133]. Furthermore, vortioxetine was proposed to have a direct pro-cognitive effect by its 5-HT_{1A}, 5-HT_{1B}, 5-HT_3 and 5-HT_7 receptor independent of restoring the affective symptoms[133,134]. Therefore, we administered vortioxetine to CMS anhedonic-like rats and monitored their hedonic state as well as tested their cognitive abilities in association with the hedonic state and examined brain gene expression. We assumed that vortioxetine rescues the hedonic state in a proportion of rats possibly similar to escitalopram treatment[122]. Furthermore, we predicted that anhedonia-associated cognitive impairments would renormalize in all treated rats due to the direct pro-cognitive efficacy. Finally, we anticipated that expression of genes relevant in the stress response and psychiatric disease would be similar in the non-stressed control group and in rats responding well to vortioxetine. Genes involved in the neuronal growth and structure were expected to be higher expressed in treated than non-treated anhedonic-like rats.

a) Can the antidepressant vortioxetine rescue the hedonic state in CMS exposed rats?
b) Can vortioxetine improve cognition in depressive-like rats?
c) Is the pro-cognitive effect of vortioxetine dependent on the affective state?
d) Do hedonic state or treatment alter expression of brain genes relevant for stress response, psychiatric disorders and neuronal structure?

Study IV

BDNF is strongly suggested to be involved in the stress response, MDD pathology and recovery. However, preclinical studies in mice yielded contradictory results. This can possibly be explained by humans but not mice producing peripheral BDNF[243], which was shown to alter brain gene expression and behaviour[244]. Rats, like humans, produce peripheral BDNF and, therefore, we used the recently introduced $BDNF^{+/-}$ rat to evaluate the effect of lower BDNF levels on affective-like behaviours, cognitive performance and brain gene expression. We predicted that $BDNF^{+/-}$ rats would display increased depressive- and/or anxiety-like behaviours, lower cognitive abilities and altered gene expression relevant in psychiatric diseases and the stress response.

a) Do reduced BDNF levels induce pathologic behaviour, such as depression, anxiety or cognitive impairments?

b) Do reduced BDNF levels alter expression of genes relevant for psychiatric disorders and stress response?

3 METHODS

3.1 Summary of methods

A short overview of the methods employed in each study is provided in the following sections. Detailed material and methods (including *n*-numbers throughout the experiment) can be found in the respective manuscripts.

3.1.1 Study I

In our facility in Aarhus, the CMS paradigm has been established using Wistar albino rats. However, albinism is associated with poor vision and might interfere with cognitive testing in the vision-based touchscreen tasks. The impact of rat strain on touchscreen testing was determined by comparing the performance of naïve Wistar ($n = 12$) and pigmented Long Evans (LE; $n = 12$) rats in the pairwise discrimination (PD) and reversal learning task. Reversal learning was re-tested for two sessions following a 10-day hiatus to assess long-term memory. In addition, we explored if LE rats exhibit a phenotype similar to Wistar rats when subjected to CMS. The effect of stress on the LE rats ($n = 16$) was evaluated with the sucrose consumption test (SCT) assessing anhedonia. At the end of the stress paradigm; anxiety-related behaviour was assessed in the elevated-plus maze (EPM) and open field test (OF) and spatial working memory in the spontaneous alternation behaviour (SAB) test. Finally, the effect of CMS on cognition was investigated by contrasting the performance of control and CMS exposed LE rats in the PD and reversal task including the retention phase. The experimental timeline of study I is provided in Figure 8.

Figure 8. Experimental pipeline study I. After acclimatization to the facility, rats were habituated to the sucrose consumption test (SCT). After acquisition of baseline sucrose consumption, the chronic mild stress model (CMS) was initiated and SCTs were carried out throughout CMS exposure. In the final week of CMS, behaviour was assessed in the elevated-plus maze (EPM), open field (OF) and spontaneous alternation behaviour (SAB) task. Subsequently, rats were gradually food deprived for ensuing touchscreen pre-training, pairwise discrimination (PD) and reversal task acquisition. Finally, rats were retested on the PD reversal task following a 10-day hiatus without touchscreen training.

3.1.2 Study II

Based on the findings in study I, LE rats were used for the CMS paradigm and subsequent touchscreen testing. Here, the strongest phenotypes of the CMS exposed rats, defined by an *a priori* criterion for the SCT[201], were employed, i.e. CMS resilient (n = 11) and susceptible (anhedonic-like; n = 10) rats; as well as non-stressed controls (n = 11). Consequently, we could identify if CMS-induced cognitive impairments are specific to the depressive-like phenotype or caused by general stress exposure. Furthermore, the different paired-associates learning (dPAL) task was applied instead of the PD and reversal task because the latter appeared to be too simple for LE rats. A cognitively demanding task is more likely to reveal impairments, which may go undetected in less challenging tests[255]. Next to being more complex, the dPAL task involves HPC function. Alterations in the HPC are central in MDD pathology and, moreover, the memory component of study I's PD task appeared to be the only part that might have been affected by CMS. A modified (over-night) CMS protocol was applied to accommodate for touchscreen pre-training and testing during the day. A two-session-lasting retention of the dPAL task was added 10 days after touchscreen acquisition. The experimental timeline of study II is provided in Figure 9.

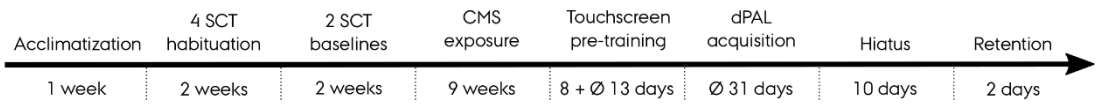

Figure 9. Experimental pipeline study II. After acclimatization to the facility, rats were habituated to the sucrose consumption test (SCT). After acquisition of baseline sucrose consumption, the chronic mild stress model (CMS) was initiated and SCTs were carried out throughout CMS exposure. Subsequently, rats were gradually food deprived for ensuing touchscreen pre-training and different paired-associates learning (dPAL) task acquisition. Finally, rats were retested on the dPAL task for 2 days following a 10-day hiatus without touchscreen training. A modified, over-night CMS protocol was applied throughout food deprivation and touchscreen testing.

3.1.3 Study III

The efficacy of vortioxetine as antidepressant with pro-cognitive efficacy was tested in the CMS paradigm by administering vortioxetine incorporated in rat chow to CMS anhedonic-like rats five weeks after CMS initiation. Treatment was continued throughout the following four weeks of CMS and throughout touchscreen pre-training and dPAL testing. Similar to study II, a modified CMS protocol was applied during touchscreen assessment. Bacon instead of sugar-coated pellets were used as reward for touchscreen operant conditioning allowing for continued SCT testing throughout touchscreen assessment. Study III included four groups:

non-stressed controls (n = 10); anhedonic-like, untreated rats (n = 10); anhedonic-like rats responding well to vortioxetine treatment and thus restored their hedonic state (responders; n = 10); and anhedonic-like rats responding low to vortioxetine treatment and thus remained anhedonic-like (low-responders; n = 10) as determined by the SCT. Cognitive performance of rats was assessed in the dPAL task. Again, a retention phase of the dPAL task was added ten days following acquisition of the task for testing long-term memory. Finally, animals were culled 1–3 days after retention and PFC and HPC gene expression was examined with real-time qPCR. Genes involved in neuropsychiatric disorders, stress response and neuronal plasticity were included in the analysis. The experimental timeline of study III is provided in Figure 10.

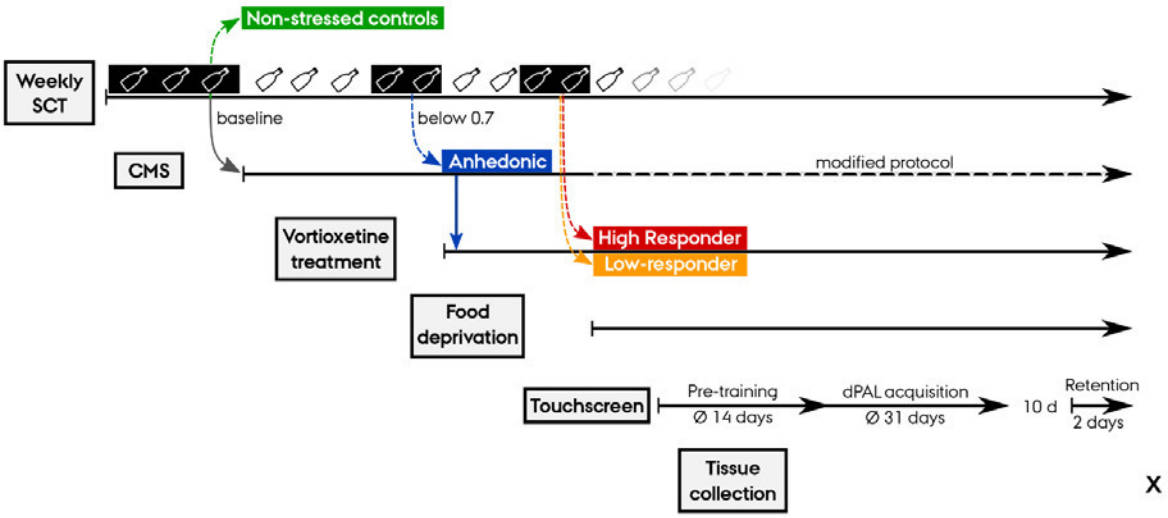

Figure 10. Experimental pipeline study III. After acclimatization to the facility, rats were habituated to the sucrose consumption test (SCT). After acquisition of baseline sucrose consumption, the chronic mild stress model (CMS) was initiated and SCTs were carried out throughout the experiment. Rats, that decreased their sucrose intake to 70% or less of their baseline, were defined anhedonic-like based on an *a prior* criterion. Two-thirds of the anhedonic-like rats were subjected to treatment with the antidepressant vortioxetine. The 30% of highest and lowest responder to treatment, determined by the SCT were subjected to pre-training, different-paired associates (dPAL) acquisition and retention together with non-treated anhedonic-like rats and controls. Touchscreen training was discontinued for 10 days after the dPAL task was acquired. Brain tissue was collected 1–3 days following dPAL retention.

3.1.4 Study IV

Stressed and depressed subjects show reduced BDNF levels, BDNF is required for antidepressant treatment efficacy and also increases in response to antidepressant treatment. To evaluate the role of BDNF in MDD pathogenesis, we used rats heterozygous for the BDNF gene ($BDNF^{+/-}$; n = 13) and WT controls (n = 14). Depression- and anxiety-related behaviour

was assessed with the sucrose preference test (SPT), novelty induced hypophagia (NIH), forced-swim test (FST), OF and EPM test. Working and spatial memory were evaluated with the SAB and Morris water maze (MWM; WT: $n = 5$; BDNF$^{+/-}$: $n = 10$) test. In naïve rats (WT: $n = 9$; BDNF$^{+/-}$: $n = 10$), PFC and HPC expression levels of genes related to neuropsychiatric disorders and stress response were investigated. The experimental timeline of study IV is provided in Figure 11.

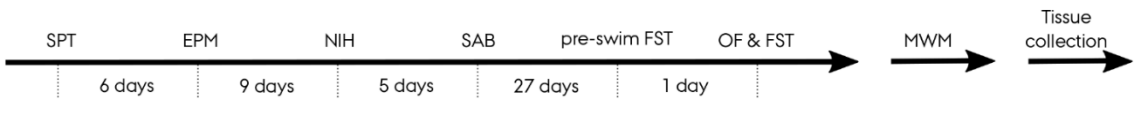

Figure 11. Experimental pipeline study IV. Three different cohorts of rats were used as indicated by the three arrows. The presented number of days illustrate how much time passed on average between the tasks.
SPT – Sucrose preference test; EPM – Elevated-plus maze; NIH – Novelty-induced hypophagia; SAB – Spontaneous alternation behaviour in the Y-maze; FST – Forced-swim test; OF – Open field; MWM – Morris water maze.

3.2 Critical evaluation of key methods

In the following sections, the most important methods and models are critically presented to allow accurate interpretation of the results.

3.2.1 Chronic mild stress model

The chronic mild stress model was presented in detail in section 1.2.3.4. In short, the CMS paradigm combines a model with good construct, aetiological, face and predictive validity and was declared as one of the most realistic models for mimicking depression in rodents[132,169,201]. A spectrum of phenotypes regarding the hedonic state follows CMS exposure. The strongest phenotypes display either the core symptom anhedonia (approximately 40%), or remain hedonic, thus, are resilient (approximately 20%)[201] allowing for separation of stress- or depression-related alterations. This is similar to humans in whom only a fraction of individuals develop depression after prolonged periods of stress[256]. Moreover, anhedonic-like animals respond to chronic but not acute antidepressant treatment and not all animals respond equally well, similar to the situation in humans. Limitations of the CMS model are its labour-intensive modelling and the high number of animals needed to obtain a sufficient amount of anhedonic-like animals after CMS exposure. Furthermore, if the CMS is discontinued animals recover after 4–5 weeks[201], which can be used to model the episodic nature of MDD but simultaneously

implies that subsequent experimental steps need to be carried out in close proximity to cessation of the CMS paradigm.

3.2.2 BDNF model

As discussed in 1.1.1, BDNF polymorphisms (e.g. Val66Met) reduce secretion of activity-dependent BDNF and predispose individuals to MDD pathology[29]. Furthermore, sufficiently high BDNF levels are required for antidepressant drugs to work efficiently[161–163]. Thus, genetically-induced lower levels of BDNF could be a valid model for affective disorders. However, studies including mice heterozygous for BDNF produced inconsistent results regarding their phenotype[257]. This might be explained by behavioural tests being designed for rats and not mice[132], and that rats, like humans, produce peripheral BDNF[243], which can influence behaviour[244]. Additionally, rats display a greater behavioural repertoire and are therefore more translational to humans than mice. Thus, $BDNF^{+/-}$ rats might be a better and more dependable model than mice for translational research. A limitation of genetic models is that only one protein is altered to provoke the phenotype, which is unlikely to mimic the complex disease aetiology in humans[132]. However, it allows us to unravel the relationship of decreased BDNF levels and the emerging phenotype.

3.2.3 Touchscreen operant platform

The touchscreen operant platform and the two tests employed in this PhD project were presented in detail in section 1.3.1. The touchscreen tasks are proclaimed to have good face validity since rodent tasks were developed to closely resemble the human tasks. However, clinical and preclinical tests are not identical. First, task rules can be explained to humans but not rodents. Therefore, rodents have to learn the tasks incrementally possibly requiring different brain areas to humans. Secondly, animals are motivated to learn the task by food reinforcement. This likely requires other brain areas, such as the striatum, to be also activated during task acquisition distinguising neuronal correlates of human and animal PAL learning[258]. Thus, although differences in construct validity of the task exist, the medial PFC as well as the HPC and dorsal HPC AMPA- and NMDA-receptor activation are required across species to successfully acquire and maintain PAL performance[224,226–229,258]. Technical differences across species could be resolved in the future by adjusting the human version of the task, i.e. including non-communication of task rules[213] and reward. Generally, it was shown that comparable brain areas are involved in preclinical and clinical touchscreen tasks assessing cognition and motivation[213,259,260]. Regarding predictive validity, few studies tested treatment in preclinical models using the touchscreen platform. An example is the study by Romberg *et al.*[261], which

demonstrated that the symptomatic Alzheimer's drug donepezil rescued accuracy in a mouse model of Alzheimer's disease (3xTgAD). More studies are required to generalize the predictive validity of touchscreen testing in disease models.

Practical aspects of the touchscreen operant platform should be considered besides its validity. Although animals can theoretically be tested in a battery of touchscreen tests, tasks can interfere with each other and prevent successful acquisition of the subsequent test[262]. Furthermore, some touchscreen tasks take longer to acquire than others and during a battery of tests, animals might go through adolescence, adulthood and old age, which likely influences cognitive performance. Moreover, touchscreen testing requires pre-training for the animals to acquire the operant concept of the touchscreen testing, which adds to the labour-intensive work and, specifically in this project, to a longer period between the original CMS protocol and task acquisition. Sugar-coated pellets are used as reward for operant conditioning. However, the high-sugar content of the reward pellets interfered with the low-sugar content of the sucrose solution (1.5%) used for the SCT. Therefore, the hedonic state could not be monitored during touchscreen testing. We overcame this problem in study III by using bacon instead of sugar pellets, which also led to successful operant learning in the dPAL task. Although the touchscreen testing is described as appetitive learning, food restriction and the novel touchscreen environment might be stressful for the animals (this issue is examined in study III). Finally, although Bussey *et al.*[263] found that albino as well as pigmented rats can be equally used for the vision-based touchscreen tasks, another study by Kumar *et al.*[220] suggested that albino rats are restricted in their vision and, thus, show a lower performance or fail to acquire certain tasks. Hence, touchscreen testing might be limited to pigmented rodent strains, particularly in more visual demanding tasks.

3.2.4 Classical behavioural tests

Classical behavioural tests were used in study I - IV assessing depression- or anxiety-like behaviour and cognition.

3.2.4.1 Sucrose consumption or preference test

Sucrose consumption is often used to evaluate the hedonic state in the CMS model. Willner and colleagues[70] have extensively evaluated the SCT in the context of the CMS model and shown that (1) sucrose solution but not total fluid intake is altered in stressed animals; (2) the test is not related to caloric intake of sucrose[73]; (3) results are not due to a global decrease in energy intake[70,168]; (4) food deprivation augments the difference in sucrose consumption between controls and stressed animals, but the difference in sucrose intake was also observed

in non-food-deprived animals only to a smaller extent[70]; (5) SCT is supported by other tests sensitive to rewarding properties, such as the food place conditioning paradigm[70,71]; and (6) isolated versus paired housing leads to similar results in the SCT tests, but paired housing might delay the effect onset of CMS[70]. An advantage of the SPT to the SCT is that the sucrose consumption can be compared to the animal's water consumption and, thus, is an internal control of a general change in fluid intake but also entails more labour in preparing two instead of one bottle for each animal. Thus, the SCT was applied in study I-III with an *a priori* criteria defining anhedonia (SCT index[†] ≤ 0.7) and resilience (SCT index ≥ 0.9)[201] and the SPT in study IV.

A caveat of the sucrose test is the requirement for single-housing if the hedonic state of each animal needs to be determined. Since animals are commonly single-housed in the CMS paradigm, this was no constriction for study I-III, but it may have induced a stressful element in animals that are usually group-housed (study IV). Overall, the SCT and SPT are non-invasive, easy to execute and appear to be a reliable measure of anhedonia.

3.2.4.2 Elevated plus-maze

The EPM is commonly used to assess anxiety behaviour in rodents. The fear of the open and lit area of the open arms of the EPM competes with the explorative drive within an animal. Thus, more time spent on the open arms relates to anxiolytic behaviour. External settings, such as light intensity can influence the time spent or percent distance travelled in the open arm, i.e. if the light settings are too bright, the time spent in the open arms might be too small to observe an effect between groups.

3.2.4.3 Novelty-induced hypophagia

The animal is introduced to a novel environment and presented with a familiar food reward, for example a chocolate chip. A longer time taken to consume the food reward relates to the depressive-like state of the animal. However, it is difficult to ascertain if this behaviour results from increased anxiety in a novel environment or from a reduced motivation to consume the reward[264]. Still, this test discloses depression-related behaviour and is an easy to execute supplement to other tests, such as the EPM and SPT.

[†] SCT index = respective sucrose intake during CMS/ baseline sucrose intake prior to CMS

3.2.4.4 Open Field

Animals are allowed to explore an open area (round or square) to assess anxiety as well as locomotor activity. Animals that are more anxious spend more time in the peripheral zone than in the open area of the centre. The test is also used to assess locomotor activity. If differences in locomotor activity exist, percent distance travelled in zones should be analysed additionally to time spent in a zone.

3.2.4.5 Forced swim test

The FST is commonly used to assess behavioural despair and is primarily used for testing antidepressant drug efficacy. The concept behind the test is that a rodent placed in a cylinder filled with water (24 ± 1 °C) will try to escape but eventually accepts the unavoidable situation. A longer time of passively coping with the situation, i.e. floating immobile in the water is an index of behavioural despair; whereas active escape attempts demonstrate non-depressive behaviour. Although the FST is often used in preclinical depression research, the test is also under critic. Critical points are that the test is sensitive not only to chronic but also to acute antidepressant treatment, which is unlike the human situation.[265] Moreover, swimming behaviour can be co-founded by differences in locomotor activity, which need to be assessed in another test, for example the OF[265]. Furthermore, the test is highly stressful for the animals and can interfere with subsequent tests. Therefore, the FST was placed last in the behavioural test battery of study IV.

3.2.4.6 Spontaneous alternation behaviour

Spatial working memory can be assessed with the SAB test. It is based on the assumption that rodents prefer to explore a novel over a familiar area. If the previously visited arms are remembered well, the animal should sequentially alternate between the three arms of a Y-maze. However, if an animal shows a generally low number of arm visits, the calculated alternation ratio (sequential arm visits/(arm entries-2)) can be distorted. Thus, animals with a low number of arm entries might be better excluded from the analysis[134,266].

3.2.5 Drug treatment

Vortioxetine is a relatively new drug on the market and falls under the category of "other antidepressants". Several preclinical and clinical studies have shown the antidepressant efficacy of vortioxetine (see section 1.1.4.1). Furthermore, a direct pro-cognitive effect was ascribed to this drug due to its multimodal mechanism of action. The beneficial effect of vortioxetine was observed in the domains of memory, executive function and attention and

included improvements in tests relevant for the dPAL task, such as object placement test and object recognition[144–146].

In study III, vortioxetine was given mixed into rat chow. This oral administration of the drug is similar to oral intake of medication in humans and, thus, increases comparability between preclinical and clinical studies. Although preclinical and clinical studies provide similar results regarding vortioxetine, it should be noted that the binding affinity for vortioxetine in the rat is different to humans. The 5-HT_{1A} and 5-HT_7 receptors in the rat have an affinity of only 7–10% that of humans for vortioxetine[267]. Furthermore, the elimination half-life of vortioxetine in the rat is considerably shorter than in humans: 2.9–3.9 h in rats and 57 h in humans[268]. Finally, the bioavailability differs between species with 10% in rats and 75% in humans[268].

3.2.6 Real-time qPCR

Real-time qPCR is a highly sensitive method and used for assessing mRNA expression levels of genes. The extracted mRNA is reverse transcribed to complementary DNA (cDNA). From the cDNA, the target gene is amplified to which the TaqMan probe then binds. The binding of the TaqMan probe results in the release of a fluorophore, which fluoresces. The fluorescence intensity is monitored and detected once it is greater than the background fluorescence and marked by the cycle threshold (C_t). The Ct value is used to compute the relative concentration of target gene in each sample using a standard curve. Pivotal is the normalization of target genes to reference genes. The latter should be present in a consistent amount throughout the sample tissue and thus allow for corrections of RNA integrity, quantity of tissue or experimental treatment[269]. Incorrect normalisation might give inaccurate data across all target genes. Although gene expression allows for assessment of gene transcription, mRNA levels might not translate consistently to resulting protein levels due to posttranscriptional and posttranslational modifications but show in which tissue or region gene expression is altered.

4 RESULTS

In the following, key results of the four studies are presented briefly. Detailed results, including statistics, can be found in the four manuscripts, respectively.

4.1 Strain and stress (Study I)

Study I aimed to answer if LE rats respond similarly to CMS exposure as Wistar rats; the suitability of albino Wistar rats in the vision-based touchscreen tasks compared to pigmented LE rats; and, finally, the impact of CMS exposure on the rats' cognition in translational touchscreen tests.

We found that LE rats react similarly to the CMS paradigm as Wistar rats did. Some rats reduced their sucrose intake, thus became anhedonic-like, whereas others remained resilient. Furthermore, CMS increased the time that rats spent in the open arms in the EPM and increased the number of central crossings in the OF demonstrating anxiolytic behaviour of CMS exposed rats. Working memory of the CMS group was indifferent to controls assessed in the SAB test. Thus, the CMS paradigm is applicable in albino and pigmented outbred rat strains once it is established in a facility.

Next, the cognitive performances of Wistar and LE controls were compared in the PD and reversal task and in the additional retention task. Wistar rats displayed inferior task performance in number of sessions to acquire the PD (Figure 12A) and PD reversal task (Figure 12B), which was supplemented by a shallower learning curve in both tasks (Figure 12C–D). Furthermore, accuracy in the first retention session was lower in Wistar compared to LE controls (Figure 12E), but memory or relearning performance was similar between the two rat strains (Figure 12F). Thus in all three task components, Wistar rats performed poorer than LE rats.

The impact of stress exposure was evaluated by comparing CMS exposed LE rats to LE controls. Stress did not impact PD and reversal learning (Figure 12A–D). However, CMS exposed rats were the only group that decreased their average accuracy below criterion on retention session one (Figure 12E). Although not significant, memory performance was lower in CMS rats compared to LE controls, whereas a trend of increased relearning performance was observed between the two LE groups (Figure 12F).

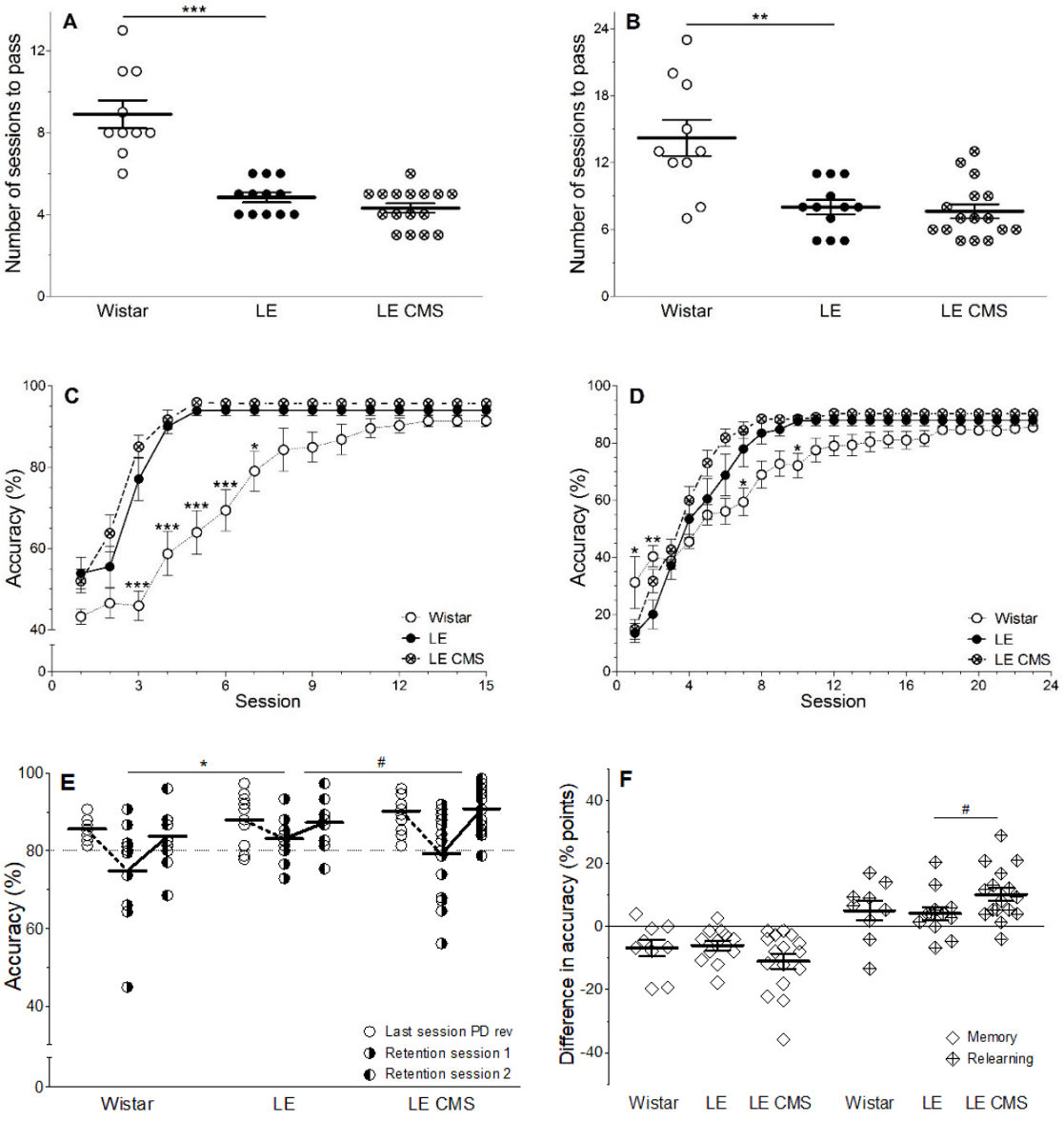

Figure 12. Pairwise discrimination (PD) and reversal touchscreen task. Strain comparison (Wistar versus Long Evans (LE) rats) as well as stress effects (LE controls versus chronic mild stress (CMS) LE) are illustrated. Number of session required to learn the **(A)** PD task and **(B)** PD reversal task. Learning curves of acquiring the **(C)** PD and **(D)** PD reversal task. **(E)** Percent correct answers of the final PD reversal session and the two retention sessions. Passing criterion of 80% accuracy is indicated with dotted line. **(F)** Difference in percent correct of the final PD reversal and first retention session (memory); and first and second retention session (relearning). Individual data points as well as group mean (± SEM) are displayed. Post-hoc comparisons are indicated with ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, #$p < 0.06$.

4.2 Resilient and susceptible (Study II)

In study II, we were interested in distinguishing general effects of stress exposure and specific effects of depression on cognitive performance. Thus, the performance of CMS exposed anhedonic-like and resilient rats was compared. In this study, the touchscreen dPAL task was applied, which involves HPC function. Task selection was based on the findings of study I, in which stress did not affect task acquisition but impacted memory (trend), a cognitive function in which the HPC is centrally involved.

First, SCTs revealed a segregation of CMS exposed anhedonic-like rats compared to both CMS resilient rats and non-stressed controls (Figure 13A). Furthermore, anhedonic-like rats needed on average more trials to acquire the dPAL task compared to non-stress controls, whereas resilient rats performed comparable to the control group indicated by a trend in the one-way ANOVA (Figure 13B). Using effect size (0.415)[‡] and sample size calculation[§], data

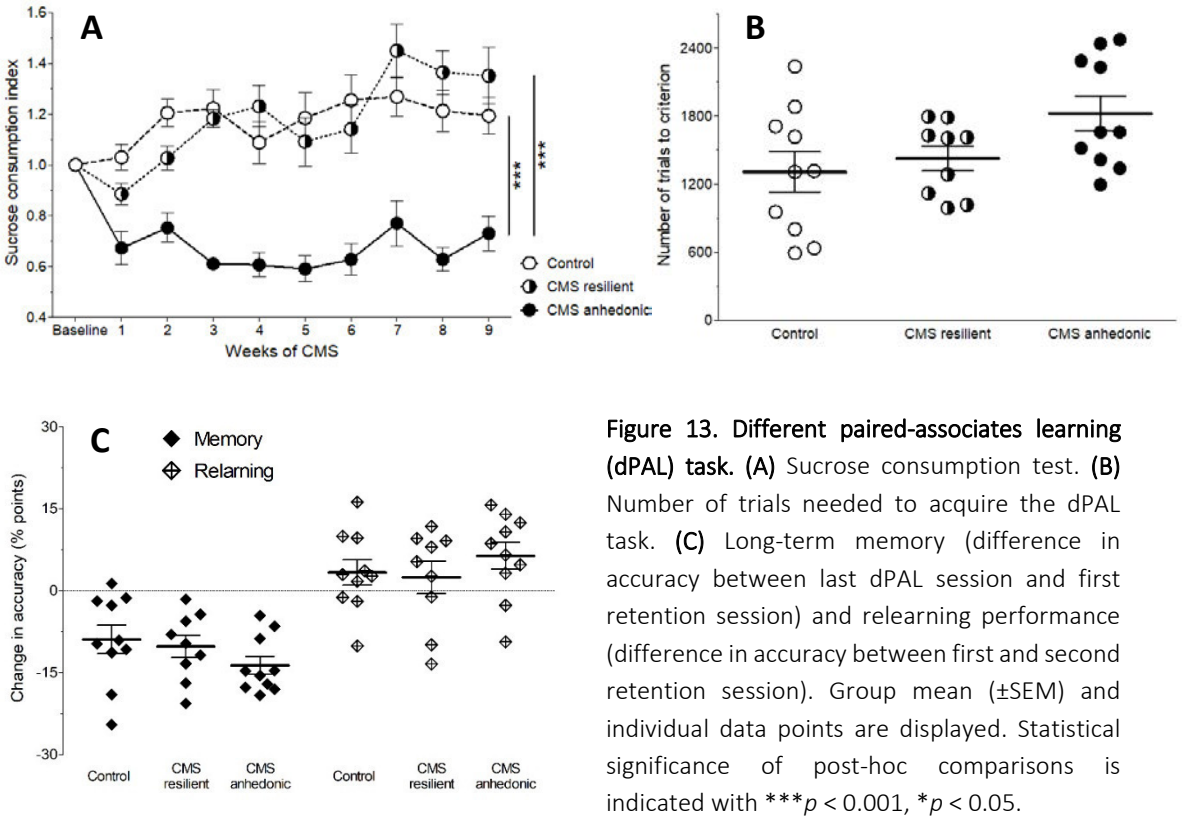

Figure 13. Different paired-associates learning (dPAL) task. (A) Sucrose consumption test. **(B)** Number of trials needed to acquire the dPAL task. **(C)** Long-term memory (difference in accuracy between last dPAL session and first retention session) and relearning performance (difference in accuracy between first and second retention session). Group mean (±SEM) and individual data points are displayed. Statistical significance of post-hoc comparisons is indicated with ***$p < 0.001$, *$p < 0.05$.

[‡] https://www.psychometrica.de/effect_size.html#fvalue: effect size using ANOVA (calculation 7)

[§] https://www.anzmtg.org/stats/PowerCalculator/PowerANOVA: power = 0.8, α = 0.05

from this study indicates $n = 20$ per group would have been required to show significance. Thus, anhedonic-like but not resilient rats' performance was seemingly poorer in the dPAL task compared to non-stressed controls. Furthermore, resilient rats showed different cognitive alterations in the dPAL task, such as an increased number of redundant screen touches, i.e. impaired response inhibition. Moreover, CMS anhedonic-like rats showed a non-significant trend for lower performance than controls and resilient rats regarding long-term memory (Figure 13C). Overall, we showed that stress affected cognitive function, however only the depressive-like group showed inferior cognitive performance.

4.2.1 Statistical rational

CMS exposed animals were divided into subgroups depending on their change in their baseline sucrose intake in response to stress. Commonly, the extremes to both ends are taken to investigate stress-susceptibility and stress-resilience[201]. In Figure 14, the whole spectrum of changes in sucrose intake in CMS animals is shown ($n = 148$), which appears normal distributed. However, Shapiro-Wilk test indicates deviation from normal distribution ($p = 0.004$). Considerations are given to two different ways to analyse this data. First, the extremes of both ends could be used to study possibly different behavioural and cellular mechanisms of resilient and susceptible animals. This approach has been previously used in the CMS[201,205,207] as well as in other stress models[193]. A disadvantage of only analysing the extremes is that the intermediate phenotypes are discarded, which increases the number of animals required for the experiment. Alternatively, all animals could be included in the data evaluation performing a regression analysis. However, it is possible that the dependent variable is discontinuous. Consequently, difficulties in fitting a linear regression could arise, especially if the

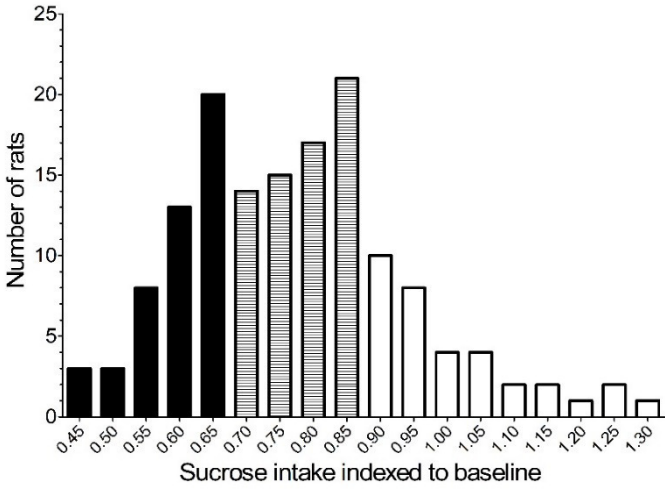

Figure 14. Sucrose intake of chronic mild stress (CMS) exposed rats. The average sucrose intake of CMS week eight and nine indexed to baseline is displayed as frequency distribution. Rats below an index of 0.7 are categorized anhedonic-like and above an index of 0.9 as resilient according to an *a priori* criterion.[204]

intermediate group's readout is similar to one of the extreme phenotypes and more complex statistics needs to be applied.

4.3 Pharmacological intervention (Study III)

In study III, we aimed to investigate the efficacy of vortioxetine in the CMS model. Particularly, we asked if vortioxetine could restore the hedonic state in the anhedonic-like rats, normalize depression-associated cognitive impairments and we wanted to determine if any pro-cognitive effects were dependent on concomitant restoration of the hedonic sate in response to vortioxetine. Finally, expression of genes relevant in neuropsychiatric disorders, the stress response and neuronal growth were measured in brain regions involved in depression and cognitive processing, the PFC, and the dorsal and ventral HPC.

SCTs revealed that vortioxetine restores the hedonic state in a proportion of anhedonic-like rats whereas others responded only marginally (Figure 15). Different to study II, anhedonic-like rats did not take longer to acquire the dPAL task than control rats (Figure 16A). However, in the control and both treated groups only one rat per group failed to acquire the dPAL task, whereas in the anhedonic-like group three rats failed task acquisition and thus, were not included in the primary readout of number of trials to pass (Figure 16A). Control rats had a longer response latency (Figure 16B), which may resemble enhanced evaluation of the stimuli before making a choice. Vortioxetine treated rats displayed an increase in the number of redundant screen touches and a greater number of correction trials, suggesting increased spontaneous, stereotypic behaviour after vortioxetine treatment (Figure 16C–D).

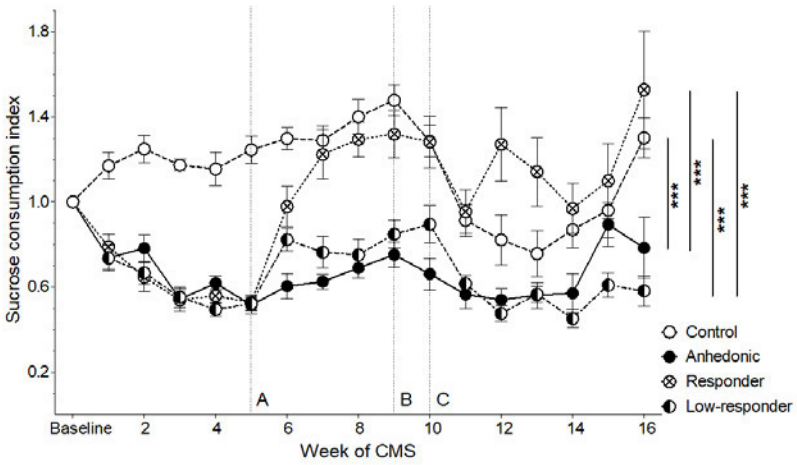

Figure 15. Sucrose consumption test. The sucrose index (respective SCT / sucrose baseline) is displayed as group mean (±SEM). Treatment start is indicated with A, food reduction commenced at time point B and touchscreen pre-training and training was initiated at C. Post-hoc statistical significance between groups is indicated with ***$p < 0.001$.

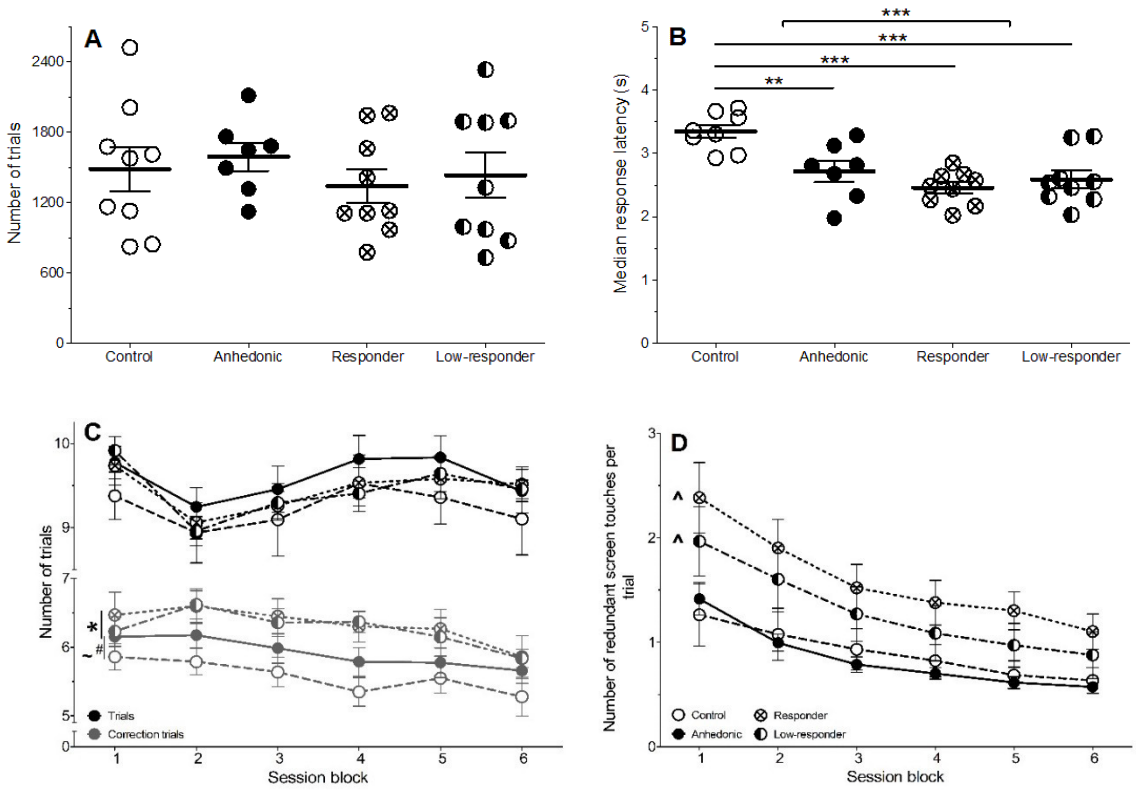

Figure 16. Different paired-associates learning (dPAL) task. (A) Number of trials required to learn dPAL (one rat per group, but three rats of the anhedonic group had to be excluded due to failure of acquiring dPAL within 46 sessions). **(B)** Median latency to respond to stimuli on the screen. **(C)** Within session learning curve showing the mean number of trials and correction trials per session block for each group. **(D)** Learning curve showing the mean number of redundant touches per trial. In (A–B) individual data points are shown. In all graphs, group means (±SEM) are displayed and statistical significance of Bonferroni corrected post-hoc comparisons is indicated with ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, #$p < 0.07$, '~' indicates a significant difference of the respective group to the vortioxetine treated groups and '^' to the untreated control and anhedonic-like group.

Unexpectedly, memory performance seems impaired in vortioxetine responders and hedonic state affected memory performance as well (Figure 17). Although not significant, the anhedonic-like rats were the only group not to improve performance on the second day of retention, i.e. during the relearning step (Figure 17). Impaired memory in the responder group can potentially be associated with overexpression of *Gsk3b*, which is involved in spatial memory and usually reduced after antidepressant treatment, in the dorsal HPC of vortioxetine responders. Furthermore, vortioxetine treatment tended to increase *Bdnf* levels in the dorsal HPC, to decrease *Homer3* in the ventral HPC and to decrease GR in the PFC. The anhedonic state was associated with increased expression of *Cofilin 1*, participating in neuronal growth, in the PFC; with lower *Homer*2 levels, a dendritic protein, and with a trend of decreased transcription factor MR expression in the dorsal HPC. Finally, *Cofilin 1* was higher expressed

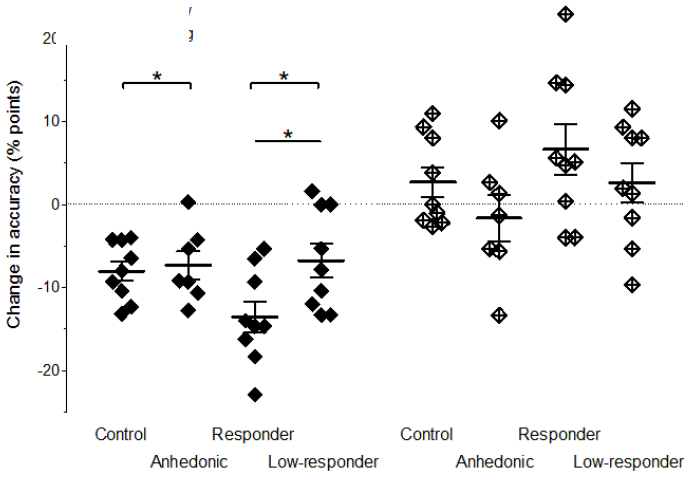

Figure 17. Retention of different paired-associates learning (dPAL) task. Acquisition of dPAL was followed by a 10-day hiatus and two days of dPAL retention. Memory resembles the difference of percent correct answers of the last dPAL before the hiatus to the first retention session. Relearning is the difference in percent correct answers of the first to the second retention session. Individual data points and group means (±SEM) are shown. Statistical significance of post-hoc comparisons and main effect treatment (angular brackets) is indicated with $^{**}p < 0.01$, $^{*}p < 0.05$.

in anhedonic-like rats compared to controls in the PFC. Thus, expression of genes involved in stress response, affective disorders and neuronal plasticity were altered in response to vortioxetine as well as the hedonic state. Overall, this study showed that vortioxetine was successful in restoring the hedonic state in the CMS model, but also induced a shift from cognitive demanding appraisal to stereotypic, habit-like behaviour. Behavioural changes were associated by alterations in gene expression due to treatment and hedonic state.

4.3.1 Statistical rational

We were interested to investigate the relationship of the affective symptom "anhedonia" and cognitive performance. Therefore, rats were grouped into responding low or well to vortioxetine ($n = 34$) by their respective change in sucrose intake (Figure 18) and the 30% of highest and lowest responder were subjected to be tested in the dPAL task.

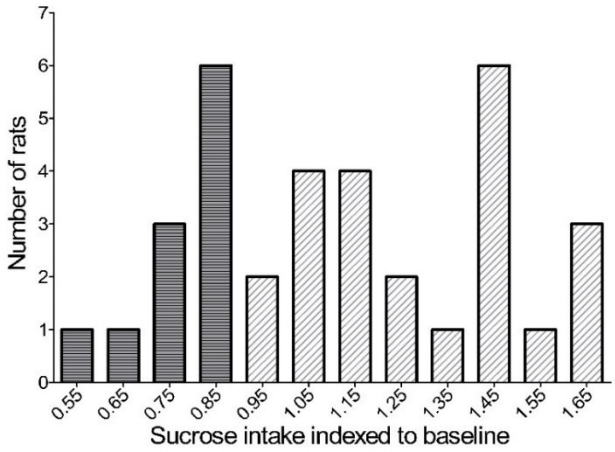

Figure 18. Sucrose intake of chronic mild stress (CMS) anhedonic-like vortioxetine treated rats. The average sucrose intake of CMS week eight and nine, i.e. week three and four of treatment is displayed as frequency distribution. Rats above an index of 0.9 are regarded as recovered from anhedonia.

Performance in the dPAL task as well as gene expression levels were analysed by two-way ANOVA to investigate if the respective changes were caused by treatment (responder and low-responder versus control and non-treated anhedonic-like rats) or by the hedonic state (control and responder versus anhedonic-like rats and low-responder). This was important to investigate since vortioxetine treatment is proclaimed to have a pro-cognitive effect independent of recovering the affective symptoms of depression[133,134].

However, the data appears not to be bimodal distributed and Shapiro-Wilk test does not indicate a significant deviation from normality ($p = 0.164$). Therefore, a regression analysis within the treatment group might be more appropriate. Consequently, a one-way ANOVA would be applied to investigate difference in performance between controls, anhedonic-like rats and vortioxetine treated rats (responders and low-responders). Additionally, a regression analysis is performed, which is exemplified here for number of trials to criterion and the mean sucrose index of treatment week two to four (i.e. CMS week seven to nine). No relationship between number of trials to reach criterion and SCT index was found within the combined treatment group ($R^2 = 0.03$, $p = 0.495$, Figure 19) after testing normality of residuals.

In theory, there should be no difference regarding the question answered by the two analysis. However, problems with regression analysis can arise when the dependent variable is discontinuous as described in 4.2.1.

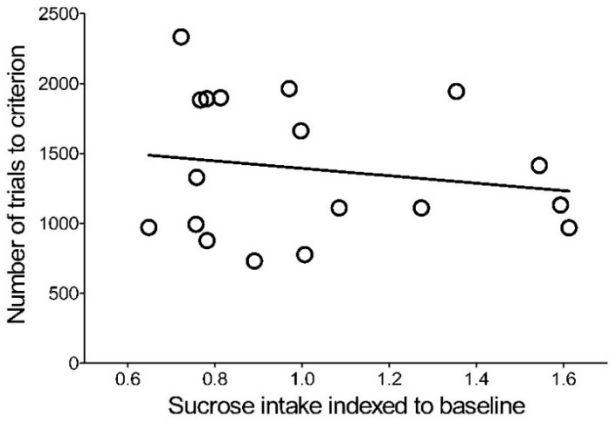

Figure 19. Linear regression of number of trials to criterion and sucrose consumption index. Given are the data points for all vortioxetine treated rats (responder and low-responder) and the sucrose intake as average of treatment week two to four.

4.4 Anhedonia and cognitive performance (Study II and III)

CMS anhedonic-like and non-stressed control rats were pooled from study II and III to increase the *n*-number per group, and thus statistical power[**]. Here, results of statistical analysis are provided since they cannot be found in any of the manuscripts.

Anhedonic-like rats tend to require more trials to acquire the dPAL task than non-stressed control rats ($t(34) = 4.10$, $p = 0.0507$; Figure 20A). Effect size[††] (Hedges' $g = 0.68$) and sample size calculations[‡‡] from the current data suggest $n = 36$ would have been required to show significance. Furthermore, anhedonic-like rats also needed more correction trials for task acquisition compared to controls ($t(34) = 4.56$, $p = 0.040$; Figure 20B). These impairments in the anhedonic-like group were not due to motivation since the time to collect the reward was not statistically significant between groups (Figure 20C). However, anhedonic-like rats showed increased spontaneous and, thus, less appraised behaviour demonstrated by the shortened median response latency in this group ($t(34) = 6.69$, $p = 0.014$; Figure 20D) and increased number of redundant screen touches per trial ($t(32) = 9.56$, $p = 0.004$; Figure 20E). The highest number of correct trials executed in a row per session was not statistically significant between controls and anhedonic-like rats. Memory and relearning performance did not differ between the groups. Thus, the pooled dPAL data confirms that cognitive impairments are present during task acquisition in anhedonic-like rats whereas memory is spared.

[**] Summary statistics were performed on animals that acquired the task and outliers (Grubbs' test, $\alpha = 0.05$ and ROUT test, Q = 1%) were removed.

[††] http://www.socscistatistics.com/effectsize/Default3.aspx

[‡‡] https://www.anzmtg.org/stats/PowerCalculator/PowerTtest: power = 0.8, $\alpha = 0.05$, independent, two-sided

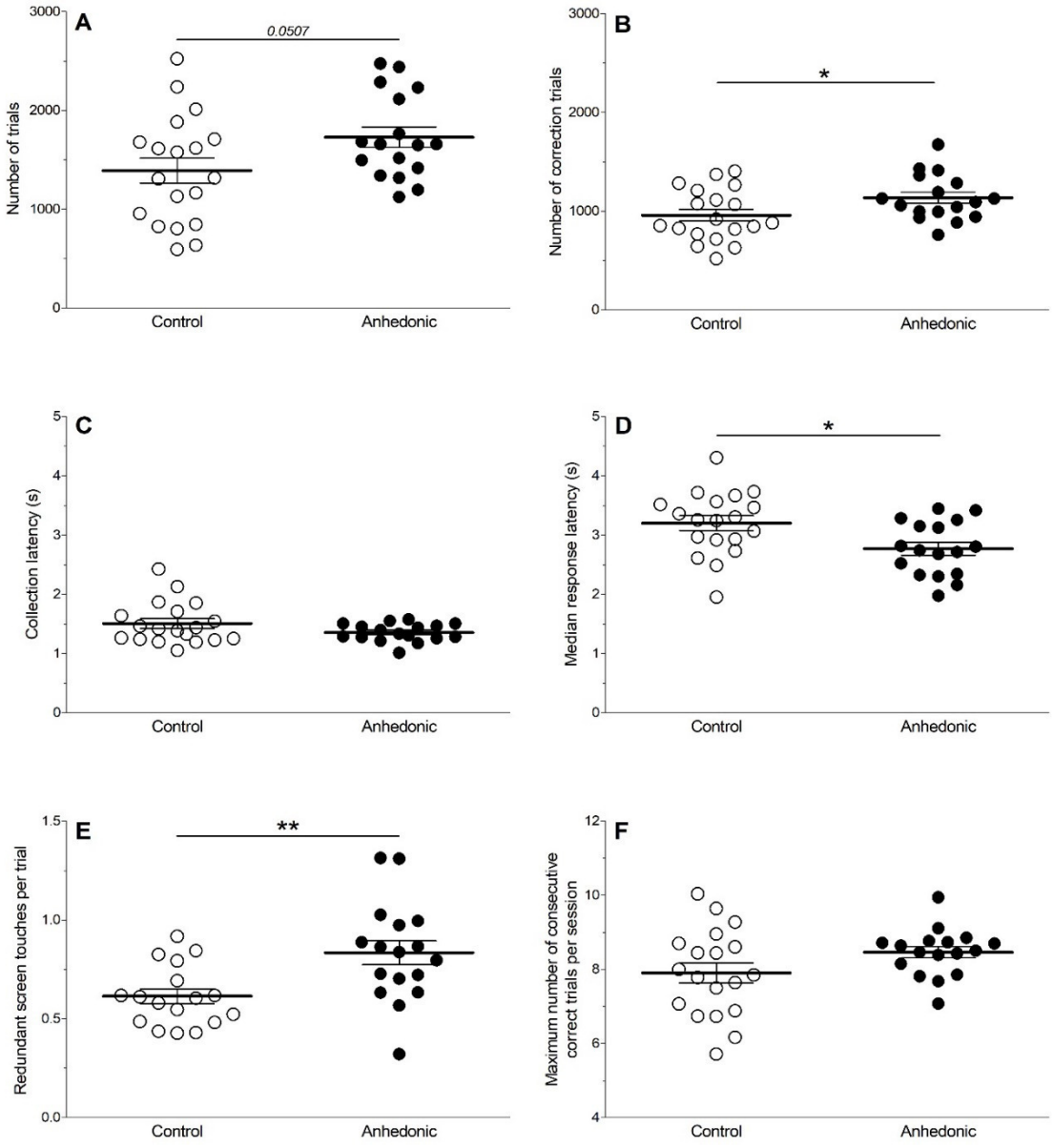

Figure 20. Combined data of study II and III. The data for non-stressed controls and CMS exposed anhedonic-like rats (untreated) is pooled to increase *n*-number per group and thus statistical power. **(A)** Number of trials to acquire the dPAL task. **(B)** Number of correction trials. **(C)** Time taken to collect reward pellet. **(D)** Median latency to respond to stimuli on the touchscreen. **(E)** Number of redundant touches executed to the screen per trial. **(F)** The highest number of trials correctly executed in a row per session. Individual data points and group means (± SEM) are shown. Significance is indicated with $^{**}p < 0.01$, $^{*}p < 0.05$.

4.5 Brain-derived neurotrophic factor (Study IV)

Reduced cerebral BDNF levels are found after stress exposure and in depressed patients. Thus, we investigated if a genetic reduction in BNDF provoked affective-like behaviour and/or cognitive impairments. Furthermore, PFC and HPC expression levels of genes relevant for neuropsychiatric disorders and in the stress response were examined. BDNF$^{+/-}$ rats displayed anhedonic-like behaviour in the SPT (Figure 21A) and anxiety behaviour in the OF (time spent in centre; Figure 21B). No difference in behaviour between BDNF$^{+/-}$ and WT rats occurred in the EPM, NIH, FST, SAB or MWM. The transcription factor GR, disrupted in schizophrenia 1 (*Disc1*), which is attenuated in psychiatric disease, and neuregulin 1 (*Nrg1*), involved in learning and memory, were upregulated in the PFC of BDNF$^{+/-}$ rats, whereas *Fkbp5*, a regulator of the HPA axis' negative feedback sensitivity, was downregulated in BDNF$^{+/-}$ rats in the HPC (Figure 22). Thus, reduced BDNF levels can be associated with a mild phenotype related to affective disorders which may be underpinned by altered gene expression in the brain.

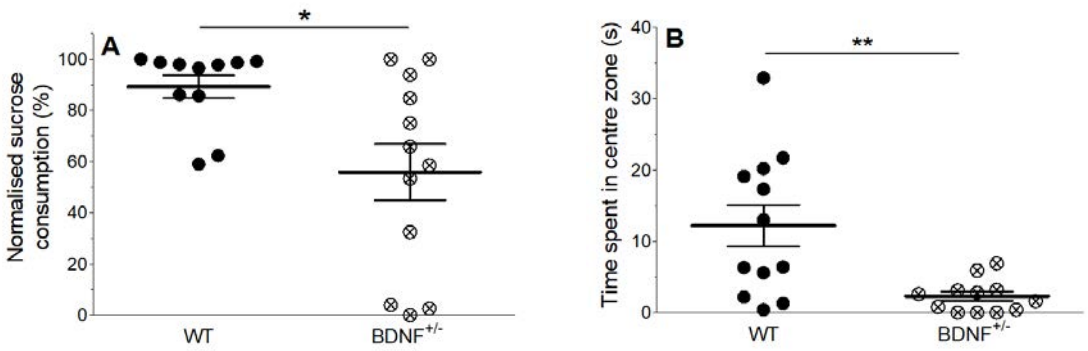

Figure 21. Sucrose preference test and open field (OF) test. (A) Sucrose intake normalised to total fluid intake (sucrose solution plus water consumption). **(B)** Time spent in the centre of the OF arena. Individual data points and group means (±SEM) are displayed. Statistical significance is indicated with $^{**}p < 0.01$, $^{*}p < 0.05$.

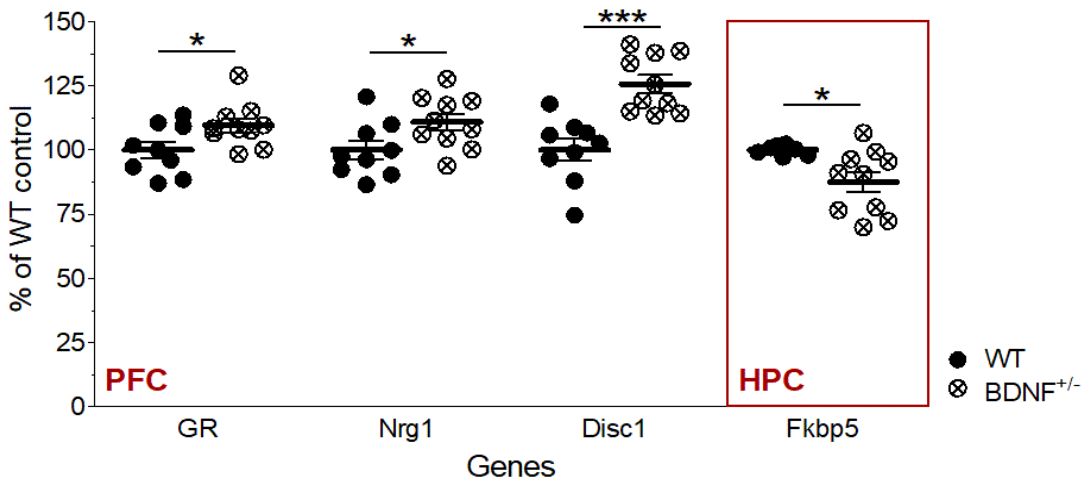

Figure 22. Prefrontal cortex (PFC) and hippocampal (HPC) gene expression. Individual gene expression levels are normalised to the respective group mean of wild type (WT) rats. Individual data points and group mean (±SEM) are displayed. Statistical significance is indicated with ***$p < 0.001$, *$p < 0.05$.

GR - Glucocorticoid receptor, Nrg1 – Neuregulin 1, Disc1 – Disrupted in Schizophrenia 1, Fkbp5 – FK506 binding protein 5

5 DISCUSSION

A detailed discussion regarding the results of each study is given within the respective manuscripts. In the following, the results of all four studies are discussed collectively.

5.1 Strain differences in touchscreen testing

The first study focused on methodological as well as scientific research questions. One of them aimed to determine if rodent strain, specifically albinism, which is associated with poor vision, affects performance in the vision-based touchscreen tasks. We found that Wistar albino rats performed more poorly than pigmented LE rats in the PD and the PD reversal task, as well as during the retention phase (study I). Thus, strain evidently affected touchscreen performance. Literature has been conflicting on this topic. For example, one study concluded that albino rats' performance was comparable to pigmented rats applying the PD and reversal task in Sprague Dawley and Lister Hooded rats[263], whereas another study found that albino rats (Wistar and Sprague Dawley) perform significantly worse than the pigmented strains (Lister Hooded and Long Evans)[220]. Our results support the latter study and confirm that albinism is accompanied by inferior touchscreen task performance. It is postulated that this is due to poor vision in albino strains[220], however, results from study I cannot decisively confirm this. In fact, albino rats were able to acquire the simple PD and reversal task and therefore were able to recognize the stimuli. However, errors could have been made more easily if it was difficult for the rats to distinguish between the stimuli. During the PD reversal learning, Wistar rats' performance was superior to LE rats in the first two sessions. Thus, Wistar rats must have incorporated the stimulus-reward association from the preceding PD task less strongly or, alternatively, they display greater cognitive flexibility. Yet, Wistar rats' superior performance in the PD reversal task was not continued in subsequent sessions, thus, it is more likely that Wistar rats had a weaker stimulus-reward association of the PD task. Consequently, Wistar rats and possibly other albino strains exhibit inferior touchscreen task performance, which might be caused by a combination of poor vision and inferior cognition. This may not necessarily be an exclusion criteria for albino strains in vision-based touchscreen task, as long as albino strain characteristics are considered in task choice, e.g. tasks with easy distinguishable visual stimuli.

5.2 The chronic mild stress model

The CMS model was applied to acquire a better understanding of the risk factor 'stress' and its effect on cognitive performance. Additionally, the influence of hedonic state, i.e. being resilient or anhedonic-like, on cognitive performance was investigated.

5.2.1 Modelling the affective state

First, we demonstrated that exposure to stressors, a main environmental risk factor for developing MDD in humans, resulted in anhedonic-like behaviour in rats independent of rat strain (study I). Anhedonia was only expressed in a fraction of CMS exposed LE rats (37%), whereas other LE rats remained hedonic (19%), thus are classified as resilient (study II), which is in accordance with previous studies using CMS exposed Wistar rats[201,270]. Furthermore, CMS exposed rats displayed anxiolytic behaviour in the EPM and OF. Anxiety disorders are a common comorbidity in MDD pathology[271–273], and thus anxiogenic instead of anxiolytic behaviour in CMS was expected. However, previous studies show that stressed animals exhibit both anxiogenic[274,275] as well as anxiolytic behaviour[202,276] and, hence overall indicate altered emotional processing linked to stress exposure.

5.2.2 Modelling cognitive impairments

First, a general effect of stress on cognition was assessed comparing CMS exposed LE rats with LE controls (study I). Both groups had similar spatial working memory ability, as tested in the SAB task. A previous study using Wistar rats showed that CMS anhedonic-like and resilient rats are impaired in the SAB task[205]. Discrepancies between the two studies might be explained by differences in rat strain or, alternatively, the SAB test needs to be supported by other tasks to provide a reliable result. Furthermore, the effect of stress on cognitive performance was evaluated by applying the PD and PD reversal task (study I). Results from this study suggest that stress does not affect cognition. However, our experimental design comprised a heterogeneous stress group, including anhedonic as well as resilient rats, which may has masked anhedonia-related cognitive impairments. Furthermore, stressed as well as non-stressed LE rats required very few sessions to learn the PD and PD reversal task and, thus, suggest that this touchscreen task may be too simple to uncover potential mild to moderate cognitive impairments.

To avoid ceiling effects in learning, the more complex dPAL task was applied in subsequent touchscreen studies. In addition, LE CMS rats were categorized according to their hedonic

state and only anhedonic and resilient rats were used, which resemble the strongest CMS phenotypes. Anhedonic-like rats tended to take longer to acquire the dPAL task compared to controls, whereas resilient rats showed a cognitive performance comparable to the control group (study II). Thus, this study demonstrated that cognitive impairments appear to be specifically associated with the depressive-like phenotype and not a general consequence of stress exposure. Impaired cognitive performance in the anhedonic-like group might be a consequence of prolonged high levels of free corticosterone in response to CMS exposure, possibly inducing adverse effects on the brain[61,204]. Briefly, resilient rats have high corticosterone levels during stress exposure; however, this initial response is quickly terminated and therefore might induce only mild or no adverse effects on the brain[204]. Although not impaired in task acquisition, resilient rats displayed increased impulsive behaviour, observed by an elevated number of redundant screen touches compared to anhedonic-like or control rats. Impulsivity relates to impairments in response inhibition, an executive function of the PFC[277–279], which implies that stress may have impaired PFC function in CMS resilient rats. Overall, study II uncovered that cognitive impairments are not only a consequence of stress exposure *per se* but are dependent on the rats' hedonic state.

To increase group size, and therefore statistical power, data from control and anhedonic-like rats was pooled from study II and III. Results confirm that anhedonic-like rats were impaired in dPAL acquisition compared to controls, as demonstrated by increased number of trials required to acquire the dPAL task. Interestingly, the anhedonic-like group appears to be subdivided: rats that required a similar number of trials for dPAL acquisition as controls and rats that need more trials than most of the controls. This might be analogous to only a proportion of MDD patients are suffering from cognitive impairments[81]. Since severity of cognitive impairments were correlated with severity of depression in humans[98,100], the stronger cognitively impaired anhedonic-like rats might resemble a more vulnerable subtype of depression. A greater group size is required to confirm the two cognitive subtypes in the anhedonic-like group and to enable comparisons between subtypes, for example of brain structure and endocrinological processes. Still, only controls were able to acquire the dPAL task with less than 1000 trials (~32% of control rats), whereas all anhedonic-like rats needed more than 1000 trials, indicating a lower cognitive performance in anhedonic-like rats. Additionally, cognitive impairments in the anhedonic-like group are demonstrated by an increased number of correction trials needed to acquire the dPAL task.

Shortened median response latency in anhedonic-like compared to control rats in the dPAL task suggests abated cognitive appraisal before making a choice. Since MR is implicated in appraisal processes[280], a trend of decreased MR gene expression in the dorsal HPC of

anhedonic-like rats compared to hedonic rats (study IV) supports the behavioural deficits in appraisal processes in anhedonic-like rats on a cellular level. The HPC is involved in appraisal processes[280,281], which further suggests impaired HPC functioning in the anhedonic-like group. In this regard it is highly interesting that impairments in HPC function coincides with reduced inhibitory control of the HPC over the HPA axis, which is often found in MDD patients[44] and might occur in anhedonic-like rats as well[204]. The hypothesis of a changed HPC function is also supported by preclinical imaging studies detecting alterations in HPC shape and diffusion properties of CMS exposed animals[210,211]. Thus, the central role of the HPC in depression found in preclinical as well as in clinical studies strengthen translational relevance of the CMS model in depression research.

Impaired response inhibition was found in MDD patients and dysfunction correlated with severity of depressive symptoms[79]. Interestingly, we found an increased number of redundant screen touches in anhedonic-like rats, which indicates stereotypic or augmented habit-like behaviour arising from deficits in response inhibition[60]. This cognitive process is part of PFC's executive functions[60,279] and may suggest altered PFC function in anhedonic-like rats. Stress has previously been shown to induce a shift from cognitive demanding processes to habit-like behaviour, which was accompanied by atrophy in the PFC and striatum[60]. Thus, CMS anhedonic-like rats might have experienced PFC and striatal atrophy and as a consequence display increased habit-like behaviour.

The number of consecutive correct trials per session and collection latency did not differ between the two experimental groups. The latter parameter was an important finding because a longer collection latency, for example in the anhedonic-like group, could be indicative of a reduced motivation to consume the reward and likewise to participate in the touchscreen task which would have made it difficult to determine if poorer task performance originated from impaired cognition or reduced motivation.

Altogether, anhedonic-like rats showed cognitive impairments that can be attributed to alterations in executive functions, by e.g. impaired response inhibition, a domain of the PFC, whereas longer task acquisition might be associated with altered HPC function. Both brain regions are known to be altered in MDD[118,282] and lesions in these areas lead to increased number of errors and trials required to acquire the PAL task in humans[228]. Thus, our model of depression-induced cognitive impairments appears clinically relevant.

In MDD patients, memory impairments are less evident than deficits in executive function or attention[81]. Although it appears that anhedonic-like rats show deficits in memory performance in study II, this finding did not reach statistical significance and was also not evident in the pooled data set. From the above mentioned touchscreen parameters, impairments

in HPC function appear to present in anhedonic-like rats, which could have affected memory performance as well. Although the HPC is central in memory formation, once the memory is consolidated, it can become HPC-independent[283]. Rats were well-trained in the dPAL task and, thus, memory might have become HPC-independent.

5.3 Vortioxetine and the CMS model

Study III aimed to investigate the efficacy of vortioxetine to restore the hedonic state and cognitive performance in CMS anhedonic-like rats. In the majority of clinical studies, vortioxetine was superior as antidepressant compared to placebo and superior to agomelatine demonstrated in a single study[133]. In a rodent model of depression, the Flinders Sensitive Line rat, the antidepressant effects of vortioxetine were established in the social interaction test, OF and FST[133]. However, as the Flinders Sensitive Line constitutes a selectively bred rat model with inherent depression-like phenotype, it may be more relevant to investigate the effects of a novel antidepressant in a model with environmentally induced depressive-like behaviour, such as the CMS rat. The literature suggests the CMS paradigm as one of the most appropriate models for modelling MDD since it fulfils predictive, construct and face validity including the MDD core symptom, anhedonia[132]. Thus, it was important to investigate the efficacy of vortioxetine in this model. In study III, we found that vortioxetine successfully restored the hedonic phenotype in the majority of rats (65%), whereas the remaining rats responded only minimally to the treatment. This response rate appears to be similar or slightly better compared to other antidepressants, such as escitalopram (50% response rate) in the CMS model[122]. Thus, this study was first to show treatment efficacy of vortioxetine in the CMS model, which appeared to have failed in a different study (personal communication with Papp, reviewed in Sanchez *et al.*[133]).

Furthermore, study III uncovered brain gene expression levels change in response to chronic vortioxetine treatment. *Bdnf* expression was increased in the dorsal HPC in rats treated with vortioxetine compared to untreated rats, which is complementary to the literature that antidepressants elevate BDNF levels[284–286]. BDNF levels were not decreased in response to CMS exposure, i.e. the untreated anhedonic-like phenotype. This was not expected since reduced BDNF levels can be found in response to stress[284] as well as in MDD patients[152]. However, BDNF is also involved in neuronal plasticity and possible negative effects of CMS on BDNF expression might have been neutralized by learning-induced increase of BDNF[287] during dPAL testing. Surprisingly, GR expression was reduced by vortioxetine in the PFC (study III). This is unexpected according to the literature, which reports decreased GR protein levels in MDD patients.

Regarding cognition, vortioxetine has a positive effect in classical behavioural tasks related to dPAL testing, such as novel object recognition and spatial working memory in 5-HT-depleted rats[134,145] and prevents age-induced visuospatial impairments in the novel object placement test[288]. In study III, cognitive impairments associated with the untreated anhedonic-like phenotype were less prominent than in study II. However, some touchscreen parameters indicate poorer cognitive performance in anhedonic-like rats compared to controls, such as increased number of correction trials needed within a session to acquire the dPAL task, which was established as a marker of lower cognitive performance in the pooled data set. In study III, vortioxetine treatment resulted in an even higher number of correction trials needed for task acquisition, which does not support a pro-cognitive effect of vortioxetine treatment. Furthermore, stereotypic and less appraised behaviour were a robust finding in vortioxetine treated rats. Reduced median response latency as well as an increased number of redundant screen touches were characteristic for vortioxetine treated rats. These two touchscreen readouts are altered similarly in anhedonic-like rats compared to controls in the pooled data set. Taken together, vortioxetine treatment appears to have strengthened depression-associated behaviour. However, number of redundant screen touches, but not median response latency, was also increased in CMS resilient rats compared to anhedonic-like rats or controls (study II). Thus, vortioxetine treatment might introduce some features of the resilient phenotype but maybe overcompensate in its efficacy resulting in stereotypic behaviour. Surprisingly, stereotypic behaviour was not reported in other vortioxetine studies, which might be due to the type of tests applied or that stereotypic behaviour was not assessed or did not interfere with task performance.

Antidepressant treatment, including SSRIs, monoamine oxidase inhibitors and tricyclic antidepressants, inhibit *Gsk3b* expression[289,290]. This was not observed in study III and *Gsk3b* expression appear even increased in vortioxetine responders in the HPC . However, elevated *Gsk3b* expression is also associated with impairments in spatial memory, object recognition and long-term potentiation, which are elements of the dPAL task[289,291–293]. Thus, increased *Gsk3b* levels in the vortioxetine responder group substantiate behavioural results of impaired memory during retention of the dPAL task (study III). Nevertheless, it remains unresolved how vortioxetine restored the hedonic state without decreasing *Gsk3b* levels. This might be explained by tissue collection and consequent mRNA levels measurement were executed at the endpoint of the experiment whereas the antidepressant treatment effect occurred much earlier and thus, temporal dynamic changes in *Gsk3b* expression might explain the discrepancy of *Gsk3b* mRNA levels. *Cofilin 1* expression, involved in reorganization of

the neuronal cytoskeleton[294] and its dysregulation is associated with cognitive decline[295,296], was found significantly increased in the PFC of anhedonic-like rats and, although not significant, both vortioxetine treated groups displayed a comparable level of *Cofilin 1* expression as anhedonic-like rats (study III). Although this finding is unexpected for anhedonic rats, treatment with vortioxetine might induce neuroplasticity changes by upregulation of *Cofilin 1* in the PFC.

5.4 BDNF$^{+/-}$ rats for modelling preclinical depression

5.4.1 Anhedonia and anxiety in BDNF$^{+/-}$ rats

Study IV aimed to investigate a possible relationship of reduced BDNF levels and MDD symptomatology based on the findings that stress reduces BDNF levels[62] and BDNF levels are decreased in post-mortem tissue of MDD patients[152]. A battery of tests was used to uncover the potential relevance of BDNF in affective behaviours. We found no behavioural differences in the EPM, FST and NIH, however, BDNF$^{+/-}$ rats exhibited anhedonic-like behaviour in the SPT and anxiety-related behaviour in the OF. Therefore, our study supports an involvement of BDNF in the affective symptoms of MDD.

Anxiety-like behaviour in the OF is in accordance with another congenital BDNF$^{+/-}$ rat study finding that BDNF$^{+/-}$ rats spend less time in the centre of the OF but are not different to WTs in the EPM and FST[158]. In contrast a BDNF$^{+/-}$ mouse study reported the opposite finding of increased time spent in centre by BDNF$^{+/-}$ mice compared to WT mice[154]. Temporally induced attenuated BDNF levels in rats resulted in increased behavioural despair in the FST[157]. Findings of the temporal model were not observed in the present study and might be explained by compensatory mechanisms which are able to take over in the congenital but not in a temporally induced model. Still, in the same model anhedonic-like behaviour was observed in the SPT as well as decreased locomotor activity in the home cage[157], which are similar findings to observations in study IV. Thus, across different rat (including our study) but not mouse studies, complementary results were found, supporting the use of BDNF$^{+/-}$ rats to investigate the role of BDNF in affective dysfunction.

A reduction of at least 30% of BDNF levels need to be present to observe changes in HPA axis activity at basal level[297]. The BDNF$^{+/-}$ rats in our study show the respective reduction[159] and consequently, the observed changes in hedonic state and anxiety in BDNF$^{+/-}$ rats could be linked to changes in HPA axis function as dysregulation of the HPA axis is often found in MDD patients[298,299]. Finally, altered emotional processing was found in BDNF$^{+/-}$ rats

and linked to amygdala activity[159] and might explain anxiety-related symptoms in the present study as well.

Interestingly, we found altered expression of genes relevant in affective disorders in BDNF$^{+/-}$ rats. *Fkbp5* expression was downregulated in the HPC of BDNF$^{+/-}$ rats, which is usually associated with increased feedback sensitivity of the HPA axis and, thus, a healthy stress response[300]. In connection with the affective phenotype of the BDNF$^{+/-}$ rats, a desensitisation of the HPA axis' negative feedback was expected instead. Similarly, GR expression was upregulated in the PFC of BDNF$^{+/-}$ rats although MDD patients show a downregulation of GR in the PFC[301]. However, overexpression of GR in the forebrain was also associated with emotional liability, i.e. increased anxiety-like and depressive-like behaviours in mice[302] and thus, elevated PFC GR levels might contribute to the behavioural phenotype in BDNF$^{+/-}$ rats. It has been demonstrated that BDNF alters HPA axis activity by increasing CRH expression in the paraventricular nucleus[303] and the subsequent high levels of secreted glucocorticoids might reduce GR expression in the long-term[45]. Thus, opposite mechanisms may occur in animals with innately low levels of BDNF resulting in increased GR expression and the subsequent behavioural phenotype. DISC1 is associated with neuropsychiatric disorders and found to be less functional in schizophrenic patients[304,305]. Thus, *Disc1* upregulation in the PFC of BDNF$^{+/-}$ rats was unexpected but also observed in a juvenile stress model, yet in the HPC[306]. This suggests that this gene is susceptible to stress and the subsequent reduction of BDNF levels and may have a pivotal role in the gene x environment aetiology of depression-like behaviour.

Overall, our study suggests that anhedonia and moderate anxiety-related behaviour is associated with lower BDNF levels and may be mediated by the same mechanisms altering signalling pathways with GR, *Fkbp5*, *Disc1* and *Nrg1*. More studies need to be conducted to establish whether this relationship of behaviour and gene expression is of causal nature, however, our findings supplement a growing body of literature that implicates BDNF in MDD pathology.

5.4.2 Cognitive performance is not impaired

Furthermore, BDNF is involved in neuronal plasticity and might be involved in the development of depression-associated cognitive deficits. However, BDNF$^{+/-}$ rats were not impaired in the SAB, evaluating spatial working memory, or MWM, investigating spatial long-term memory (study IV). We chose these tests based on the fact that SAB and MWM are tasks with a spatial component, which requires HPC function[307–309], a brain area central in MDD pathology and high on BDNF[310,311]. However, cognitive impairments may not be

induced by congenitally low BDNF on its own, but might require a gene x environment interaction. This has been shown in MDD, where the BDNF polymorphism Val66Met modulates the impact of stressful life events in MDD pathogenesis[32]. We additionally found *Nrg1* levels to be elevated in the PFC of BDNF$^{+/-}$ compared to WT rats. NRG1 is associated with spatial learning but also increased in response to stress in the PFC[312,313]. Thus, attenuated BDNF levels evoked a similar effect as stress exposure did.

5.5 Translational testing

Translation between preclinical and clinical testing is central to be able to compare results across species, such as efficacy of a novel drug treatment. However, the nature of assessing symptoms or phenotypes is dissimilar between humans and animals. For example, MDD questionnaires in human subjects are equated to forced swim test, tail suspension test or sucrose intake in rodents. Therefore, new tests and techniques, such as touchscreen testing, which can be used in both humans and rodents, are developed to bridge this gap in translational testing.

5.5.1 Touchscreen testing

Preclinical touchscreen testing is still a rather young field. Although the task protocols are straightforward and uniformly accessible across institutions, protocols are described slightly different between studies, which might explain variation in their results. For example, Wistar rats needed more than 27 sessions to acquire the simple PD task in a study by Kumar[220], whereas in study I, Wistar rats only required nine sessions on average and the slowest Wistar rat 13 sessions to acquire the task. Possible sources of explanation for these differences in performance are (1) differences in food deprivation. Most studies use food deprivation for operant conditioning in touchscreen tasks. Often rats are food deprived down to 85–90% of their body weight[229,262,314]. However, we used percent baseline food intake as the guideline for food deprivation and payed close attention not to food deprive rats to less than 90% of their body weight and some rats did not even lose weight. Furthermore, rats were monitored during pre-training and food deprivation was adjusted to the rats' behaviour, i.e. unmotivated rats received a small decrease in their daily amount of food (- 0.5/ -1 g) or an increase if the rat appeared rushed. Thus, hunger might influence performance in touchscreen testing and should be kept to a minimum also because humans are not food deprived for touchscreen testing. Our study showed that rats learn the task well also with less food deprivation, which should be aimed for in future rodent studies. Further differences could result from (2) number of sessions

per week (with/without break); (3) number of trials per session; (4) passing criterion; (5) passing the task individually or on a group level; (5) stimuli used; (6) duration of ITI; (7) duration of punishment interval; (8) testing during light or dark phase; (9) loudness of tone; (10) house light on/off during ITI; (11) food reward (milk shake, sweet pellets, and by us newly introduced: bacon pellets). Hence, more studies and protocol transparency enable comparisons of protocol modification and results and could lead to an optimized, and consequently unified protocol.

In study III, we introduced bacon pellets and were consequently able to monitor the rats' hedonic state through SCTs throughout touchscreen testing. We observed that touchscreen testing and the accompanying food reduction is perceived mildly stressful since control rats decreased their sucrose intake in the initial phase of touchscreen assessment. Rats later habituate to be touchscreen tested and therefore initially mildly stressful phase occurred predominantly during pre-training and spared the actual task acquisition. We were first to show that although touchscreen testing operates on appetitive operant conditioning, the change in environment, the food deprivation and possibly touchscreen training itself can be stressful for rodents. This might have reduced effect size when comparing supposedly non-stressed control rats to CMS exposed animals.

Still, touchscreen tasks appear to be one of the most translatable and standardized tests at present[6,213]. Accordingly, touchscreen tests applied on clinically relevant animal models are a promising approach to close the gap between preclinical and clinical research and hopefully promote aetiological understanding of cognitive impairments in diseases and enable tailored treatment development.

5.6 Limitations

The conducted studies have some limitations, which were not avertable in the most cases due to practical matters, but should be kept in mind when interpreting results.

We did not find reduced BDNF gene expression levels in anhedonic-like compared hedonic rats (study III), which may not translate into functional changes due to possible post-transcriptional and post-translational modifications. Touchscreen testing and consequently learning processes might have increased BDNF levels[287] which may have masked any reductions in BDNF levels in response to stress.

Vortioxetine receptor occupancy is slightly different between humans and rats (5-HT_{1A} and 5-HT_7 receptor are showing an *in vitro* binding affinity of only 7-10% of the one for humans for vortioxetine)[267]. Therefore, results from rodent studies might not be exactly translational to humans.

In our study, we food-deprived rats, which may had consequences on the treatment effect of vortioxetine. Vortioxetine was incorporated into the rats' food chow, which mimics closer the clinical route of administration and is simultaneously less stressful than any other route of administration. However, rats had to be food restricted for touchscreen testing. Consequently, rats received a lower dose of vortioxetine during that period. However, rats had already recovered prior to touchscreen testing and therefore we believe that rats had reached a steady-state which they maintained even during food deprivation. Furthermore, antidepressant efficacy of vortioxetine was demonstrated even during food-deprivation by the SCTs of study III.

In study III vortioxetine was not administered to a non-stressed control group. Ideally, this should have been done to separate effects of vortioxetine on its own and in the experimental condition. We refrained from treating control rats for the benefit of including a vortioxetine low-responder group in touchscreen testing. The resources available allowed to include only 40 rats in total in touchscreen testing.

Experimental conditions, such as food deprivation, touchscreen testing and isolated housing were likely mildly stressful for control rats. This may have diminished effect size between CMS and the non-stressed control group. However, continuous SCTs testing showed that control rats never entered an anhedonic-like state and the greatest decrease in sucrose consumption occurred during pre-training and not task acquisition. Thus, the observed effects were valid but may have been smaller.

Another limitation comprises the lack of SCTs during touchscreen learning in study I and II. Thus, we cannot exclude that animals spontaneously recovered from CMS exposure during touchscreen assessment. However, study III proves that the CMS-induced state remained during touchscreen testing and we can most likely conclude that it was similar in study I and II.

Furthermore, cognitive performance could not be assessed before CMS exposure. Thus, it is theoretically possible that cognitively inferior rats predominantly entered the anhedonic-like group. However, this hypothesis would entail that cognitively impeded individuals are more likely to become depressive, which is not supported by the literature.

Finally, although preclinical touchscreen testing was developed based on the human CANTAB tasks, tests across species are not identical. This originates from the circumstance that task rules can be simply explained to human subjects but not to animals, which acquire the rules through operant conditioning. Thus, different brain areas might be required for testing. Furthermore, the nature of the reward differs between humans and animals. Animals

are presented with primary rewards, such as food whereas humans receive secondary rewards, such as money or the task might be rewarding in itself for humans.

5.7 Conclusion

When we talk about depression, we tend to primarily and predominantly think about the affective symptoms a person is suffering from. Recently, increased attention has been given to depression-associated cognitive impairments, in part due their impact on daily functioning, their persistence as residual symptom and their role in treatment response and relapse. However, this change in thinking is not currently implemented in therapy and particularly in medical treatment. A key to resolve this shortcoming is the implementation of a clinically relevant preclinical model of depression including affective and cognitive symptoms. This PhD project aimed to establish such an animal model with focus on clinical pertinence and for deepening our understanding of the aetiology of depressive symptoms.

This work suggests that reduced BDNF levels are involved in the aetiology of anhedonia, a core symptom of depression, and moderately in the emergence of anxiety, a common co-morbidity of MDD. Stress exposure resulted not only in the core symptom anhedonia in a proportion of rats, but also in cognitive impairments specifically associated with this phenotype. Stress-resilient rats, defined by their hedonic state, also displayed cognitive alterations; however, their performance in the translational touchscreen task was not impaired. Touchscreen task performance suggests that PFC function might be changed in response to stress, thus in both CMS groups; and HPC function in depressive-like rats only. Vortioxetine treatment was successful in restoring the hedonic state, thus treating the affective symptoms of MDD. The purported pro-cognitive effects of vortioxetine were less evident. Vortioxetine's effect on cognition mirrored in some ways those of resilient rats (increased impulsivity); there might have been a pro-cognitive effect (number of animals acquiring dPAL task), but it did not restore cognition to control level (number of correction trials). Finally, our touchscreen studies contributed to the young and growing field of touchscreen testing. We were first to show that the dPAL task is sensitive enough for detecting depression-associated cognitive impairments and distinguish those from resilient rats. We further established that albino strains are inferior to pigmented strains in touchscreen testing. We were also first to show that touchscreen testing and the accompanying food deprivation is mildly stressful on the rats, which need to be carefully considered when working with e.g. stress models. In addition, we were first to demonstrate that vortioxetine is effective in the CMS model. Thus, this work contributed to the field of preclinical touchscreen testing, risk factor and symptom

relationships in depression, characteristics of stress-induced cognitive impairments and drug treatment applying research tools of high clinical relevance.

We believe that the conducted studies are clinically pertinent owing to the model of choice (rat versus mouse and pigmented versus albino rats); the use of stress (main environmental risk factor for developing depression) and reduced BDNF levels (in response to stress, but also genetic predisposition (val66met)); and application of touchscreen tasks, which were developed based on the human CANTAB test battery. Furthermore, CMS exposure elicited a depressive-like phenotype in only a fraction of rats, similar to human susceptibility, where stress-experience is not inevitably leading to MDD. Moreover, the pooled data of study II and III suggests that only a proportion of depressive-like rats might be cognitively impaired, which is also comparable to the human condition. Thus, our approach mimics very closely the human situation of depression-associated cognitive impairments provoked by a main environmental risk factor of MDD.

5.8 Perspective

This project provides a solid basis for future touchscreen studies, in which various factors can be modified to further optimize the experimental design and translational value. For example, other antidepressants should be evaluated in the CMS model and touchscreen tasks since vortioxetine is a relatively new drug and less characterized in humans than other antidepressants. This would also help to identify potential other antidepressants with pro-cognitive effects and provide starting points for novel drug development.

Furthermore, other preclinical models than the CMS model should be tested with the touchscreen operant platform. Different preclinical depression paradigms may model different subtypes of MDD and thus allow tailoring of MDD subtype-specific antidepressant treatment. Furthermore, the CMS model is labour-intensive and an inbred model, such as the Wistar-Kyoto rat or $BDNF^{+/-}$ would reduce workload and costs if successful in modelling depression-associated cognitive impairments as well. It could be promising to test $BDNF^{+/-}$ rats since the role of BDNF in antidepressant action is evident and they appear to display the core symptom anhedonia. Although, we did not find cognitive impairments in those rats, this might be due to classical behaviour paradigms being less sensitive to cognitive impairments than touchscreen testing or that stress exposure is required, e.g. by 3-day pre-pubertal juvenile stress paradigm to elicit cognitive impairments in $BDNF^{+/-}$ rats.

The promising value for translational testing was already discovered by the pharmaceutical industry and requires now optimization of the experimental design for best clinical relevance. Despite preclinical touchscreen testing being more elaborate than classical

tests, the high clinical relevance might result in a more reliable drug profile, which is beneficial in the long-term, e.g. less failure of drug testing at stage three in humans accompanied with reduced numbers of human subjects for drug testing, and decreased costs in human studies.

Moreover, more women than men suffer from depression. Although female rats may introduce more variance to the results due to their hormonal cycle[314], the female-male-ratio in depressed patients strongly suggests testing of female rats in preclinical studies, though greater group sizes may be required.

Furthermore, comparisons of the resilient and anhedonic-like phenotype should be further elaborated to decipher depression aetiology and get inspiration from the resilient phenotype for antidepressant drug development. Here, touchscreen studies should ideally be combined with examination of genetic traits, neuroendocrinology and a detailed behavioural profile.

Functional magnetic resonance imaging (fMRI) is another highly translational technique that could support the profiling of resilient and anhedonic rodents. fMRI is a frequently applied tool in neuropsychological and neuropsychiatric research in humans to assess indirectly neuronal activity. Basic fMRI tests are already implemented for rodents[159,315] and could highly contribute towards a better understanding and characterisation of preclinical depression models and translation between clinical and preclinical studies.

Finally, in a very elaborate study, rats could be endocrinologically, behaviourally (affective and cognition) and genetically phenotyped before entering the CMS. This would clarify if a certain pheno- or genotype is more prone to develop affective symptoms and be cognitively impaired as result of stress exposure. This would also answer the question if cognitive predisposition contributes to the depression aetiology.

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The effect of rat strain and stress exposure on performance in touchscreen tasks

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ABSTRACT

Patients suffering from depression-associated cognitive impairments often recover incompletely after remission from the core symptoms of depression (lack of energy, depressed mood and anhedonia). This study aimed to set the basis for clinically relevant testing of cognitive impairments in a preclinical model of depression. Hence, we used the chronic mild stress (CMS) model of depression, which provokes the core symptom of anhedonia in a fraction of the stress exposed animals, while others remain resilient, and assessed the entire CMS groups' cognitive performance on the touchscreen operant platform. Specifically, we applied the pairwise discrimination (PD) and reversal task including a retention phase on Wistar and Long Evans controls and CMS exposed Long Evans rats. We observed differences between the albino Wistar and the pigmented Long Evans strain regarding performance in the PD and reversal task as well as in memory consolidation. CMS exposure did not alter learning and memory in the PD and reversal task, even though it altered affective behaviours in the elevated plus-maze and open field test. This is likely due to the heterogeneity of the CMS group, in which stress exposure elicited the expected range of phenotypes from anhedonic-like to resilient shown with the sucrose consumption test. Thus, our study suggests that pigmented rat strains, such as Long Evans, are superior to albino rats in the vision-based touchscreen studies. Furthermore, we propose investigation of the CMS subgroups in more complex, hippocampus-dependent tasks to refine a translational preclinical model of depression-induced cognitive impairments. Hence, this study increased awareness of strain-specific differences in touchscreen performance and added to the literature regarding the sensitivity of the PD and reversal task to stress-induced cognitive alterations.

1. Introduction

Major depressive disorder (MDD) is the leading cause of disability worldwide and the disease has an increasing incidence rate. Currently, 300 million individuals suffer from depression also affecting their social and economic environment [1]. Anhedonia (loss of interest or experience of pleasure), depressed mood and lack of energy are the core symptoms of depression. Additionally, patients may also exhibit other symptoms, including, suicidal thoughts, feelings of guilt and worthlessness as well as cognitive impairments [2]. Depression-related cognitive impairments have been observed in 30–50% of depressed patients affecting memory, attention and executive function [3–6]. Although cognitive impairments persist after remission from depression in 94% of these patients [6], they are often disregarded but continue affecting the patient's quality of life. Cognitive impairments can augment negative feelings and thoughts, counteract therapy and increase risk of relapse [7,8]. Consequently, development of an antidepressant treatment that also generates remission of depression-related cognitive symptoms is crucial [3,4]. Hence, it becomes critical to identify a valid animal model for treatment testing [9]. However, modelling MDD is difficult due to the heterogeneity of the disease symptoms, the introspective nature of the symptoms and the incomplete understanding of disease aetiology which arise from complex gene-environment interactions [9–12]. The literature indicates the chronic mild stress (CMS) model as one of the most ideal paradigms for rodents models of depression covering face, construct and predictive validity [10,12,13]. The CMS paradigm uses unpredictable mild stressors over weeks, mimicking daily stress exposure in humans and eliciting anhedonia, a core symptom of MDD [10]. Furthermore, cognitive impairments have been demonstrated in this model using novel object

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recognition tasks [14], spontaneous alternation behaviour and enhanced aversive memory in contextual fear conditioning [15]. Although these results are promising, their translational value and clinical relevance is debatable [12,16]. Especially when addressing such a complex entity as human cognition and its alteration in depression, application of highly translational and meaningful tests is indispensable. Consequently, we address this difficulty by employing a new technique; the touchscreen operant platform based on appetitive reinforcement learning. Major advantages over commonly used tests for rodents include its cognitive phenotyping array adapted from the Cambridge neuropsychological test automated battery (CANTAB) for cognitive assessment in humans, its accurate and objective readouts and the standardized setup [17–19]. In this study, we focused on several questions to optimize implementation of translational testing in the CMS model of depression. First, we investigated potential differences in the suitability of a pigmented rat strain (Long-Evans (LE)) and an albino strain (Wistar). The latter one has been undergoing the CMS paradigm in our laboratory for years. Yet, the Wistar albino strain bears risk for impaired vision [20–22] which could restrict their performance in the vision-based touchscreen apparatus independent of their cognitive abilities. Other studies considered this issue previously, but with contradictory results [18,22]. Furthermore, we determined the susceptibility of the LE strain to display depressive symptoms in response to the CMS paradigm. Finally, we examined the effect of CMS exposure on touchscreen performance using the LE strain. The touchscreen pairwise discrimination (PD) and reversal task was applied consisting of two steps: Visual discrimination and stimulus-reward association learning was evaluated with the PD touchscreen task, whereas the PD reversal task assessed perseveration behaviour and new stimulus-reward association learning. Perseveration behaviour is suggested to play an important role in MDD [23,24] and association learning assesses attention and executive function [25,26]. Finally, we included re-testing of the PD reversal task after a 10 day hiatus to assess long-term memory performance [16].

2. Materials and methods

2.1. Animals

Male Wister (Taconic M&B, Denmark; $n = 12$) and Long Evans rats (LE; Janvier Labs, France; $n = 28$) were 5–6 weeks of age and 100–120 g at arrival in our facility. Animals were housed in groups of four for the first week followed by single-housing for the time duration of the experiment. Rats had free access to food and water and kept on a 12 h light-dark cycle (lights on at 6 am). All experiments were conducted according to the Danish National Committee for Ethics in Animal Experimentation (2013-15-2934-00814).

2.2. Chronic mild stress protocol

A proportion ($n = 16$) of the LE rats were subjected to the CMS paradigm 6 weeks after arrival in the facility. The rats were exposed to a series of unpredictable mild stressors (Table 1) for 5 weeks as described in Jayatissa et al. [27] for Wistar rats.

The Wistar rats and the remaining 12 LE rats were not exposed to the CMS paradigm (controls).

2.3. Sucrose consumption test to assess the rats' hedonic state

Sucrose consumption tests (SCT) were carried out to assess the rats' hedonic state before and during CMS exposure. After one week of acclimatization to the animal facility, the LE rats undergoing the CMS paradigm were habituated to consume a palatable sucrose solution (1.5%) for 24 h in one week, and for 1 h following 14 h of food and water deprivation the next week. Succeeding this habituation phase, three 1 h SCT were carried out every Friday (following 14 of food and water deprivation) and sucrose intake was measured for each animal [28]. The last two sucrose consumption tests were averaged and employed as baseline sucrose consumption for each rat. Thereafter, stress exposure was commenced and succeeding SCTs were normalised to the baseline sucrose consumption creating sucrose consumption indexes. The final two SCT indexes during CMS exposure were averaged and used to evaluate the hedonic state of each animal. Animals were grouped using an a priori criteria for hedonic state [10]. Animals are categorized as anhedonic-like with a SCT index ≤ 0.7, whereas a SCT index ≥ 0.9 defines a CMS exposed rat as resilient.

2.4. Classical tests for measuring anxiety-like behaviour and working memory

The phenotype of the CMS exposed LE rats was further assessed using typical behavioural tests. Phenotyping focused on anxiety behaviour and working memory. Testing was conducted in the final week of CMS exposure in the animals' light phase. The experimental room was illuminated with red light if not otherwise stated. Testing order was randomized. Rats were acclimatized to the experimental room for at least 1 h before testing. The experimental equipment was cleaned with 70% Ethanol between animals. A person blinded to group identity scored the recorded behaviours.

2.4.1. Elevated plus-maze

Animals were placed in one end of a closed arm (40 cm high wall) of the plus-shaped maze (arms: 50 × 10 cm, 70 cm elevation from floor) facing the back wall. Light intensity was 80 lx in the open and 20 lx in the closed arms. Rats were left to freely explore the elevated plus-maze (EPM) for 10 min. Behaviour was scored for time spent in the open and closed arms, number of head dips and arm entries.

2.4.2. Open field

The circular open field (OF) arena measured 120 cm in diameter with 40 cm high wall. Animals were placed in the centre of the OF and their movement was recorded for 10 min. The number of central and horizontal crossings, time to first rearing, number of rears and grooming were measured.

Table 1
Weekly CMS protocol. The variation of stress duration (indicated in brackets) and different type of stressors used aimed to increase unpredictability of the protocol and prevent habituation to the stress regime. During the "grouping" stressor, an intruder rat (another rat undergoing the CMS protocol) was added to the cage of the resident rat. The role of the resident and intruder as well as the pairing rat was changed every week.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning	New cage,[a] body weight measurement, intermittent illumination (5 h)	Water deprivation (9 h)	Stroboscopic light (6 h)	New cage	SCT (1 h), alternating each week: food or water deprivation (7 h)	Alternating each week: water or food deprivation (9 h)	Cage tilt 45° (9 h)
Evening	–	Cage tilt 45° (14 h)	Wet bedding (14 h)	Remove food and water (14 h)[a]	Grouping (14 h)	Cage tilt 45° (14 h)	Wet bedding (14 h)

[a] For CMS and non-stressed controls; SCT = sucrose consumption test.

2.4.3. Spontaneous alternation behaviour assessing working memory

The y-maze consisted of three arms (50 × 18 cm) surrounded with dark Plexiglas walls (35 cm). The room was dark, but a light was fixed above the centre of the y-maze resulting in 5 lx illumination in the centre and 1–2 lx in the arms. The rat was placed at the end of one arm facing the back wall and recorded for 12 min during free exploration of all three arms. An arm entry was scored if all four paws of the rat entered the arm. Visiting all three arms consecutively was counted as one correct alternation. The maximum possible number of correct alternations was the total number of arm entries minus two. The alternation ratio was calculated as the number of correct alternations made normalised to the maximum number of possible alternations.

2.5. Translational testing of visual discrimination learning and long-term memory with the touchscreen operant platform

2.5.1. Apparatus

The sound- and light-attenuating Bussey-Saksida touchscreen operant chambers (Campden Instruments Ltd., Loughborough, UK) contained a trapezoid shaped interior chamber (height 30 cm, length 33.2 cm, width screen 24 cm, width magazine 12.6 cm) with a touch-sensitive screen on one side and a reward delivery system (magazine) on the opposite side. A mask covered the screen leaving two windows (10 × 10 cm) for the rat to touch the screen in a defined area. A spring-hinged shelf in front of the mask prevented unintentional and hasty touches by slowing the rat down before reaching the screen. A fan ensured sufficient ventilation and masking of external noise. The chambers were further equipped with a grid floor, house and magazine light, and a tone generator. The touchscreen program was controlled by Whisker Server and Abett II software (Campden Instruments Ltd.).

2.5.2. Touchscreen pre-training

Rats were gradually food restricted to 80% of their individual free feeding consumption and body weight was checked daily. Forty stimuli (white on black background) were randomly used during pre-training and displayed one at the time leaving the other touchscreen window blank. Pre-training followed the protocol of Horner et al. [17]. Briefly, rats underwent “habituation” (free exploration of touchscreen chamber), “initial touch” (exposure to stimuli, tone and automatic delivery of reward pellets (sugar coated 45 mg dustless precision pellets, BioServ, NY, USA)), “must touch” (response towards the stimulus is required for reward delivery), “must initiate” (initiation of new trial by nose poke in food magazine) and “punish incorrect” (response to a blank touchscreen window is punished by 5-s time out with house light turning on) to step-wise guide the rats on operating the touchscreen system. Each trial was followed by an inter-trial-interval (ITI) of 20 s. Each session lasted maximum 45 min or 75 trials (except “habituation”). Passing “punish incorrect” by completing 75 trials within 45 min with at least 60 correct choices (≥ 80% accuracy) on two consecutive days completed pre-training and rats were individually moved on to PD acquisition (Fig. 1).

2.5.3. Pairwise discrimination and reversal learning

In the pairwise discrimination (PD) task, rats had to learn association of one touchscreen symbol with a sugary reward pellet (S +) and another symbol (S −) with a mild punishment (5 s house light on). The association was independent of symbol location on the screen. Achieving 80% or more correct choices out of 75 trials within 45 min on two consecutive days was equipollent with passing the PD task. Rats were then individually moved on to PD reversal learning. In that task, the other symbol (S −, PD task) became the rewarding one and the previously learned rewarding symbol (S +, PD task) would entail the 5-s time out period with house light on. The same criteria for passing applied as in PD. Hence, we tested visual discrimination and stimulus-reward association learning in the PD task, whereas PD reversal assessed perseveration behaviour and new stimulus-reward association learning.

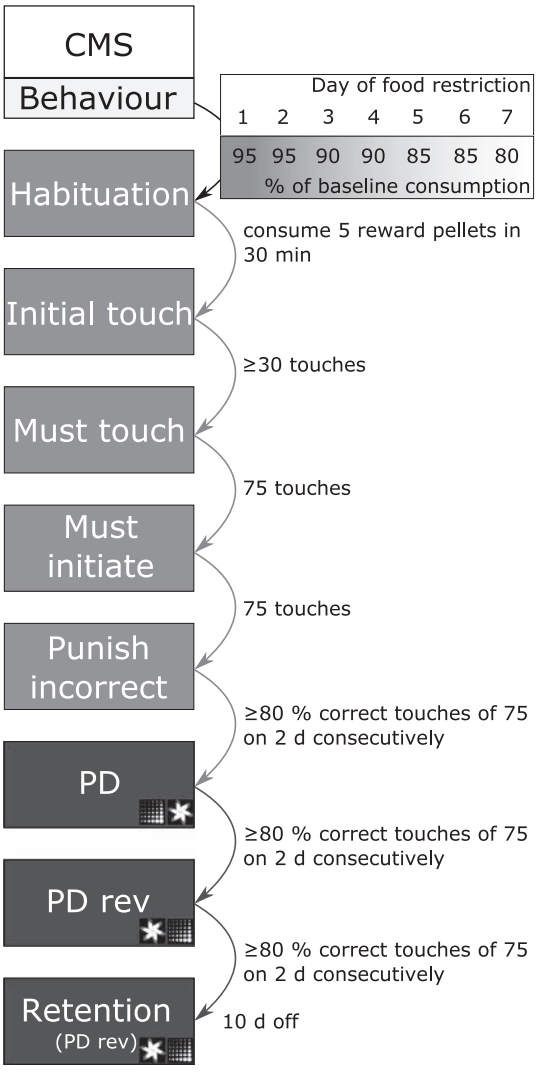

Fig. 1. Overview of experimental pipeline. Consecutive experimental steps including details of food deprivation and passing criteria for each step of touchscreen pre-training and training are given.

2.5.4. Retention

After acquiring PD reversal learning, the rats received 10 days without touchscreen testing and an increase in food that substituted for the daily amount of reward pellets. Next, rats were re-tested on the PD reversal task for two days to evaluate the rats' long-term memory (Fig. 1).

The three groups (Wistar control, LE control, LE CMS) were balanced across testing chambers preventing possible chamber-specific differences (illumination, tone intensity or pitch, odour, stimulus appearance, reward delivery) as cofounding factor on group performance. Furthermore, the individual testing time-point of the day was changed daily to avoid daytime specific performance. Rats of all groups occupied the touchscreen chambers at the same time of the day. On the following days, the same rats were tested each time 45 min earlier than the day before. Rats tested first of the day were moved to be tested last on the next day. Touchscreen sessions were carried out seven days a week avoiding a possible change in performance due to days without touchscreen training.

2.6. Statistical analysis

Data was analysed using Stata 14 (StataCorp LP, Texas, USA). Summary statistic was applied for number of sessions to pass PD and PD reversal task as well as EPM, OF and SAB using student's *t*-test or Welch's *t*-test accounting for violation of homogeneity of variance. Normality was tested with QQ-plots and Shapiro-Wilk test and violation lead to application of non-parametric Mann-Whitney test. Outliers were removed according to Grubb's test ($\alpha = 0.05$; GraphPad Software Inc., California, USA). SCT index was correlated with behavioural data of EPM, OF and number of sessions to pass PD and PD reversal learning. For the correlation, data was rank transformed. Repeated measurements data of accuracy and reaction time (RT) were analysed by two-way repeated measurements ANOVA and possible interaction effects were post-hoc analysed with Bonferroni-corrected pairwise comparisons for each time point. To not distort the mean over time, animals that passed the touchscreen tasks were kept in the analysis by including their last readout of accuracy in the mean over time. Outlier performances were kept in the analysis because they did not alter significance level of the results. Data was checked for normality and homogeneity of variance with QQ- and residual plots. All RT readouts had to be log-transformed and outlier RTs determined by Grubb's test ($\alpha = 0.05$; GraphPad Software Inc.) were removed. Two animals of the Wistar group were unable to pass pre-training and hence were excluded from the PD and reversal task.

3. Results

3.1. The effect of CMS on the rats' hedonic state

On an individual basis rats were differently affected by stress and could be classified as anhedonic-like (3/16), resilient (7/16) or intermediate (6/16) depending on their sucrose consumption (Fig. 2). This was expected based on previous studies from our laboratory in Wistar rats [10,15,27]. Hence, once established, the CMS model can be effectively used across strains.

3.2. Classical behavioural phenotyping of CMS exposed rats reveals altered anxiety-like behaviour but no changes in working memory

Additionally to the SCT, we assessed anxiety behaviour and working memory in the whole LE CMS group by comparing them with non-stressed LE controls in the EPM, OF and spontaneous alternation behaviour task.

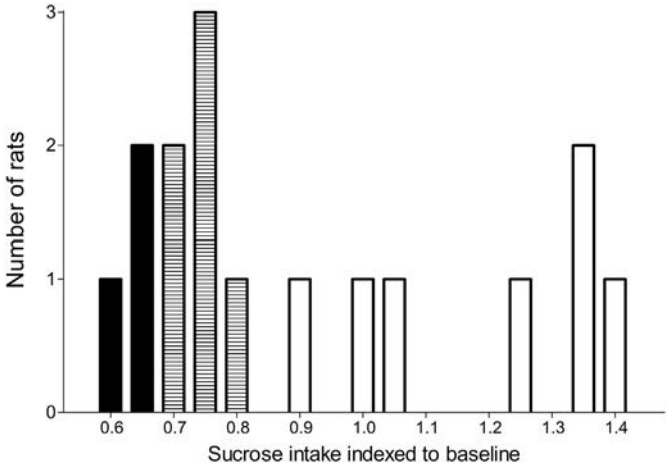

Fig. 2. Sucrose intake of CMS exposed LE rats. The frequency distribution of the rats' hedonic state was calculated as the mean sucrose intake for the last two weeks of CMS indexed to prior to stress baseline. A sucrose intake index below 0.7 defines rats as anhedonic-like (black bars), above 0.9 as resilient (empty bars) and in between as intermediate phenotype (striped bar).

Significant differences between LE controls and LE CMS were observed in the EPM. CMS exposed rats spent less time in closed arms ($p < 0.0001$) and more time on open arms ($p < 0.0001$) than non-stressed controls. CMS exposed rats also carried out more head dips into open arms ($p < 0.0001$) and entered the open arms more frequently than control rats ($p < 0.0001$; Fig. 3). Within the CMS group, the SCT index did not correlate with the time spent in the open or closed arms, the number of entries into the open arms nor the number of head dips. The EPM results indicate decreased anxiety in the rats exposed to CMS.

In the OF, LE CMS rats carried out significantly more central crossings ($p = 0.026$) and number of grooming bouts ($p = 0.034$) compared to non-stressed LE rats. Furthermore, the number of rears ($p = 0.026$) and time spend till first rearing ($p = 0.040$) was significantly decreased in CMS exposed LE rats than in controls (Fig. 4). The apparent decreased anxiety could not be explained by altered locomotor activity between groups ($p = 0.935$) and did not correlate with SCT index within the CMS group.

Hence, CMS exposed rats exhibited an anxiolytic rather than an anxiogenic phenotype.

No effect of stress was observed on the alternation ratio in the spontaneous alternation behaviour test indicating intact working memory of CMS LE rats.

3.3. Cognitive performance of Wistar and LE rats and the consequences of CMS exposure

First, we wanted to assess if strain alone (albino Wistar and pigmented LE rats) alters performance in the translational PD and reversal touchscreen tasks. Secondly, the effect of CMS on cognitive performance was assessed by comparing LE controls to LE CMS rats.

3.3.1. Touchscreen task acquisition

Wistar rats took significantly longer to acquire the PD task ($t(11.29) = 5.68$, $p < 0.001$) and the PD reversal task ($t(11.87) = 3.54$, $p = 0.004$) compared to LE controls. No effect of CMS on number of sessions needed to acquire the PD and reversal task was observed (Fig. 5A, B).

3.3.2. Learning curves

Detailed analysis, evaluating the rats' performance over time, was carried out to detect possible changes during learning that may have been missed by solely analysing the number of sessions needed to acquire the touchscreen tasks.

No effect of CMS exposure was found in PD or PD reversal task comparing the learning curves of CMS exposed LE rats to non-stressed LE rats. A significant interaction effect of group x time on accuracy was found for the learning curves between Wistar and LE controls in the PD ($F(12, 240.00) = 8.78$, $p < 0.0001$) and PD reversal tasks ($F(22, 440.00) = 4.14$, $p < 0.0001$). Post-hoc analysis was Bonferroni corrected and determined the sessions in which Wistar and LE controls differed significantly in their performance (Fig. 5C, D).

3.3.3. Reaction time

We further investigated the mean RT (time between rats initiating a new trial and making a choice by touching the screen) to check for group differences in time taken to reach a decision. An interaction effect of group x time on reaction time was observed for strain comparisons in the PD task ($F(12, 231.15) = 2.04$, $p = 0.022$) and PD reversal task ($F(22, 414.23) = 3.43$, $p < 0.0001$) and for stress in the PD ($F(5, 125.47) = 3.40$, $p = 0.007$) and PD reversal task ($F(12, 299.26) = 2.39$, $p = 0.006$) suggesting differences between groups in processing speed of decision-making. In the PD task, Wistar rats showed a longer RT than LE controls and this effect diminished over time. In PD reversal, LE rats needed longer to make their choice until session eight after which Wistar rats displayed a longer RT. For both PD and reversal

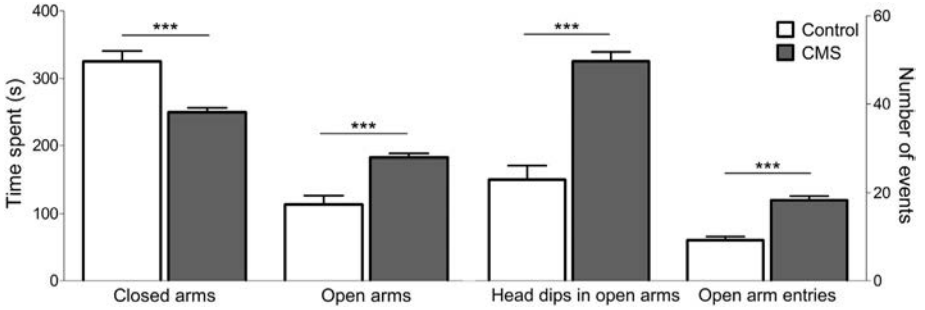

Fig. 3. Behavioural differences in EPM. Shown are time spent in the closed and open arms as well as number of head dips in the open arms and open arm entries for both non-stressed (empty bars ± SEM) and CMS exposed LE rats (filled bars ± SEM). (***Student's *t*-test, $p < 0.001$).

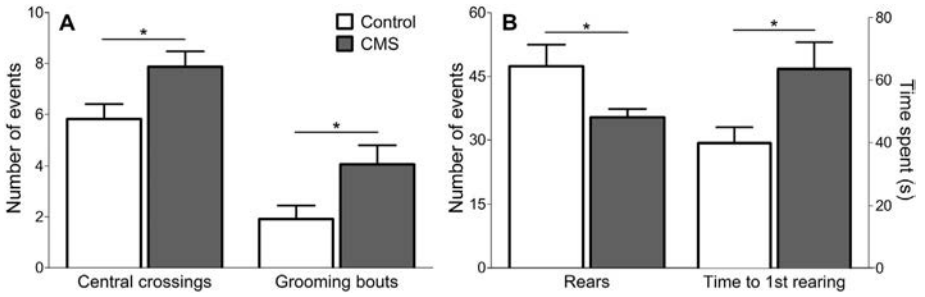

Fig. 4. Behavioural differences in the OF. (A) Number of central crossings and grooming bouts. (B) Number of rears and latency to first rearing behaviour. Group mean (± SEM) is displayed for LE controls (empty bars) and CMS exposed LE rats (filled bars). (*Student's *t*-test, $p < 0.05$).

task, CMS exposed rats had a longer RT compared to non-stressed LE rats in the first session after which RT duration was reversed suggesting quicker decision-making due to stress exposure.

3.3.4. Retention

Finally, long-term memory was assessed by re-testing the PD reversal task following a 10 d hiatus. Two-way repeated measures ANOVA on the last PD reversal session, retention session one and retention session two reveals a main effect of strain on accuracy ($F(1, 16.47) = -2.35$, $p = 0.032$) with a simple main effect at retention session one ($p = 0.049$, Fig. 6A) suggesting that Wistar rats have an impaired long-term memory compared to LE rats. No effect of stress was

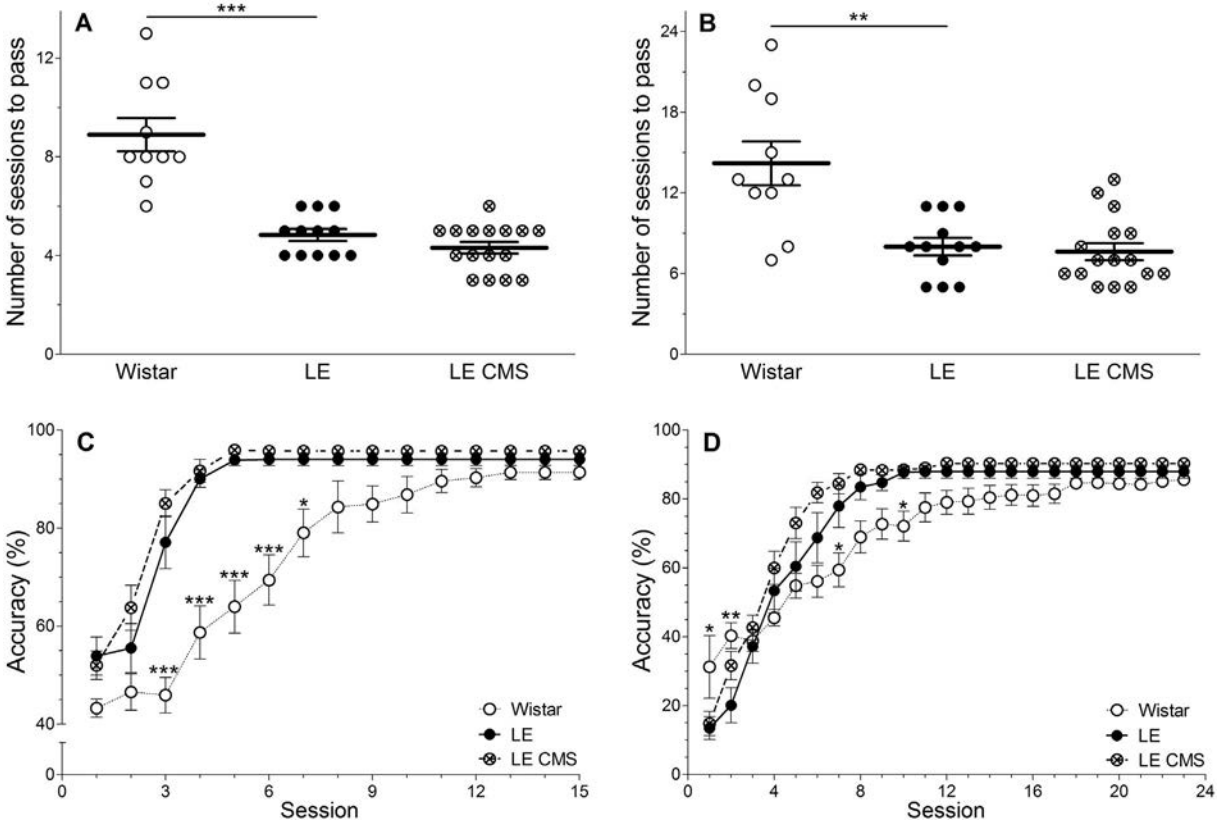

Fig. 5. Results for PD and PD reversal touchscreen tasks. The number of sessions needed to pass the PD (A) and PD reversal task (B) are displayed as group average (± SEM) and as individual scores (analysed by Welch's *t*-test (Wistar vs LE)) or student's *t*-test (LE control vs CMS). Mean accuracies over sessions (learning curves) are displayed for PD (C) and PD reversal (D) learning (analysed by two-way repeated measures ANOVA). Statistical results of Bonferroni post-hoc test comparing LE and Wistar controls at each session are indicated with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

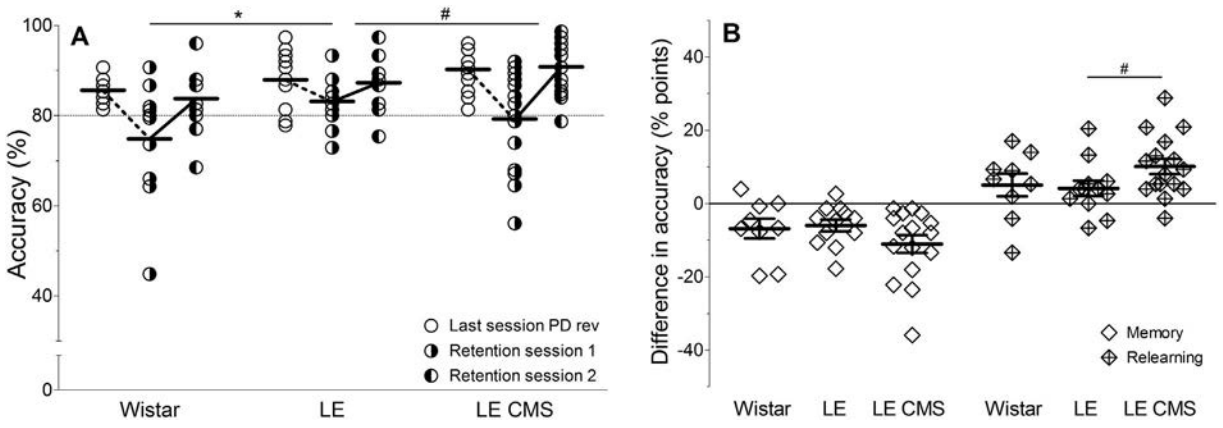

Fig. 6. Retention of PD reversal task. (A) Accuracy of the last session in PD reversal, the first and second session of retention following the 10 d hiatus are shown as mean group performance as well as the individual performances. Passing criterion of 80% correct choices is indicated with a dotted line. Memory (difference in accuracy between last PD reversal session and first retention session is indicated with ···) and relearning (difference in accuracy between first and second retention session is indicated with —). Memory and relearning are shown again in more detail with individual and group results (± SEM) in (B). Differences are indicated with $*p < 0.05$ (two-way repeated measurements ANOVA); $\#p < 0.060$ (student's *t*-test).

observed for retention (accuracy over time).

Separate analysis of retention was carried out to distinguish two different cognitive processes: first, memory was calculated as the difference in accuracy between the last PD reversal session and the first retention session comparing performance before and after the break. Second, relearning was calculated as the difference in accuracy between the first and second retention session. Analysis revealed a trend for relearning performance between the LE control and LE CMS group with $t(26) = -1.98$, $p = 0.058$ (Fig. 6B) maybe indicating altered retention processing due to stress exposure.

4. Discussion

4.1. General

First, we demonstrated that the hedonic state of LE rats was altered in response to CMS exposure comparable to Wistar rats. All three categories of the hedonic state (anhedonic-like, resilient and intermediate) reported in former studies on Wistar rats [10,29–31] and in others [32,33] were present in the CMS exposed LE rats. Hence, we could confirm the applicability of the CMS paradigm to different rat strains without the need for paradigm adjustment emphasizing its robust and comparable outcome of the MDD core symptom anhedonia.

Moreover, in classical behavioural rodent tests for anxiety, stressed LE rats displayed less anxiety-related behaviours than non-stressed controls. CMS rats executed more central crossings, more grooming bouts, less rears, and showed increased latency to first rearing in the OF. In the EPM, CMS rats spent more time on the open arms and less on the closed arms, they showed increased numbers of entries to the open arms and increased number of head dips. Overall, these findings suggest that CMS attenuates anxiogenic behaviour in LE rats. The apparent decreased anxiety behaviour did not correlate with the hedonic state of the CMS group. Although this co-occurrence is not imperative, an anxiogenic effect of CMS exposure could have been expected du the comorbidity of depression and anxiety disorders in humans [34–36]. However, reports have shown contradictory findings regarding anxiogenic [37,38] versus anxiolytic [39,40] behaviour in animals after stress exposure. The latter two studies interpreted the anxiolytic effect observed in the stressed group as dampened emotional processing of sensory input, and, thus, a loss of interest in the environment. Furthermore, the rodent strain, type of stressor, stressor duration and length of protocol differ greatly between studies, likely driving the different outcomes and making comparisons difficult. The present CMS protocol, unlike in the above studies, does not involve electric foot shock, forced swimming, restraint stress or stressors lasting over 14 h, which could explain the non-emergence of an anxiogenic phenotype due to a milder stress protocol in this study. Moreover, the above mentioned papers did not report about segregation into stress-susceptible, stress resilient or intermediate subgroups in their stress paradigm, but only of a stress group that seems to be equivalent with the depressive-like phenotype. Parameters of the CMS protocol, such as stressor intensity, determine the proportion of rats exhibiting a susceptible phenotype and, hence, the heterogeneous composition and overall phenotype of the group. A stressed group, in which all rats are susceptible, models traumatic rather than common daily stressful situations in humans. Such a model seems inadequate for depression research since only a proportion of humans develop depression after experiencing stressful events [41].

Furthermore, there was no significant difference between CMS exposed and control LE rats in the spontaneous alternation behaviour test. This result conflicts with the study of Henningsen et al. [15], in which resilient and anhedonic-like rats showed a significantly lower alternation ratio than controls. It could be that stress-induced impairments in working memory are rat strain-specific. However, Henningsen et al.'s study [15] did not include rats that are in between these marginal phenotypes of resilience and susceptibility making comparisons to our study difficult.

Stress exposure had no effect on cognitive performance in the PD and PD reversal task examining number of sessions needed to acquire the task as well as differences in the learning curves (accuracy over time). Hence, there seem to be no alterations due to stress exposure in the domains for visual discrimination learning, perseveration behaviour and stimulus-reward association learning. Non-stressed LE rats needed very few sessions to acquire the PD and reversal tasks indicating the simplicity of these tests for the rats and, hence, likely accounting for a missing effect of stress exposure. Thus, PD and reversal learning might not be sufficiently challenging for detecting mild cognitive impairments but only drastic alterations in cognition (e.g. lesion or pharmaceutical studies). However, a trend was observed of stress influencing the relearning ability during retention. Although both memory and relearning performance during retention lead to an average decrease or increase of over 10% respectively (Fig. 6B), only relearning reached a close to significant difference between groups. Interestingly, the average performance of non-stressed LE rats in the first retention session after the hiatus still met the passing criterion of 80% for the PD reversal task, whereas the LE CMS group's average performance was slightly below criterion (Fig. 6A). This finding could indicate an alteration in long-term memory and hence hippocampal function. The

hippocampus plays a key role in memory formation and is known to be structurally and functionally altered in depressed patients [42] and stress-exposed animals [43].

Non-stressed Wistar rats performed inferior to non-stressed LE rats in the PD, PD reversal and retention of the PD reversal task. Wistar rats needed more sessions to acquire the PD and the PD reversal task and their learning curve was in both cases significantly shallower. The lower performance in the two tasks could be due to impaired cognition as well as to restricted visual capability. Our results do not allow a definite differentiation. However, all Wistar rats were able to acquire these tasks and, hence, their vision was sufficiently efficient to discriminate the visual stimuli. Nevertheless, reduced vision in albino rats [22] could increase the likelihood for spontaneous involuntary mistakes. It should be considered that Wistar rats perform significantly better than LE controls in the first sessions of PD reversal task, indicating a weaker internalisation of the recently passed PD task. This is unlikely to be caused by greater cognitive flexibility since superior performance in the PD reversal task discontinued after the second session. Hence, we likely observe a phenomenon of decreased cognitive abilities in the Wistar albino strain combined with poor vision.

After the 10 day hiatus, the first retention session was significantly lower in accuracy in Wistar compared to LE controls indicating a lower memory capability in the albino strain. Wistar rats perform below the criteria (≥ 80% accuracy) in the first retention session (Fig. 6A). They regain a performance level above criterion on retention session two ruling out any other interference than cognitive impairments.

RT was predominantly longer for Wistar than for LE controls and shorter for CMS exposed LE rats than for LE controls (except session one of PD and reversal tasks). The RT can hint on the processing speed of decision-making leading to the conclusion of impaired processing speed in the Wistar strain since a longer RT did not result in improved task performance. On the other hand, LE controls and CMS exposed rats showed a similar performance during touchscreen task learning. Given that controls are considered normal, the decreasing effect of CMS on RT should be regarded as spontaneous or impulsive behaviour rather than superior cognitive processing [44–46].

In a similar study by Kumar et al. [22] aiming to make comparisons between pigmented and albino rat strains, the group found superior performance in the pigmented to the albino rat strain in the visual discrimination and reversal task emphasizing the reproducibility of touchscreen testing. However, in Kumar et al.'s study [22] no differences in RT were observed between Wistar and LE rats but a longer RT for the pigmented Lister-hooded as to albino Sprague-Dawley and Wistar rats. The similar RT between Wistar and LE controls in Kumar et al.'s study [22] might be caused by a slightly intensified food restriction in Kumar et al.'s study [22]. Ideally, food restriction should be avoided, but it is deemed necessary to motivate the rats engaging with the touchscreen setup [17]. However, intensified food restriction may lead to spontaneous, unconsidered decision-making precipitated by hunger and interfering with diagnostic cognitive assessment.

Interestingly, in Kumar et al.'s study [22], Wistar rats did not acquire reversal learning and overall performance of both LE and Wistar rats was poorer compared to the current study. For example, LE controls needed seven sessions (plus three criterion sessions) to achieve the visual discrimination task on a group level whereas in this study only four sessions (plus two criterion sessions) were needed for the slowest LE control rat to pass the PD task. Furthermore, in visual discrimination reversal learning, Wistar rats were abolished from testing at session 14 because their accuracy level scarcely reached 60% [22]. In our study at session 14 of PD reversal learning, Wistar rats performed at an average accuracy level of 80% (Fig. 5D). These pronounced differences in performance level might be due to small differences in the experimental setup of our study to Kumar's such as longer ITI, different stimulus symbols, house light off during stimuli presentation, increased number of trials per session and a consistent session duration of 45 min throughout pre-training and training, two instead of four stimuli locations and stringency of pre-training (inclusion of a "punish incorrect" step during pre-training). These parameters may have allowed an $n = 10$ to be sufficient to produce clear results between strains. Kumar et al. [22] suggested an increased N number might have been responsible to track differences between strains in their study compared to the study of Bussey et al. [18], which did not find differences in performance between albino and pigmented strains. Hereby, we could confute this possibility and further shed light on the influence of intrinsic touchscreen parameters on task performance.

Hence, the current study adds to the growing resource of data in the rather young touchscreen field. Comparisons between studies reveal which parameters may increase performance in the touchscreen chambers and, thus, accelerating optimization of protocols. A well evaluated standard protocol for touchscreen testing should be the objective, enabling comparisons across institutions, reproducibility of studies and giving possibility of increased quality in meta-analysis.

4.2. Limitations

A limitation of this study was that CMS exposure was not continued during touchscreen pre-training and training. Hence, spontaneous recovery from stress effects may have occurred. Literature reports spontaneous recovery from CMS exposure by 4–5 weeks [10]. However, introduction to the new element of touchscreen pre-training might have prolonged stress experience and extended the period to recovery.

Furthermore, the low number of animals in the stressed group did not allow for subdividing them into the three categories of the hedonic state. Hence, the heterogeneity of the stressed group including anhedonic-like, intermediate and resilient animals may have masked depression-related cognitive impairments. Moreover, the correlation of hedonic state with cognition or anxiety was inconclusive due to the low animal number and the high variance of the data.

4.3. Conclusion

This study replicated the findings of Kumar et al. [22] emphasizing the reproducibility of touchscreen testing in different labs. Further, our study underlined the validity of the CMS model across strains without the need of additional strain-specific adaptations. Moreover, key points of implementation were successfully disclosed allowing optimized testing of CMS animals' cognition with the touchscreen task: Pigmented rats should be used for testing in a vision-dependent touchscreen setup to be able to distinguish cognitive deficits from visual ones. Further, stress alone results only in a trend during retention phase but not during task acquisition. This suggests testing of the distinctive subgroups of the CMS paradigm since cognitive impairments might be specific to the depressive-like phenotype and not observable in the heterogeneous CMS group. Moreover, a complex touchscreen task should preferably be used for detecting even mild cognitive impairments. In particular, a hippocampus-dependent task would be relevant based on the consistent literature matching alterations in hippocampal function and structure in individuals with depression.

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Declaration of interest

We have no conflict of interest to declare.

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Manuscript II

Resilient and depressive-like rats show distinct cognitive impairments in the touchscreen paired-associates learning (PAL) task

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Depression-associated cognitive impairments persist after remission from affective symptoms of major depressive disorder (MDD), decreasing quality of life and increasing risk of relapse in patients. Conventional antidepressants are ineffective in restoring cognitive functions. Therefore, novel antidepressants with improved efficacy for ameliorating cognitive symptoms are required. Translational animal models are in demand for tailoring such antidepressants. The chronic mild stress (CMS) model is a well-validated preclinical model of depression and known for eliciting the MDD core symptom "anhedonia" in stress-susceptible rats. Thus, cognitive performance was assessed in rats susceptible (depressive-like) or resilient to CMS and in unchallenged controls. The rodent analogue of the human touchscreen Paired-Associates Learning (PAL) task was used for cognitive assessment. Both stress groups exhibited a lack of response inhibition compared to controls while only the depressive-like group was impaired in task acquisition. The results indicate general stress effects, as well as specific depression-associated effects on cognition in the CMS model. Hence, we propose that the application of a translational touchscreen task on an etiologically relevant, preclinical MDD model, displaying depression-associated cognitive impairments, provides a novel platform for pro-cognitive and clinically pertinent antidepressant drug screening.

Introduction

Major depressive disorder (MDD) is the leading cause of disability worldwide affecting 300 million people and constituting a major socio-economic burden to society[1]. The core symptoms of MDD are lack of energy, depressed mood and anhedonia, a decreased sensitivity or anticipation to reward[2,3]. Additionally, depressed patients can exhibit a plethora of other manifestations including feelings of guilt and worthlessness, altered sleep architecture, change in body weight, suicidal thoughts, or impairments in cognition, primarily in attention, executive function and memory[4,5]. After remission from the affective symptoms of MDD, these cognitive impairments still persist in 30–60% of patients[4–7] and were found to be the longest present residual symptom[8]. Cognitive impairments are a major contributor to the disabling impact of MDD[9] and, thus, in patients with persistent cognitive impairments quality of life is decreased and risk of relapse elevated[4,10]. Accordingly, treatment of depression associated cognitive impairments in addition to the affective symptoms is considered crucial for complete remission[4,5,7,10].

Although many resources have been directed towards depression research, the causal mechanisms of MDD remain unknown due to a variety of symptoms emerging from the complex gene x environment interaction. A major environmental risk factor for developing MDD is the exposure to stress[11]. Stress can cause neuropsychological changes which can lead, in predisposed individuals, to an excessive or prolonged stress response and increased risk for mental diseases, such as depression[11–13]. The hypothalamic-pituitary-adrenal (HPA) axis is hyperactive in most MDD patients. Increased amounts of circulating glucocorticoids are released to cope with stressors and to endeavour maintenance of homeostasis. The hippocampus, which is central in memory formation, is sensitive to prolonged high levels of glucocorticoids. MDD patients show memory impairments and a decreased hippocampal volume, which is associated with the duration and number of depressive episodes[14–18]. Both, hippocampal atrophy and memory impairments might be a direct consequence of stress in MDD patients. Moreover, chronically elevated cortisol levels, as a consequence of prolonged stress exposure, can impair cognition in non-depressed individuals[19]. This highlights the possibility that stress is a causal factor in the development of depression-associated cognitive impairments. To gain further insight into the relationship of stress and cognitive impairments in depression, a preclinical stress model exhibiting depression associated cognitive impairments is indispensable[6]. A number of preclinical models of depression apply stressors (etiological validity) to provoke a depressive-like phenotype. Some milder paradigms, such as the chronic mild stress (CMS) model, also enable the segregation of a stress-resilient

subgroup, which allows investigation of distinct stress- and depression-related effects as well as the study of potential resilience mechanisms. Comparable studies are impossible in humans since stress intensity, nature and duration, as well as time point in life of stress experience, differ greatly between subjects. Depressed patients are often medicated and a "resilient" group with comparable stress experience is difficult to identify. These confounding parameters and obstacles are controlled for in preclinical MDD models applying defined stress paradigms. The CMS model, mimicking daily stress experience in humans, is a highly validated preclinical model of depression, well known for the manifestation of the MDD core symptom of anhedonia (face validity). Additionally, CMS exposed rats exhibit other depressive-like symptoms such as changes in sleep architecture, changes in body weight, decreased sexual activity and altered aggression behaviour[20–22]. Impaired CMS-induced working memory and increased conditioned contextual fear response were shown with classical rodent behavioural tests[20]. Such studies indicated that impaired cognition is associated with a stress-induced or depressive-like phenotype. However, the translational value and clinical relevance of the classical behavioural tests used in these studies is poor. Therefore, a highly translational method, the touchscreen operant platform, for assessing cognition in rodents was applied in the present study. These touchscreen tasks were developed based on the Cambridge Neuropsychological Test Automated Battery (CANTAB), the most frequently applied cognitive test battery in MDD patients[6]. Further advantages of the rodent touchscreen platform include standardized experimental equipment and tasks, objective readouts, minimization of experimenter's bias, a cognitive test battery and high throughput[23,24]. In the present study, we applied the different Paired-Associates Learning (dPAL) task which has been used in preclinical models of schizophrenia and Alzheimer's disease, and is known for being a hippocampus-dependent task[25,26]. Hence, we investigated if the highly translational touchscreen platform is sensitive for detecting cognitive impairments in stress exposed rats. Furthermore, we determined if the impairments observed are the consequence of general stress exposure or specifically associated with the depressive-like phenotype by including stress-susceptible and stress resilient rats in the study. This will provide insight in the relationship of stress, mood (anhedonia) and cognitive symptoms. The aim of this study was to establish a clinically relevant platform for developing and tailoring pro-cognitive antidepressant treatments.

We hypothesized to observe cognitive impairments in both stress exposed groups in the dPAL task. Additionally we expected the stress-susceptible, depressive-like, rats to be impaired in a different cognitive area or more severely than the CMS resilient rats. These

cognitive impairments might possibly be observed in attention, executive function or memory.

Results

Hedonic state changes in response to stress

Rats exposed to CMS segregated into anhedonic-like and resilient phenotypes based on their sucrose consumption test (SCT) index. The CMS groups responded differently to stress in respect to their sucrose consumption (interaction effect of group x weeks of CMS: $\chi^2(16) = 41.84$, $p = 0.0004$; Figure 1). The CMS anhedonic-like group significantly decreased in sucrose intake over the course of stress exposure compared to non-stressed controls (Bonferroni-corrected group-wise comparisons $p < 0.0001$) and CMS resilient rats (Bonferroni-corrected group-wise comparisons $p < 0.0001$). The non-stressed control and CMS resilient group did not differ statistically significant from each other. The SCT results show that stress provoked clearly distinct phenotypes. Only a fraction of rats became anhedonic-like, thus depressive-like, in response to stress, whereas another subgroup of rats was stress-resilient.

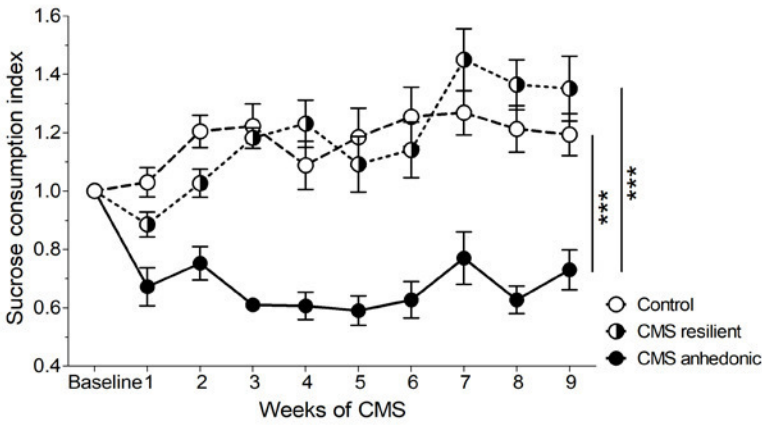

Figure 1. Sucrose consumption during CMS. The weekly sucrose consumption, normalised to baseline, is shown as group mean (± SEM). Statistically significant group-wise Bonferroni-corrected comparisons over all time points are indicted by ***$p <$ 0.001 (Control: $n = 11$, Resilient: $n = 11$, Anhedonic: $n = 10$).

Paired-associates learning touchscreen task

Learning of the dPAL task

Learning behaviour until attaining dPAL acquisition criterion was evaluated with summary statistics comparing non-stressed controls, CMS anhedonic-like and resilient rats.

Anhedonic-like rats might have needed more trials (*Mean (M)* = 1821.40 trials, *SD* = 153.56) to acquire the dPAL task than controls (*M* = 1305.80 trials, *SD* = 176.11; trend in

main effect of group: $F(2,26) = 3.26$, $p = 0.054$; Figure 2A). Although the total number of correction trials to acquire the dPAL task was higher in the anhedonic-like group (M = 1176.90 trials, SD = 263.90) than in controls (M = 953.90 trials, SD = 316.63) or resilient rats (M = 992.89 trials, SD = 192.67; Figure 2B), the difference was not statistically significant.

The time to collect the touchscreen reward pellet (collection latency) did not differ significantly between groups, nor did the median time to respond to the stimuli on the screen (response latency; Figure 2C) or the number of screen touches additionally to the one for making a choice (redundant screen touches per trial).

We examined the highest number of correct trials that the rats were able to carry out in a row within a session. This parameter was used to assess sustained attention and is in the following referred to as "maximum consecutive correct trials per session". CMS anhedonic-like rats carried out significantly more maximum consecutive correct trials per session (M = 8.55 trials, SD = 0.24) than controls (M = 7.43 trials, SD = 0.29; LSD post-hoc $p = 0.005$; main effect of group: $F(2,26) = 4.65$, $p = 0.019$; Figure 2D).

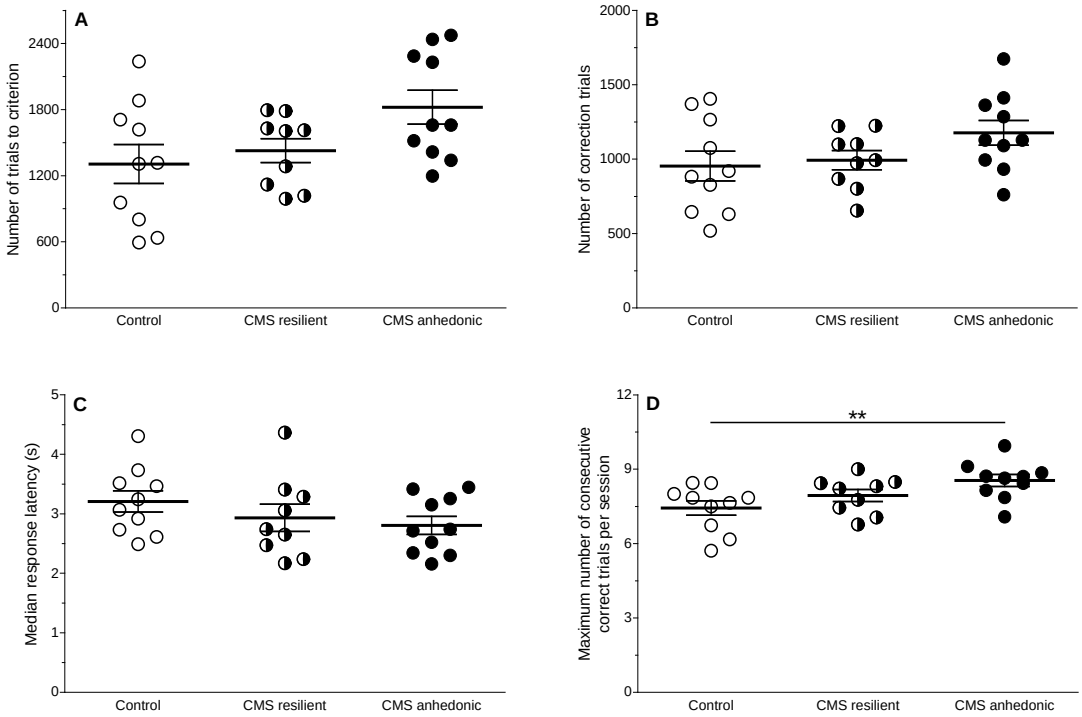

Figure 2. Summarized touchscreen parameters of dPAL task acquisition. (A) Absolute number of trials needed to pass the dPAL task. **(B)** Absolute number of correction trials needed for learning the dPAL task. **(C)** Median response latency to touchscreen stimuli. **(D)** Average number of maximum consecutive correct trials per session. Group means (± SEM) and individual results are shown. LSD post-hoc comparisons are indicated with $*p < 0.05$, $**p < 0.01$.

These results suggest that anhedonic-like rats have a different strategy for learning the touchscreen task compared to non-stressed controls and CMS resilient rats. Overall, CMS anhedonic-like, but not resilient rats, exhibited impaired learning behaviour.

dPAL task acquisition over time

The total number of trials (trials plus correction trials) required to learn the dPAL task was split into ten equal bins. Thus, the variable number of sessions, and consequently the total number of trials, between individual rats was normalised to ten time points (bins) for each rat. This permitted a more direct comparison of individual rats and the progress of different behavioural parameters in the learning task as well as statistical analysis with repeated measurements ANOVA.

The accuracy of learning ($F(5.72,165.88) = 63.04$, $p < 0.0001$; Figure 3A) and number of trials ($F(2.76,80.10) = 46.91$, $p < 0.0001$; Figure 3B) significantly increased over time with increasing bin number. No differences for these parameters were observed among groups. The number of correction trials ($F(2.75,79.68) = 47.44$, $p < 0.0001$) significantly decreased over time. There is therefore no apparent difference between groups on the performance in the learning phase of this task.

During the initial trials learning the dPAL task the CMS resilient rats executed more redundant screen touches than controls or CMS anhedonic-like rats (interaction effect of group x bin: $F(5.04,73.12) = 3.35$, $p = 0.009$; Figure 3C). Furthermore, the number of redundant screen touches per trial significantly decreased over time for all groups ($F(2.52,73.12) = 10.92$, $p < 0.0001$).

Interestingly, the CMS anhedonic-like animals executed more consecutive correct trials ($M = 12.42$ trials, $SD = 5.99$) than non-stressed controls ($M = 10.58$ trials, $SD = 5.18$; LSD post-hoc $p = 0.016$) or CMS resilient rats ($M = 10.92$ trials, $SD = 5.25$; LSD post-hoc $p = 0.048$; main effect of group: $F(2,29) = 5.64$, $p = 0.009$; Figure 3D).

A trend in group x bin interaction was observed for collection latency ($F(5.56,80.59) = 2.18$, $p = 0.058$). Collection latency decreased significantly with increasing bin number ($F(2.78, 80.59) = 9.07$, $p < 0.0001$).

Median response latency also decreased significantly over time ($F(3.13,90.64) = 12.99$, $p < 0.0001$). Maximum number of consecutive correct trials ($F(4.78,138.75) = 17.16$, $p < 0.0001$) significantly increased over time with increasing bin number. Both parameters indicate task improvement over the course of dPAL task acquisition.

These results show that all groups were able to learn the task over time, but differences in learning strategies between groups were evident.

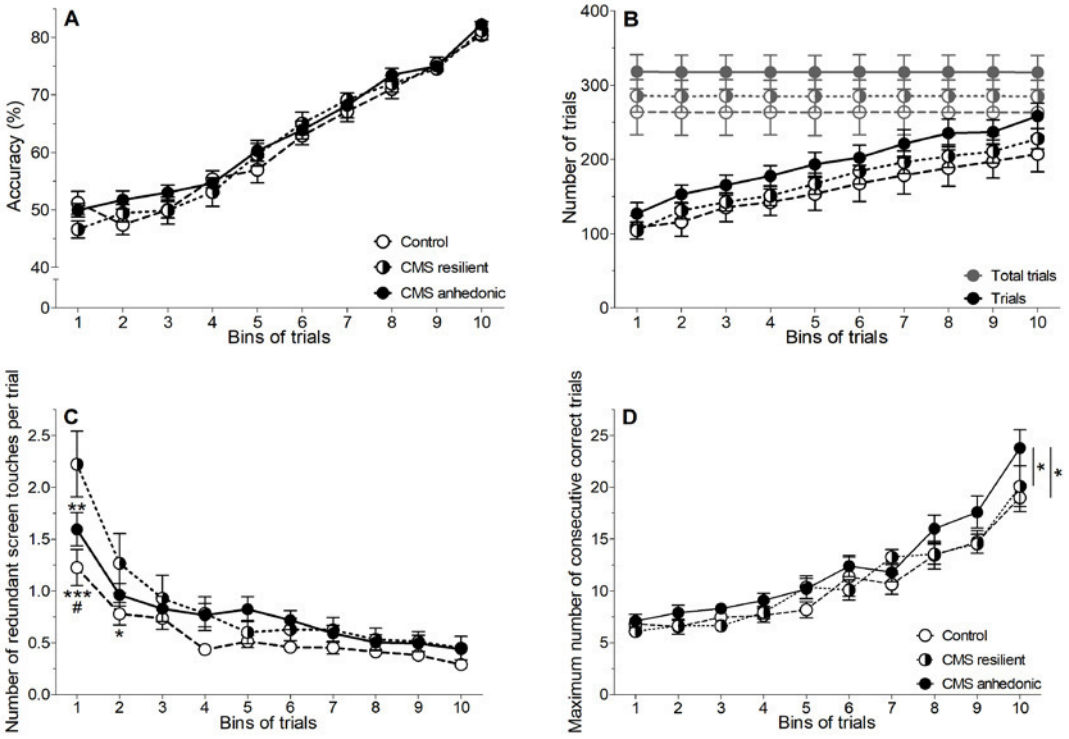

Figure 3. Learning of the dPAL task over time. Total number of trials (trials plus correction trials) are split into bins of ten. **(A)** Accuracy over time. **(B)** Number of trials (black) and total number of trials (trials plus correction trials; grey). **(C)** Number of redundant screen touches per trial. Post-hoc comparisons compared to the CMS resilient group respectively are indicated by ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and controls versus anhedonic-like by $^{\#}p < 0.06$. **(D)** The maximum number of consecutive correct trials. LSD post-hoc comparisons between groups are indicted by *$p < 0.05$. Group means are shown (± SEM).

Learning behaviour within the course of an average dPAL session

All sessions of one animal were averaged to a single session. This session was then split into six equal blocks by the total number of trials (trials plus correction trials). This allowed for the analysis of learning behaviour within the course of a session.

Accuracy (Figure 4A) and number of trials were not significantly altered over the course of a session or between groups. However, the number of correction trials decreased significantly with increasing session block ($F(5,145) = 3.18$, $p = 0.009$).

Non-stressed controls executed less redundant touches per trial than CMS resilient and anhedonic-like rats in the first third of a session (interaction effect of group x session block: $F(4.78,69.34) = 3.40$, $p = 0.009$). The number of redundant touches per trial decreased within the course of a session ($F(2.39,69.34) = 7.22$, $p = 0.0007$; Figure 4B).

During the progression of a session, thus with increasing block number, maximum number of consecutive trials increased significantly ($F(5,145) = 14.61$, $p < 0.0001$; Figure

4C) as well as median response latency ($F(1.59,46.16) = 10.19$, $p < 0.0001$; Figure 4D). Collection latency varied with block number ($F(3.22,93.38) = 2.25$, $p < 0.0001$).

Thus within a session, primary readout parameters, like accuracy and number of trials, seemed not to change, but secondary parameters did, such as decreased number of correction trials and redundant touches, increased number of consecutive correct trials and median response latency.

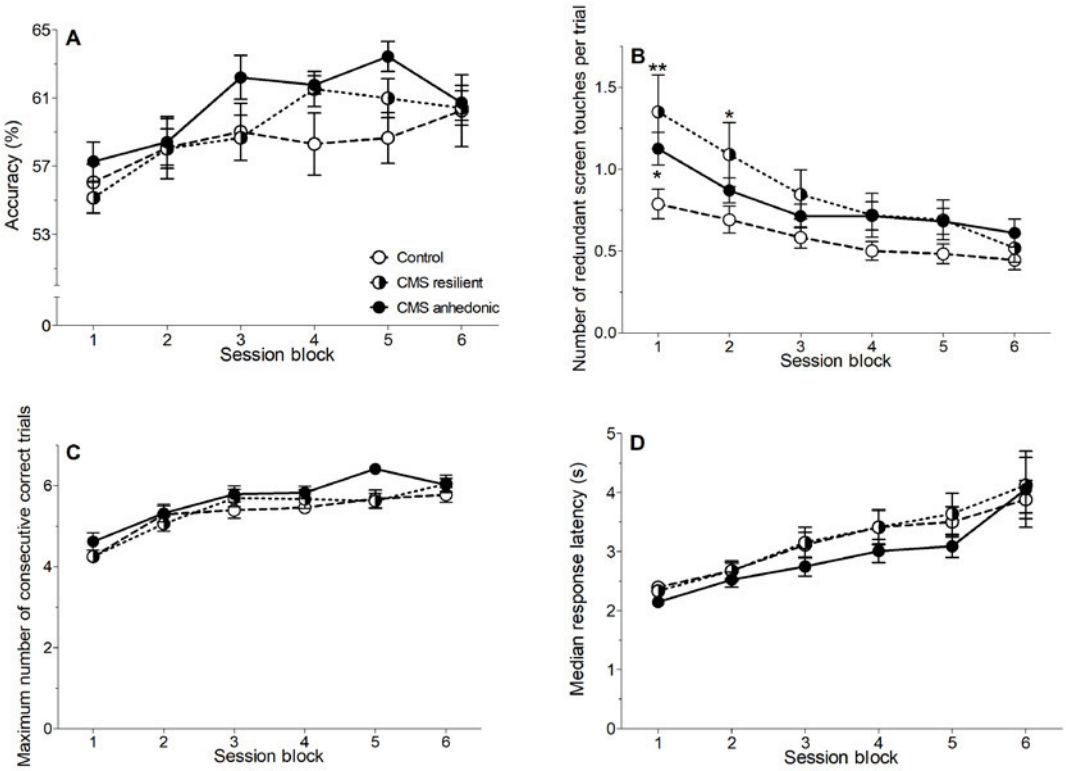

Figure 4. Learning parameters within the course of a session. (A) Percent of correct choices. **(B)** Number of redundant screen touches per trial. Post-hoc group-wise comparisons are indicated by $^{**}p < 0.01$, $^{*}p < 0.05$ comparing to the control group, respectively. **(C)** Maximum number of consecutive correct trials. **(D)** Average median response latency. Group means (± SEM) over the course of session blocks are displayed.

Retention of the dPAL task assessing long-term memory

Following dPAL acquisition and a 10-day hiatus, animals were retested on the dPAL task over two days to assess long-term memory performance. The final session of dPAL acquisition as well as the two retention sessions were included in the analysis (mixed model repeated measurements ANOVA).

Accuracy of performance was significantly decreased in the first retention session after the hiatus ($M = 74.30\%$, $SD = 6.42$) compared to accuracy at time of acquisition ($M =$

80.27%, *SD* = 6.21; post-hoc p = 0.002). However, accuracy increased from the first retention session to the second retention session (*M* = 80.47%, *SD* = 5.83; post-hoc p = 0.0001; main effect of session: $\chi^2(9) = 16.17$, $p = 0.0003$; Figure 5A).

Next, memory (difference in accuracy between the last session passing dPAL criterion and the first retention session) and relearning (difference in accuracy between the first and second retention session) were analysed separately with one-way ANOVA. Neither memory nor relearning performance differed statistically between groups. Individual changes in accuracy are shown in Figure 5B.

Hence, results show changes in performance due to the 10-day hiatus, but long-term memory differences were not observed between groups.

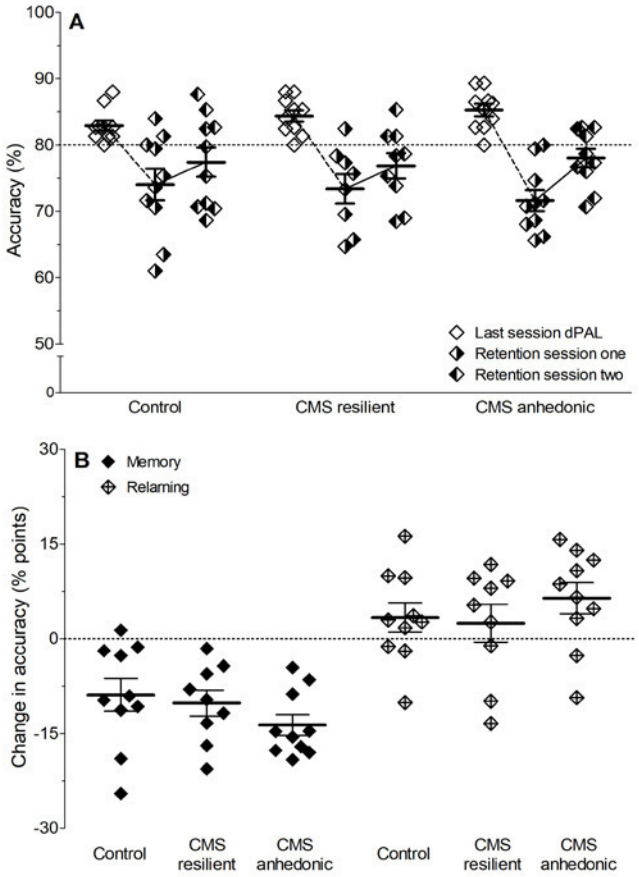

Figure 5. Long-term memory and relearning performance in the dPAL task. (A) Accuracy is shown for the last session before the 10 d hiatus and the two retention sessions afterwards. Changes in group accuracy are displayed for memory (····) and relearning (—). Passing criterion is indicated at 80% accuracy. **(B)** The rats' individual changes in accuracy from last dPAL criterion session to the first retention session (memory) and first to second retention session (relearning). Group means (± SEM) and individual results are displayed.

Discussion

In the present study, translational testing applying the touchscreen operant platform revealed cognitive impairments in the anhedonic-like, but not in the resilient subgroup of CMS exposed rats. This was mainly apparent from the trend that anhedonic-like rats appear to be

slower at acquiring the dPAL task compared to controls, whereas resilient rats appear to required comparable time to learn the dPAL touchscreen task as controls. However, CMS resilient rats displayed increased impulsive behaviour, as suggested from a higher number of redundant screen touches, than non-stressed controls and anhedonic-like rats. This suggests a differential but still efficient learning ability in the resilient group compared to controls. The results show that the cognitive impairments are specific to the depressive-like phenotype making it an excellent model for testing antidepressant drugs aiming to target both depressive and cognitive symptoms of MDD.

To our knowledge, the use of translational touchscreen testing in depression and anxiety models is not established[6] and, hence, the different parameters of touchscreen testing are discussed in detail here.

As shown previously[27–29], CMS induces reduced reward sensitivity, which is demonstrated by reduced sucrose consumption in a subgroup of stress exposed rats, whereas another subgroup is resilient and remains hedonic. Reduced reward sensitivity is believed to be the biological underpinning of the MDD core symptom anhedonia[30].

This study aimed to determine whether cognitive ability is altered in response to stress generally or specifically in association with the anhedonic-like phenotype, which appears more susceptible to the detrimental stress effects. The anhedonic-like rats tended to require more trials to acquire the dPAL task than non-stressed control rats, however, stress exposed resilient rats performed similar to controls. Hence, a trend of impaired learning seems specific to the depressive-like phenotype and not a consequence of stress exposure in general. Interestingly, the anhedonic-like group appears to split into two subgroups with respect to their touchscreen performance. Four rats needed evidently more trials than the remaining six rats of the group, which performed around the mean level of the resilient group (Figure 2A). These distinct performances within the anhedonic-like group may model that only a proportion of depressed patients display cognitive impairments. These findings potentially endorse the CMS model as preclinical model of MDD, because the cognitive impairments seem to be specifically associated with the incidence of anhedonia and not as a general consequence of CMS exposure.

It could be argued that prolonged dPAL acquisition in the anhedonic-like group is due to reduced motivation. However, reward collection latency did not differ between groups indicating similar motivation to consume the reward and perform the touchscreen task. Similarly, cognitive impairments in MDD patients are ascribed to deficits in cognition and not to a lack of motivation[7,31].

Median response latency did not differ significantly amongst groups although non-stressed controls appear to take longer to respond than resilient and anhedonic-like rats (Figure 2C). A similar trend of prolonged response latency in the dPAL task was found in sham compared to hippocampus lesioned mice[32]. Furthermore, temporal deactivation of the dorsal hippocampus with lidocaine significantly decreased response time in the dPAL task accompanied with decreased accuracy[33]. These findings may suggest impaired hippocampal functioning of CMS exposed rats in the dPAL task. This is supported by another study from our group, in which non-stressed controls took longer to respond than CMS exposed rats in a pairwise discrimination touchscreen task[29]. Hence, we interpret increased response latency in non-stressed controls as more comprehensive cognitive appraisal before making an active response in the touchscreen task.

Surprisingly, on average, anhedonic-like rats were able to perform a higher number of maximum consecutive correct trials than non-stressed controls. This was also evident from the learning curve, where anhedonic-like rats executed more consecutive correct trials than controls and resilient rats although all three groups increased the number of consecutive correct trials in the course of learning. This finding is counterintuitive since anhedonic-like rats tended to show overall inferior performance in the dPAL task acquisition. Furthermore, the opposite result was found in the human continuous performance test where untreated MDD patients reached a lower score of correct responses than treated MDD patients or healthy controls indicating decreased vigilance[34]. Still, maximum consecutive trials is parameter that reveals cognitive differences between the CMS resilient and the depressive-like phenotype.

The total number of correction trials needed to acquire the dPAL task was not significantly different between groups. However, anhedonic-like rats needed on average more correction trials than controls or resilient rats (Figure 2B), which might indicate learning deficits and is concordant with faster task acquisition in the controls and resilient rats. Regarding the learning curves, the number of correction trials decreased over time, as well as within a session, indicating improved task comprehension over time (Figure 3B).

Added numbers of redundant screen touches may suggest increased impulsive or habit-like behaviour and decreased control of executive function, which is a feature of the prefrontal cortex (PFC)[35,36]. The number of redundant screen touches per trial did not differ in summary statistic, although the average number of touches was lower in controls than in the two CMS groups. However, over the course of task acquisition resilient rats showed an increased number of redundant screen touches per trial, followed by anhedonic-like rats and then controls. This significant difference was evident in the beginning of dPAL learning and

diminished towards passing criterion in the dPAL task (Figure 3A). Similarly, within a session, the number of redundant screen touches decreased over time with controls executing the least number of redundant touches (Figure 4B). Ideally, we would expect only one touch per trial. Contrary to Talpos et al.[26] suggesting that the dPAL task may not be sufficiently sensitive for detecting failures in response inhibition as an effect of LSD treatment, we suggest from our present findings that an increased number of redundant touches display a failure in response inhibition in CMS exposed rats. In a classical operant learning study, stress exposed rats shifted from effortful decision-making to increased habit-like behaviour, which was accompanied by atrophy in the medial PFC and associative striatum and hypertrophy in the sensorimotor striatum[37]. Dias-Ferreira et al.[37] explained this behavioural shift as a coping strategy to avoid demanding, goal-directed behaviour during stress exposure. Thus, the present findings might suggest redundant screen touches as an indicator of utilization of different coping strategies and PFC functioning. Both CMS groups, especially the resilient group, seem to abandon demanding in favour of habitual behaviours whereas unchallenged controls confide to a greater extent on appraisal.

All groups showed a decrease in accuracy in the first retention session compared to their performance in the final dPAL session before the 10-day hiatus. Although the anhedonic-like group showed a greater decrease in accuracy (-13.6%) than CMS resilient (-10.2%) or controls (-8.9%; Figure 5B), no effect of group on memory performance was observed, indicating intact long-term memory in the CMS groups or failure to reach significance due to the high variance, especially in the control group.

It was unexpected that there was no effect of stress or anhedonia on long-term memory. Formation of long-term memory and object-in-place tasks are hippocampus-dependent[38,39] and both are main components of the rodent dPAL task[25]. It was shown that dPAL retrieval is impaired in rodents with dysfunctional dorsal hippocampi[33] as well as dPAL performance was impaired in mild cognitive impairment patients displaying altered hippocampal function in an fMRI version of the PAL task[40]. The hippocampal structure is known to be altered in depressed patients, e.g. decreased hippocampal volume in MDD patients, and memory is known to be impaired in these patients as well[17,41]. Moreover, subtle substructural changes in the hippocampus of CMS exposed rats exist[42], neurogenesis is decreased in the dentate gyrus by CMS exposure and recovered after antidepressant treatment[43]. Hence, altered long-term memory in the CMS depressive-like group may have been expected in the dPAL task. However, CMS exposure commenced long before dPAL retention and alternative brain structures could have taken over the role of the hippocampus. This theory is supported by a study in which only post- but not pre-acquisition hippocampal

lesioning severely impaired dPAL performance in mice[44]. Furthermore, lesioning is a severe manipulation compared to general stress exposure and, thus, likely led to smaller effects in the present study. The finding by Kim et al.[44] most likely explains why only minor impairments in hippocampus-relevant readouts were observed in the present study.

Another brain region that was shown to be involved in dPAL acquisition[45] and altered in MDD patients[46–48] is the PFC. In the present study, the increased number of redundant touches and the tendency of shortened response latency in the CMS groups indicate a lack of response inhibition, a function of the PFC. Hence, we conclude that the PFC was likely impaired by CMS exposure and such effects were observable in the dPAL touchscreen task.

Structural changes in the hippocampus[16,17,49,50] and frontal cortex[51–53] have been observed in depressed patients using neuroimaging. Consistent with this, preclinical imaging of CMS resilient and anhedonic-like animals suggests reorganization of the hippocampus depending on the hedonic state[42]. These findings are supported by our behavioural data showing an increased acquisition time of anhedonic-like rats in the hippocampus-dependent dPAL task. The present study has demonstrated that stress exposure directly induced cognitive impairments, especially in susceptible individuals. This may shed more light on the causal relationship of stress as a risk factor in disease development.

The behavioural changes observed in the present study were salient in visuo-spatial learning and attention. These processes appear to be a major contributor to disability in life functioning in humans even after half a year of remission from depression[7]. Hence, the present study is of high clinical relevance. Moreover, we applied chronic stress, which is a major risk factor in MDD, to provoke a depressive-like phenotype. Thus, clinical relevance and translational value of the present study is further supported.

A limitation of the present study is that the SCT test was abandoned during the touchscreen testing due to the sugary touchscreen pellets desensitising the rats for consumption of a dilute 1.5% sucrose solution. Hence, anhedonic-like rats could have recovered from their depressive-like state. However, it is known that rats recover spontaneously only after 4–5 weeks following cessation of CMS[21]. Furthermore, it is likely that the continuation with a modified CMS protocol during touchscreen testing delayed spontaneous recovery. Moreover, food restriction accompanying touchscreen testing may have added to the delaying effect of the modified CMS protocol. Muscat and Willner[54] have shown that a two-week over-night stress protocol elicited comparable hedonic phenotypes as the original CMS protocol. They applied similar over-night stressors as in our modified CMS protocol. Hence, it appears

likely that spontaneous recovery after cessation of the original CMS protocol was prevented by the modified version in the present study.

Another limitation is that baseline cognition was not assessed in the rats before stress exposure commenced for logistic reasons, thus we cannot exclude that a cognitive predisposition directed the development of a certain stress phenotype.

In summary, the present study demonstrated that cognitive impairments tend to be specifically associated with the depressive-like phenotype. These impairments were not a result of lacking motivation but can be attributed to cognitive deficits. In both CMS groups, response inhibition was impaired indicating deficits in executive functions as result of stress exposure. Surprisingly, anhedonic-like rats showed superior sustained attention, which was, however, not reflected in their overall performance. Furthermore, outcomes of the stress resilient group revealed differences in cognitive strategies compared to the depressive-like group, which potentially may be integrated in human studies and therapy.

To our knowledge, this is the first study to show that the touchscreen dPAL task can potentially be applied to detect depression-associated cognitive impairments in a preclinical MDD stress rat model. Accordingly, the present study suggests CMS anhedonic-like rats, assessed with touchscreen tasks, as a translational, standardized and well-validated platform for developing and screening novel pro-cognitive antidepressant treatment regimens, which are deemed necessary for obtaining higher remission rates of MDD and reducing risk of relapse.

Materials and Methods

Animals

Male Long Evans rats (LE; Janvier Labs, France) were 5–6 weeks and 100–120 g at arrival to our facility. Originally, 174 LE rats (n (control) = 24, n (CMS) = 150) were purchased and used for different studies.

Animals were housed four per cage for one week and afterwards they were single-housed. Rats were kept on a 12-h light/dark cycle (lights on at 6:00 am) with free access to food and water (otherwise stated). All experiments were conducted according to and approved by the Danish National Committee for Ethics in Animal Experimentation (2013-15-2934-00814).

A timeline of the experiment is shown in Figure 6.

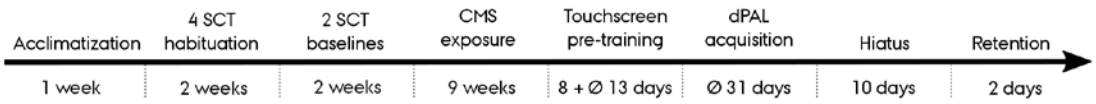

Figure 6. Experimental timeline. Depiction of the different stages of the experiment and their duration. Touchscreen pre-training included 8 days of gradual food restriction to 80% of *ad libitum* intake, followed by operant conditioning in the touchscreen setup. The acquisition of the touchscreen dPAL task was conducted until passing criterion was reached and retention was determined in two additional dPAL sessions after 10-day hiatus without testing. (SCT–sucrose consumption test, CMS–chronic mild stress, dPAL–different paired-associates learning, Ø–average time for rats to learn the relevant stage).

Chronic Mild Stress paradigm

Baseline sucrose consumption test

The SCT was carried out to assess the rats' hedonic state during stress exposure. Animals were acclimatized to the facility for one week. In the next two weeks, rats were habituated to SCTs by drinking a palatable sucrose solution (1.5%) semi-weekly for 1 h following 14 h of food and water deprivation. Thereafter, weekly SCTs were carried out twice and averaged to a baseline sucrose consumption for each rat individually (Figure 6). Animals were split in two groups with equal group mean and standard deviation (SD) of their baseline sucrose consumption. CMS exposure was initiated for one of the groups and the other group was housed in a separate room and left unchallenged. Weekly SCTs were conducted throughout the CMS paradigm.

CMS paradigm and hedonic state

Rats entering the CMS paradigm were exposed to a series of stressors lasting between 5–14 h[43]. Stress duration and type of stressors were varied across a two-week protocol (Figure 7) to increase unpredictability of stressors and avoid habituation. During the stressor "grouping", a CMS rat was transferred to the home cage of another CMS rat (resident-intruder). Grouping partners were exchanged weekly and individual rats were alternated in being resident or intruder. After 10 weeks of CMS, the stress exposed group was divided in subgroups depending on their sucrose index (mean of last two SCTs during CMS / baseline SCT). Rats were categorized as stress-susceptible, thus anhedonic-like, with a SCT index $\leq$ 0.7 and as stress resilient with a SCT index $\geq$ 0.9 based on an *a priori* criteria used in previous studies[21]. Rats with a change in sucrose consumption in between these marginal phenotypes were excluded. According to these criteria, approximately 19% of CMS rats

showed a resilient phenotype and 41% an anhedonic-like phenotype. Consequently, 58 rats would have been needed to undergo the CMS paradigm to obtain the number of rats subjected to this touchscreen study.

Once touchscreen training began, the CMS protocol was modified (Figure 7) to avoid interference with training. Food and water deprivation were abandoned since this would be a confounding factor on the motivation for obtaining sugar pellet rewards, furthermore stressors were only applied during the night.

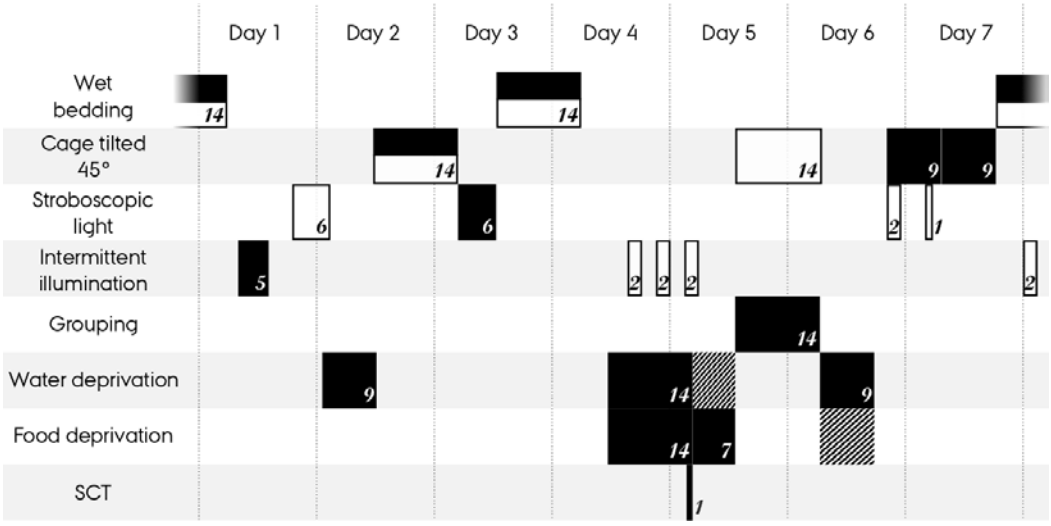

Figure 7. Original and modified two weeks CMS protocol. The listed stressors were applied for the duration (h) indicated by the respective number. Black bars show the original stress protocol and white bars the touchscreen adapted CMS protocol. Crosshatched bars indicate that these stressors were applied in the second week of the two-week repeated schedule replacing the black bars shown for the same time point. The SCT and the 14 h food and water deprivation on day 4–5 were applied to the stress as well as control group.

Touchscreen operant platform

Learning and memory was assessed with the translational touchscreen platform applying the PAL task.

Apparatus

The Bussey-Saksida operant chambers (Campden Instruments Ltd., Loughborough, UK) are sound- and light attenuated boxes including a trapezoid shaped interior (height 300 mm, length 332 mm, width screen 240 mm, width magazine 126 mm). A touch-sensitive screen was covered by a mask leaving three windows open (height 100 mm, width 60 mm) and built in opposite a reward delivery system (magazine). A spring-hinged shelf (90°) was installed

below the mask windows to slow the rat down before touching the screen and avoiding hasty choices. The chambers were further equipped with a house and magazine light, a metal grid floor, a tone generator and a fan, which ensured sufficient ventilation and masked external noise. The touchscreen program was controlled by Whisker Server Abett II (Campden Instruments Ltd.).

Touchscreen pre-training

Initially rats were pre-trained using the touchscreen setup before learning the actual dPAL task. Rats were food restricted to reinforce operant conditioning. Food was gradually decreased by 5% every second day to 80% of free feeding consumption. On the last two days, rats were habituated to consume five touchscreen reward pellets (sugar coated, 45 mg dustless precision pellets, Bio Serv, Flemington, NJ, USA) in their home cage. Body weight was monitored daily. Touchscreen training was carried out every day during the light phase in a session of 45 min or 75 trials maximum (except for "habituation" step). All rats were moved to the testing room 30 min prior to touchscreen training. Pre-training consisted of five steps[24]. First, in the "habituation" step, rats were left in the touchscreen box with house light off for 30 min and had to consume five reward pellets from the food magazine. Second, "initial touch", rats automatically received one reward pellet every 30 s or three reward pellets if the rat touched the stimulus (randomly, one of the three touchscreen windows was illuminated). Reward collection was followed by a 20 s inter-trial-interval (ITI) after which the next trial would automatically start. Rats were moved on to "must touch" if they touched the stimulus ≥ 30 times in one session (passing) or, alternatively, if they touched the stimulus ≤ 5 times per session on two consecutive days (failing). In "must touch", the rat had to touch the stimulus in order to receive a reward pellet. If the rat was new to "must touch" or had less than 40 touches the day before, peanut butter was introduced to each screen window prior to session start to draw attention to the screen. If the rat touched the stimulus ≤ 5 times per session on two consecutive days (failing), it was moved back to "initial touch" (only if it had failed "initial touch") or passed on to "must initiate" if it touched the stimulus 75 times within a session. "Must initiate" was similar to "must touch", additionally the rat had to initiate each trial by nose poking in the food magazine. Finally during "punish incorrect", a touch on the two non-illuminated windows on the screen resulted in a 5 s time-out period with house light on, followed by the ITI. To pass, the rat had to accomplish 75 trials within 45 min with at least 60 correct touches to the illuminated window (≥ 80% accuracy) for two consecutive days. The rat was stressed again for 3 h ("grouping") the day following pre-

training as a reminder of the original stress protocol and hence received one day without touchscreen testing.

Different paired-associates learning task

dPAL training[24] began the day after grouping. In this task, three symbols (white on black background) should be associated with one of the touchscreen windows, respectively (Figure 8A). A session followed the same rules as in "punish incorrect", but instead of one illuminated and two blank windows in each trial, two windows displayed two of the three symbols. One of the symbols would be in its correct window, whereas the other one in an incorrect window. The remaining window was left blank (Figure 8B). This resulted in six different experimental trials, which were randomly balanced within a session. A correct response was registered if the rat touched the symbol that was displayed in the correct location. An incorrect response was followed by a 5 s time-out with house light on. After the ITI, a correction trial was initiated, meaning the previous incorrectly responded trial was displayed again. If the rat responded incorrectly to the correction trial, another one would be displayed until the rat managed a correct choice. Criterion to pass was accomplished by completing 75 trials (not counting correction trials) within 45 min, with 80% accuracy, on two consecutive days.

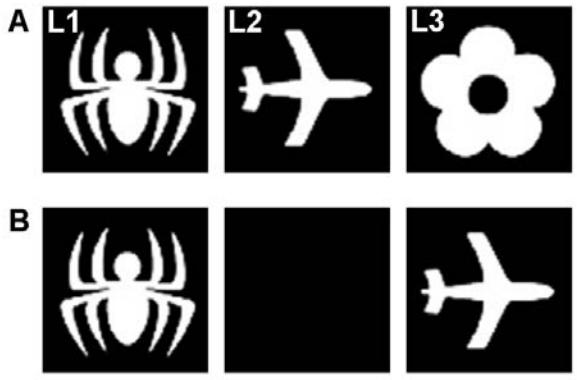

Figure 8. Different paired-associates learning task scheme. (A) Each symbol is shown in its correct location (L): spider-L1, plane-L2, flower-L3. (B) An example trial is displayed with one symbol (spider) in its correct location, and one symbol (plane) in an incorrect location.

Retention

Passing dPAL was followed by 10 days without touchscreen testing and an increase in food accounting for the lack of reward pellets. After the 10-day hiatus, rats were retested on the dPAL task for two days.

Statistical Analysis

SCT data were analysed without the baseline SCT applying mixed effects model for repeated measurements with post-hoc Bonferroni-corrected pairwise group comparisons. dPAL

summary statistics was evaluated with Shapiro-Wilk test for residual normality and Levene's test for homogeneity of variance with non-significant results allowing for statistical analysis by ANOVA and LSD post-hoc analysis. dPAL repeated measurements data were analysed using univariate repeated measurements ANOVA and Greenhouse-Geisser correction if sphericity was violated. Retention was analysed applying multivariate repeated measures ANOVA. Moreover, memory and relearning performance were analysed by one-way ANOVA, and assumption of normality and homogeneity were reviewed. Rats that did not pass the dPAL task were removed from summary statistics and retention only (two CMS resilient and one control rat). Data of summary statistic and retention were analysed for outliers with Grubb's test ($\alpha = 0.05$) and ROUT test (Q = 1%; GraphPad Prism 6, GraphPad Software Inc., California, USA) and revealed no outliers. Statistical analysis was conducted with RStudio (RStudio Inc., Massachusetts, USA) and rdata.online (Montreal, Canada). Data was displayed with GraphPad Prism 5.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

L.-S.M. and O.W. designed the experiment. L.-S. M. and C.B. performed the experiment. L.-S.M. conducted data analysis and prepared figures. L.-S.M. wrote the manuscript with input from M.C.H. and O.W. All authors reviewed the manuscript.

Competing financial interest

The authors declare no competing financial interests.

Manuscript III

Vortioxetine recovers anhedonic-like behaviour and promotes strategic cognitive performance in a rodent touchscreen task

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Abstract

Depression-associated cognitive impairments affect daily functioning in major depressive disorder (MDD). Furthermore, cognitive impairments are the most prevalent and persistent symptom during remission from a depressive episode and thus increase the risk of relapse. Consequently, development of antidepressant drugs alleviating cognitive impairments is vital. The multimodal antidepressant drug, vortioxetine, has been approved for having a pro-cognitive effect on top of mitigating the affective symptoms of depression. In the present study, chronic mild stress (CMS) exposed, anhedonic-like rats were submitted to chronic treatment with vortioxetine. In more than half of the treated rats, the affective symptom, anhedonia, was relieved. These treatment high responders as well as low responders, untreated anhedonic-like and non-stressed controls were subjected to translational touchscreen testing for assessing their cognitive performance. Rodent touchscreen testing was developed based on the human Cambridge Neuropsychological Test Automated Battery (CANTAB) test battery, the most frequently used tool for assessing cognition in depression research. Our data show that vortioxetine improves some aspects of learning, but not memory performance. Notably, vortioxetine induces a shift from appraised cognitive evaluation to stereotypic, habit-like behaviour in the touchscreen paired-associates learning task. To supplement the observed neurobehavioral results, expression of genes important for

stress responses, neuropsychiatric disorders and synaptic plasticity were investigated. Minor alterations in prefrontal cortex and hippocampal gene expression patterns were found in association with anhedonia or vortioxetine treatment. The present study benefits from using clinically relevant approaches, such as the CMS paradigm and translational touchscreen testing. In summary, our findings suggest vortioxetine mediates an antidepressant-like effect in the validated CMS model and affects cognition in some domains, but further investigations are needed to clarify this further.

1 Introduction

Worldwide, around 300 million people suffer from depression, constituting major depressive disorder (MDD) as the leading burden of disability worldwide[1] and additionally impacting the patients' socio-economic environment. The relapsing nature of the disease as well as the insufficient treatment responses of only 50%, to a two-step treatment regimen, add to the devastating burden of the disease[2]. Core symptoms of MDD are lack of energy, depressed mood and an attenuated anticipation or experience of pleasure (anhedonia). Additionally, patients suffer from a variable number of associated symptoms, such as impaired cognitive abilities, primarily in attention, executive functions and memory. These cognitive symptoms persist in 30–60% of treated patients after remission from the affective MDD symptoms. Furthermore, cognitive impairment is the most persisting residual symptom of depression and, hence, it continues to decrease daily functioning and quality of life after remission[3–6]. Moreover, persistent cognitive impairment augments risk of relapse and is increasingly regarded as core component rather than an epiphenomenon of depression[7,8]. Recovering from cognitive symptoms is associated with a rapid remission from depression[9], further underlining the importance of restoring cognitive impairments when treating depression.

However, current antidepressant treatment focuses mainly on alleviating the affective state, thus, neglecting cognitive impairments[10]. Treated MDD patients show improved cognitive performance compared to untreated patients, but fail to accomplish the performance level of healthy controls[11]. Therefore, development of novel, pro-cognitive antidepressants are vital for complete remission of MDD. Thus, the demand is high for clinically relevant drug screening in a well-validated preclinical depression model exhibiting depression-associated cognitive impairments. In a previous study (Martis, L.-S., Brision, C., Holmes M., Wiborg O.; unpublished data), we demonstrated that the chronic mild stress (CMS) paradigm fulfils exactly these criteria. The CMS model is well known for exhibiting the MDD core symptom anhedonia (face validity) evoked by stress exposure (etiological validity). Additionally, CMS anhedonic-like rats display depression-associated cognitive

impairments. They take longer to acquire a translational touchscreen task than non-stressed controls. Hence, cognitive impairments are specific to the depression-like phenotype and in the present study we follow up by assessing the novel antidepressant drug vortioxetine in the CMS model.

Vortioxetine was approved in 2013[12]. On top of alleviating mood symptoms, a direct pro-cognitive effect was ascribed to vortioxetine via its multimodal mechanism of action[13]. Vortioxetine operates as a serotonin (5-HT) transporter (SERT) inhibitor; 5-HT_3, 5-HT_7 and 5-HT_{1D} receptor antagonist; 5-HT_{1B} receptor partial agonist and 5-HT_{1A} receptor agonist[14]. In rodents, vortioxetine improves spatial working memory, visuo-spatial memory and contextual fear memory besides increasing synaptic plasticity and decreasing behaviour despair[15–19]. In MDD patients, executive functions, attention, speed of processing, verbal learning and memory functions, as well as affective symptoms, have been shown to be recovered by vortioxetine treatment[20]. The CMS model shows good predictive validity with antidepressant, such as desipramine or escitalopram[21,22] and thus, we investigated in the present study if vortioxetine can restore the hedonic phenotype of CMS exposed, anhedonic-like rats. Furthermore, cognition of non-stressed controls, untreated and vortioxetine treated CMS anhedonic-like rats was assessed in the different paired-associated learning (dPAL) touchscreen task, a standardized and translational tool in clinical as well as in preclinical research[23,24]. The rodent touchscreen platform involves appetitive operant condition and was developed based on the human Cambridge Neuropsychological Test Automated Battery (CANTAB); the most frequently applied cognitive assessment tool in depression research[4]. Finally, hippocampal (HPC) and prefrontal cortex (PFC) gene expression was analysed to link neurobehavioral alterations with underlying molecular changes. Genes that are known to play a role in MDD and psychiatric disorders and/or the stress response, such as the mineralocorticoid receptor (*Mr*), glucocorticoid receptor (*Gr*), FK506 binding protein 5 (*Fkbp5*), glycogen synthase kinase 3 beta (*Gsk3b*), disrupted in Schizophrenia 1 (*Disc1*), homer scaffolding protein 1-3 (*Homer1-3*) and brain-derived neurotrophic factor (*Bdnf*) as well as genes important in cognition and neuronal plasticity, such as neuroregulin 1 (*Nrg1*), *Shank 1*-3, *Spinophilin* and *Cofilin 1* were analysed.

In short, this study aimed to investigate the effect of vortioxetine on the affective state, cerebral gene expression and cognitive performance in a translational touchscreen task. Furthermore, the relationship between the affective state and cognitive functions was evaluated.

2 Materials and Methods

2.1 Animals

Male Long Evans (LE) rats (n = 242; Janvier Labs, France) were 5–6 weeks of age weighing 100–120 g at arrival in our facility (Translational Neuropsychiatry Unit, Aarhus University). Rats were acclimatized housed in groups of four for one week, followed by single housing for the remainder of the experiment. Animals had free access to food and water (otherwise stated) and kept on a 12 h light-dark cycle (lights on at 6 am). All experiments were conducted according to and approved by the Danish National Committee for Ethics in Animal Experimentation (2013-15-2934-00814).

2.2 Chronic mild stress paradigm

2.2.1 Sucrose consumption test

Sucrose consumption tests (SCTs) were carried out to assess the hedonic state of each rat during stress exposure and antidepressant treatment. First, animals were habituated to drink a palatable sucrose solution (1.5%) by exchanging their regular drinking water with sucrose solution for 15 h in one week and for 1 h in the following week. The 1 h exposure was preceded by a 14 h food and water deprivation. Thereafter, three weekly baseline measurements were conducted (1 h SCT following 14 h of food and water deprivation) and averaged to a SCT baseline for each rat individually. After removing animals with a baseline ≤ 10 mg due to a potential floor effect, rats were split (same baseline group *mean* and *SD*) in a control (n = 44) and CMS group (n = 175). Stress exposure for the latter group commenced after SCT baseline acquisition. Weekly SCTs were continued throughout the experiment. In the following, SCT indexes (SCT normalised to baseline) were used to determine CMS susceptible, i.e. anhedonic-like, rats and eventually high and low responders to antidepressant treatment. For clarification of the experimental pipeline, a simplified outline is illustrated in Figure 1.

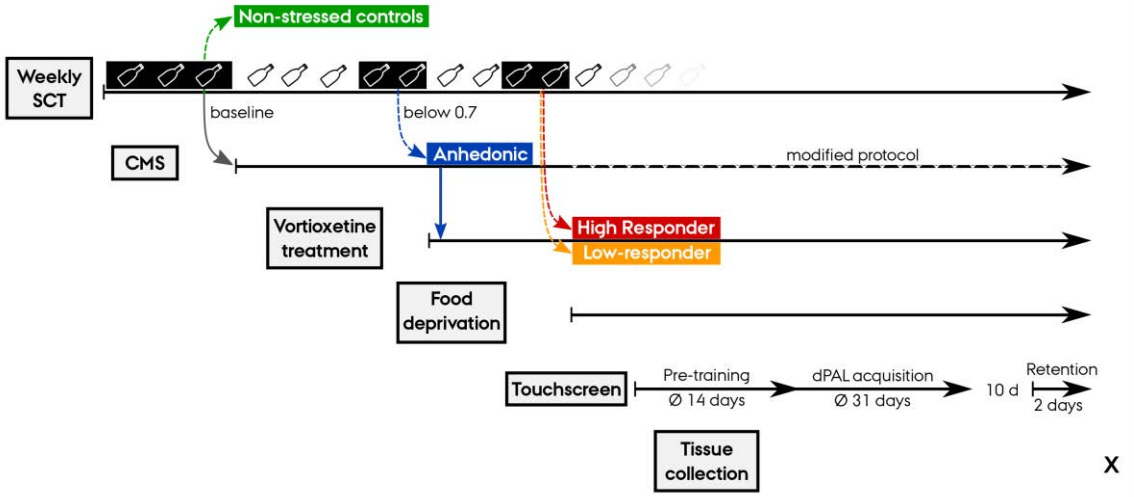

Figure 1. Simplified experimental pipeline. Sucrose consumption tests (SCTs) were conducted throughout the experiment to measure baseline sucrose intake, alterations in sucrose consumption due to chronic mild stress (CMS; determining anhedonic-like rats) and subsequent antidepressant treatment with vortioxetine (determining the 30% highest and lowest responders to treatment). SCTs also revealed the rats' response to touchscreen testing, which included food reduction, pre-training, dPAL task acquisition and retention. Brains were collected for gene expression studies at the end of the behavioural testing.

2.2.2 Stressors

The CMS paradigm was initiated after the third SCT baseline measurement (Figure 1) and the animals' body weight was monitored weekly. Rats placed in the CMS group were exposed to several mild stressors in a 2-week repeated protocol (Table 1) to provoke a depressive-like phenotype. Variation of stressor duration and exposure time point increased the unpredictability of the stressors, hence, decreased habituation to the stress protocol.

Table 1. Two-week chronic mild stress (CMS) protocol. Mild stressors and their duration are shown. During "grouping", one CMS rat (intruder) was introduced to the home cage of another CMS rat (resident). Grouping pairs as well as acting as resident or intruder was alternated weekly.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning	New cage*, intermittent illumination (5 h)	Water deprivation (9 h)	Stroboscopic light (6 h)	New cages	SCT (1 h)*, alternating food or water deprivation (7 h)	Water or food deprivation⁺ (7 h)	Cage tilted 45° (9 h)
Evening		Cage tilted 45° (14 h)	Wet bedding (14 h)	Remove food and water (14 h)*	Grouping (14 h)	Cage tilted 45° (14 h)	Wet bedding (14 h)

*For CMS and non-stressed controls, ⁺Alternating weekly.

After five weeks of CMS, stress exposed rats with a SCT index (SCT/baseline SCT) ≤ 0.7 for both, the last SCT and the average of the last two SCTs were categorized anhedonic-like according to an *a priori* cutoff[25,26] and remained in the study together with the non-stressed control rats (SCT index ≥ 0.9).

After nine weeks of CMS, a modified CMS protocol was applied to adjust for touchscreen training during daytime (Figure 1). The stressors "water and/or food deprivation" were removed from the modified CMS protocol to avoid interference with the touchscreen food reward and food deprivation accompanying touchscreen training. Furthermore, stressors were only applied during the night leaving the day for touchscreen assessment. Every Friday, the SCT was carried out followed by 4 h of grouping and light stressors. Thus, touchscreen testing was discontinued for that day. The modified CMS schedule (Supplementary Table 1) was changed every second week to prevent habituation to the milder stress protocol.

2.3 Drug administration

After five weeks of CMS, two-thirds of the 54 anhedonic-like animals (i.e. 34 rats with an average SCT index of week four and five ≤ 0.7) were treated with the antidepressant vortioxetine (Figure 1). Standard rat chow (Altomin 13324, Brogaarden, Denmark) was supplemented with vortioxetine (Carbosynth Ltd., UK) at a concentration of 1.8 g/kg rat chow. The original CMS paradigm was continued during treatment for all anhedonic-like rats for four more weeks. Then, treated rats were subdivided into high responders (responders; the 30% of animals with the greatest recovery determined by the weekly SCT indexes, i.e. $n = 10$) and treatment poor responders (low-responders; the 30% of animals with the lowest response to treatment determined by the weekly SCT indexes) and subjected to touchscreen testing.

2.4 Touchscreen operant platform

2.4.1 Apparatus

The sound- and light-attenuating Bussey-Saksida touchscreen operant chambers (Campden Instruments Ltd., Loughborough, UK) contained a trapezoid shaped interior (height 300 mm, length 332 mm, width screen 240 mm, width magazine 126 mm). A touch-sensitive screen was located at the wide side of the interior box and a reward delivery system (magazine) at the opposite small side. A mask covered the touchscreen leaving only three windows (100 x 60 mm) for the rat to touch the screen. A spring-hinged shelf (90°) in front of the mask

slowed the rats down preventing hasty and unconsidered choices to the screen. A fan ensured sufficient ventilation and masking of external noise. The chambers were further equipped with a grid floor, house and magazine light, and a tone generator. The touchscreen program was controlled by Whisker Server and Abett II software (Campden Instruments Ltd.).

2.4.2 Food reduction and touchscreen pre-training

Three baselines of *ad libitum* food intake were obtained for each rat. Rats were gradually food deprived to 75% of their individual baseline consumption (Table 2). The rats' body weight was monitored daily to ensure they maintain at least 90% of their body weight during food deprivation. Additionally, rats were introduced to peanut butter (Bilka, Denmark) and bacon pellets (45 mg dustless precision pellets, Bio Serv, Flemington, NJ, USA) used for operant conditioning during touchscreen testing.

Table 2. Gradual food restriction regime prior to touchscreen pre-training. Parallel to food restriction, rats were habituated in their home cage to peanut butter (PB) as well as to bacon pellets (BP) used as reward in touchscreen (TS) testing.

Day of food restriction	1	2	3	4	5	6	7	8	TS 1
% of baseline food intake	95	95	90	90	85	80	80	75	75
Habituation to						BP	BP	PB	PB

Pre-training began after day eight of food restriction. In four steps, the rats were conditioned to operate the touchscreen chamber (Figure 2). Each rat was tested once daily Saturday to Thursday with a session lasting maximum 45 min or 75 trials. In the first pre-training step, "initial touch", rats received automatically a bacon pellet after 30 s of stimulus presentation (randomly one of the three windows was illuminated). Three instead of one reward pellet would appear if the rat touched the screen during stimulus presentation. Reward pellet dispensation always coincided with stimuli disappearance, a tone and food magazine illumination throughout touchscreen assessment. After reward collection a 20 s inter-trial-interval (ITI) started. A new trial followed. Rats passed "initial touch" by executing 30 or more screen touches. Rats, which failed to touch the screen, were also moved on to the next step to encourage them in an active participation and with the option to return them to "initial touch" (Figure 2). In "must touch", rats had to touch the screen to receive one reward pellet. Before session start, peanut butter was applied to all three windows to attract the rats to the screen. Peanut butter was not applied anymore if rats completed ≥ 40 trials in a previous "must touch" session. Rats passed by completing 75 screen touches, thus 75 trials,

within 45 min. Rats with a low number of trials were moved back to "initial touch" to increase their motivation if they had not passed "initial touch" before (Figure 2). In the third step "must initiate", rats had to initiate each trial after the ITI by poking into the illuminated food magazine. Otherwise, the trial followed the concept of "must touch". Finally, in "punish incorrect", rats only received the reward touching the illuminated window. A touch to a blank window was punished by a 5 s time out interval with house light on and followed by the ITI. Such an incorrect trial was ensued by a "correction trial" in which the same window as before was illuminated. Passing "punish incorrect" by completing 75 trials (excluding correction trials) within 45 min with at least 60 correct choices (≥ 80% accuracy) on two consecutive days equalled the end of pre-training.

Rats were kept on 80% of their baseline food intake for pre-training and the dPAL task. Only during initial touch, food was reduced further by 5% accounting for the high availability of reward pellets during this step.

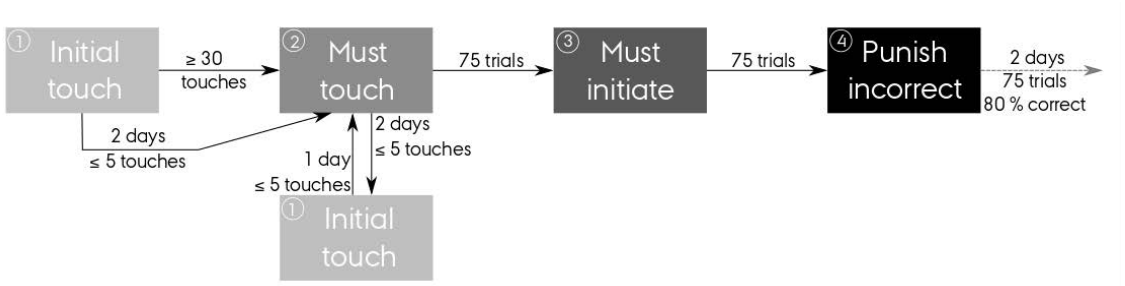

Figure 2. The four stages of touchscreen pre-training (1-4). Passing criteria to move on to the next stage are indicated next to the arrows. Peanut butter was added to the screen when the rat entered "must touch" or if it performed ≤ 40 touches in the last "must touch" session.

2.4.3 Paired-associates learning touchscreen task

Cognitive performance was assessed applying the dPAL task. The rat had to associate a symbol (white on black background) with a specific location on the touchscreen. In each trial, only two of the three symbols (spider, flower, plane) would be displayed, one at its correct location (S+) and the other symbol at an incorrect location (S-). The third window was left blank (Figure 3). A touch to S+ resulted in reward pellet delivery followed by the ITI. S- was ensued by a 5 s time out with house light on, the ITI and a correction trial. The six trial types resulting from the stimulus-location association pairs were balanced over the course of a session. dPAL criterion was achieved by completing 75 trials (excluding correction trials) with at least 60 correct trials (≥ 80% accuracy) within 45 min on two consecutive days. Rats that did not acquire the task within 46 session were marked as failing

the task by an *a priori* criterion from a previous study (Martis, L.-S., Brision, C., Holmes, M., Wiborg, O.; unpublished data).

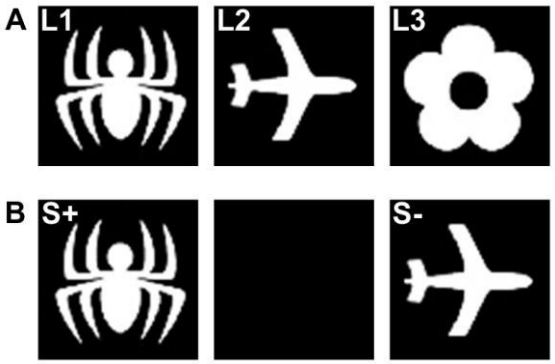

Figure 3. The different paired-associates learning task's object-location association pairs. (A) The three symbols are shown in their correct location (L) on the touchscreen (Spider-L1, Plane-L2, Flower-L3). (B) An example trial of the six possible trial types. The spider is displayed in its correct location L1 (S+), whereas the plane is presented in an incorrect location (S-). The third window is blank.

2.4.4 Retention of the dPAL task

Passing the dPAL task was ensued by a 10-day hiatus without touchscreen testing and an increase in food substituting for the touchscreen pellet deficiency. Next, rats were re-tested on the dPAL task for two days assessing long-term memory.

2.5 Cerebral gene expression

2.5.1 Tissue collection

Rats were decapitated within 1–3 days (*Mean* = 1.3 days) after completing the dPAL retention testing. Culling took place in the afternoon (2–4 pm) and the last feeding the day before at 1 pm. The brain was removed. Prefrontal cortex (PFC), dorsal and ventral hippocampus (HPC) were collected and frozen with powdered dry-ice immediately.

2.5.2 RNA extraction and cDNA synthesis

Tissue was stored at -80 °C until extraction of RNA with the ParisTM RNA and protein isolation kit (Ambion, TX, USA). The isolation procedure is well-established in our laboratory and was processed as previously described[27]. The RNA concentration and the purity were determined by a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Delaware, USA). Before cDNA synthesis, the RNA concentration of the samples was adjusted to match the sample with the lowest concentration determined by the NanoDrop spectrophotometer. RNA was reversely transcribed using random primers and Superscript IV Reverse Transcriptase (Invitrogen, CA, USA) following manufacturer's instructions and with a RNA input concentration per reaction of 129 ng/µl and 12.8 ng/µl for PFC and HPC, respectively. The cDNA samples were stored undiluted at -80 °C until quantitative real-time

polymerase chain reaction (real-time qPCR) analysis. Samples from PFC were diluted 1:48 and samples from HPC 1:10 with DEPC-treated water prior to real-time qPCR analysis.

2.5.3 Real-time qPCR

Real-time qPCR was carried out on individual samples in 96-well PCR-plates using the Mx3005P (Stratagene, La Jolla, CA, USA) and SYBR Green as described previously[28]. The gene expression of eight different reference genes (*18s rRNA, ActB, CycA, Gapd, Hmbs, Hprt1, Rpl13A* and *Ywhaz*) and 15 different target genes (*Gr, Mr, Bdnf, Fkbp5, Disc1, Gsk3b, Nrg1, Shank1-3, Homer1-3*, *Spinophilin* and *Cofilin 1*) were investigated. Essential gene specific data about primer sequence and amplicon sizes are given in Supplementary Table 2. Briefly, each SYBR Green reaction (10 µl total volume) contained 1 × SYBR Green master mix (Sigma-Aldrich, St. Louis, MO, USA), 0.5 µM primer pairs, and 3 µl of diluted cDNA. The thermal conditions for the PCR were 3 min at 95 °C to activate the hot-start iTaqDNA polymerase, followed by 40 cycles of 10 s denaturation at 95 °C, 30 s annealing at 60 °C, and 60 s extension at 72 °C. Each run was completed by dissociation curve analysis to confirm the amplification specificity and absence of primer dimers. A standard curve performed in duplicate was included on each plate. For data normalization, we first measured mRNA levels for the reference genes. Stability comparison of the expression of the eight reference genes was conducted with the Normfinder software (http://www.mdl.dk)[29] and the best combination selected. Values for each individual were normalized with the geometric mean of the reference genes *ActB* and *Rpl13A* (PFC) as well as *Hprt* and *CycA* (HPC), respectively.

2.6 Statistical Analysis

SCT data were analysed with univariate repeated measurements ANOVA and group comparisons were Bonferroni corrected. SCT data are displayed and were included in the analysis until the time point when the first animal completed the experiment and took no longer part in the SCTs.

Number of redundant screen touches resembles the amount of additional screen touches per trial to the one needed to make a choice. Redundant number of screen touches were normalised to the total number of trials (trials plus correction trials).

Summary statistics of the dPAL task (3.2.1) were analysed applying two-way ANOVA (hedonic state x treatment) or by rank aligned two-way ANOVA if assumptions of normality (assessed with QQ-plots) or homogeneity of variance (assessed with Bartlett's test) were violated. Animals that did not acquire the dPAL task within 46 sessions (one

control, three anhedonic-like rats, one responder, one low-responder) had to be excluded from this analysis due to missing data points, e.g. number of trials needed to acquire the task. Furthermore, outliers determined by Grubbs ($\alpha = 0.05$) or ROUT ($Q = 1\%$) test (Prism 6, ©2012 GraphPad Software Inc., California, USA) were excluded.

Repeated measurements data analysing learning behaviour across the task (3.2.2) and learning behaviour within a session (3.2.3) included all animals (acquiring and failing dPAL acquisition). The data were analysed with repeated measures ANOVA of type III if significant interaction effect was present, otherwise with type II. Mauchly's sphericity test, if significant, led to Greenhouse-Geisser ($\varepsilon < 0.75$) or Huynh-Feldt correction. Post-hoc comparisons were carried out with Fisher's least significant difference (LSD).

Retention data (accuracy of the final session of dPAL acquisition and both retention sessions) were analysed with multivariate repeated measures ANOVA. In a separate analysis of memory performance (difference in accuracy of the final dPAL session and first retention session) and relearning performance (difference in accuracy of first and second retention session) were analysed by two-way ANOVA just as summary statistics.

Normalised target genes were displayed as percent of control group mean (PFC data) or percent of dorsal HPC control mean (dorsal and ventral HPC data). The effect of the hedonic state and treatment on PFC, dorsal and ventral HPC gene expression was examined with a two-way ANOVA or rank aligned two-way ANOVA. Differences between dorsal and ventral HPC gene expression were analysed with Student's *t*-test.

Statistical analysis was performed with RStudio (Version 0.99.892, Boston, USA) and data displayed with GraphPad Prism 5 (©1992-2007, GraphPad Software, Inc).

3 Results

3.1 Hedonic status changes in response to CMS and Vortioxetine treatment

Non-treated CMS anhedonic-like rats consumed significantly less sucrose solution over the course of all SCTs than non-stressed control rats ($p < 0.0001$). Vortioxetine treated anhedonic-like rats responded individually to antidepressant treatment. Some rats (65%) responded well and their sucrose intake was not statistically significant different from non-stressed controls, but significantly increased compared to non-treated anhedonic-like rats ($p < 0.0001$). Rats that responded poor to vortioxetine, thus low-responders, consumed significantly less sucrose than responders ($p < 0.0001$) or non-stressed controls ($p < 0.0001$),

but were not statistically significantly different to untreated anhedonic-like rats (interaction effect of group x time: $\chi^2(45) = 187.31$, $p < 0.0001$; Figure 4).

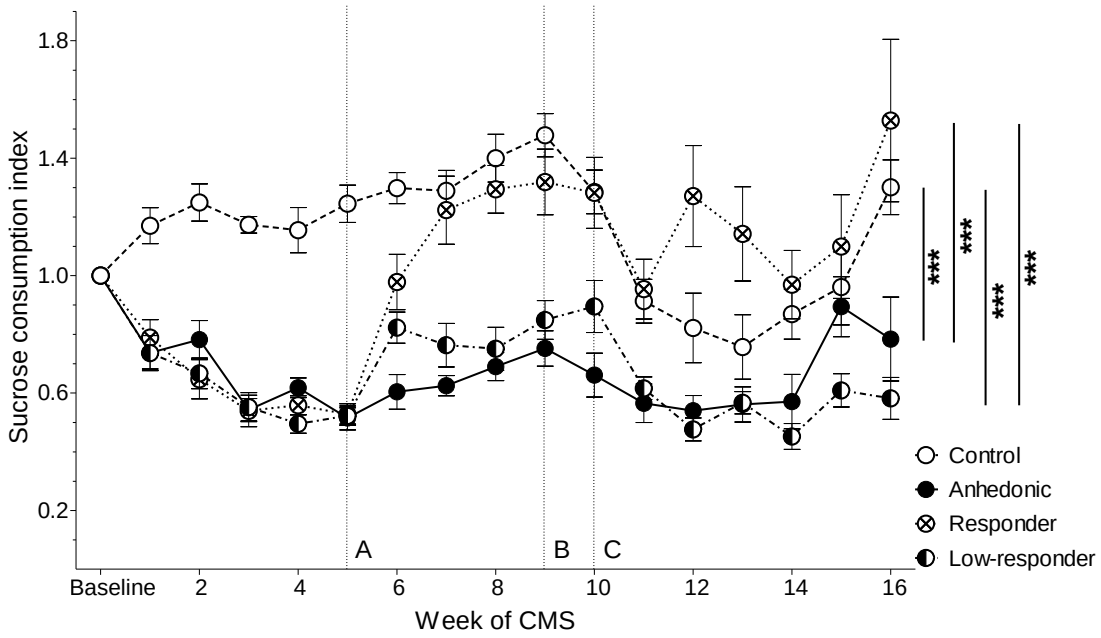

Figure 4. Sucrose consumption test throughout the experiment. The sucrose consumption index displays the sucrose consumption of the respective week of CMS normalised to baseline sucrose intake prior to CMS exposure. **(A)** Treatment start with the antidepressant vortioxetine in two-thirds of anhedonic-like rats. **(B)** Food deprivation for touchscreen testing was initiated for all four groups. **(C)** Start of touchscreen pre-training followed by dPAL acquisition for all four groups. Group means (± SEM) are displayed. Pairwise group post-hoc comparisons over the entire course of CMS exposure are indicated with ***$p < 0.001$ (Non-stressed control: $n = 10$, anhedonic-like: $n = 10$, treatment responder: $n = 10$, treatment low-responder: $n = 10$).

3.2 Paired-associates learning touchscreen task

3.2.1 Learning of the dPAL task

The period to acquire the dPAL task, indicated by the aggregated number of trials, did not differ significantly between groups (Figure 5A).

Two-way ANOVA revealed that treatment increased the number of redundant screen touches compared to non-treated animals (main effect of treatment: $F(1,28) = 9.74$, $p = 0.004$). This treatment effect is possible driven by a trend of hedonic state x treatment interaction effect ($F(1,28) = 3.93$, $p = 0.057$), where responders appear salient (Figure 5B).

Median response latency was altered due to a hedonic state x treatment interaction effect ($F(1,29) = 9.03$, $p = 0.005$; Figure 5C). Specifically, anhedonic-like rats (Bonferroni p

= 0.013), responders (Bonferroni p = 0.0001) and low-responders (Bonferroni p = 0.0010) responded faster to touchscreen stimuli than non-stressed control rats. Furthermore, treatment alone shortened median response latency ($F(1,29) = 17.58$, $p = 0.0002$; Figure 5C).

No difference for collection latency (Figure 5D), number of correction trials or maximum number of consecutive correct trials per session were observed between groups. Six animals (one non-stressed control, three anhedonic-like rats, one responder and one low-responder) did not pass dPAL and, thus, were excluded from this analysis.

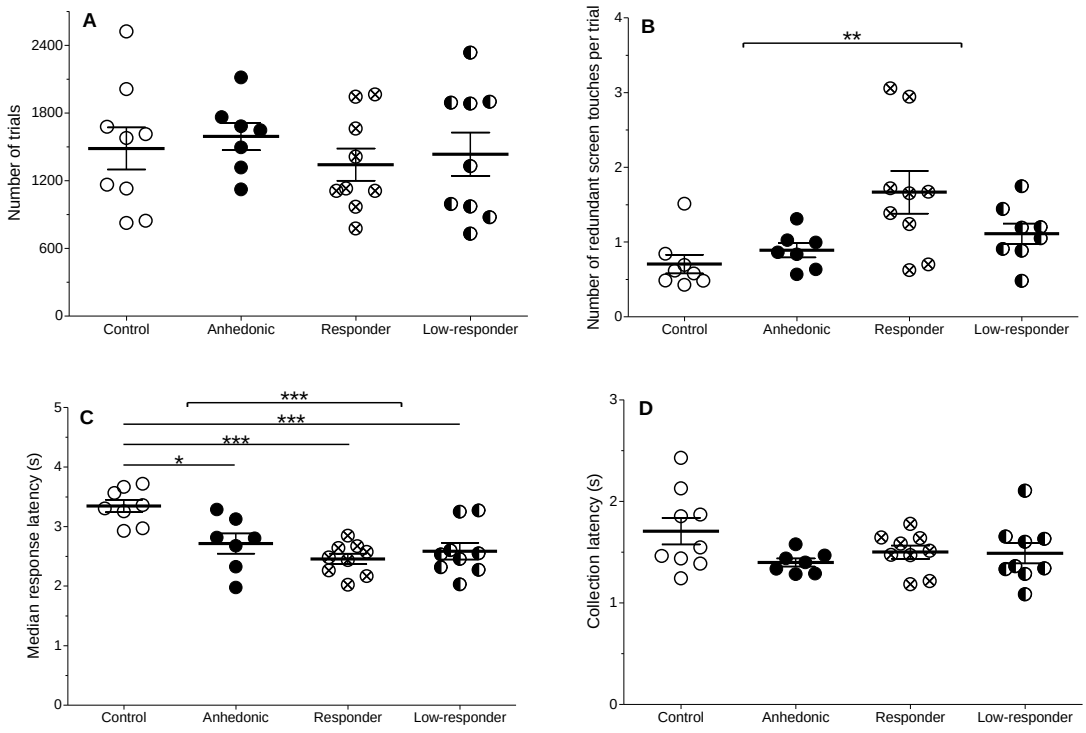

Figure 5. Acquisition of dPAL until passing criterion. (A) The overall number of trials needed to acquire the dPAL task. (B) Redundant touches executed per trial (trial or correction trial) besides the one for choosing a stimulus. (C) Median response latency to react to the stimuli. (D) Collection latency of touchscreen reward. Individual results and group means (± SEM) are shown. Two-way ANOVA main effects and LSD post-hoc comparisons are indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.2.2 dPAL task acquisition over time

Touchscreen data from the first until the last dPAL session of each rat were divided in ten equal bins by the combined number of trials plus non-correction trials. Thus, the varying number of sessions between rats to acquire the task was normalised to ten time points (bins) for each rat allowing statistical analysis with repeated measurements ANOVA.

The percent of correct trials (accuracy) increased significantly with increasing number of bins ($F(4.68,168.32) = 49.34$, $p < 0.0001$), indicating learning of the dPAL task, but no effect of group on accuracy performance was observed (Figure 6A).

The number of trials performed increased significantly over time with growing bin number ($F(3.82,137.34) = 63.09$, $p < 0.0001$), whereas the number of correction trials decreased significantly by bin number ($F(3.08,110.73) = 48.37$, $p < 0.0001$; Figure 6B), further indicating learning, however, with no statistically significant differences between groups.

Non-stressed control rats responded slower to stimuli compared to anhedonic-like rats (Bonferroni $p = 0.002$), low-responders (Bonferroni $p < 0.0001$) or responder (Bonferroni $p < 0.0001$), whereas vortioxetine responders showed the shortest median response latency compared to non-stressed controls, anhedonic-like rats (Bonferroni $p = 0.002$) and low-responders (Bonferroni $p = 0.029$; main effect of group: $F(3,36) = 3.24$, $p = 0.033$). Median response latency decreased significantly over the course of dPAL acquisition (main effect of time: $F(4.32, 155.40) = 9.14$, $p < 0.0001$; Figure 6C), thus, suggesting response latency as readout of cognitive processing rate, which decreases with increasing understanding of the touchscreen task.

Vortioxetine responders executed the highest number of redundant screen touches per trial compared to control rats (Bonferroni $p < 0.0001$) and anhedonic-like rats (Bonferroni $p < 0.0001$). Vortioxetine low-responders performed more redundant screen touches than control (Bonferroni $p = 0.022$) or anhedonic-like rats (Bonferroni $p = 0.005$; main effect of group: $F(3,36) = 3.10$, $p = 0.039$). The number of redundant screen touches decreased over the course of dPAL acquisition (main effect of time: $F(2.46,88.45) = 5.67$, $p < 0.0001$; Figure 6D).

Maximum number of consecutive correct trials per session increased over the course of dPAL acquisition ($F(4.58,165.05) = 24.19$, $p < 0.0001$), with no statistically significant difference between groups. Collection latency was not significant for group or time suggesting equal motivation to participate in the dPAL task.

3.2.3 Learning behaviour within the course of a dPAL session

All sessions of one animal were averaged to a single session. This session was then split into six equal blocks by the total number of trials (trials plus correction trials). This allowed for the analysis of learning behaviour within the course of a session.

Within the course of a session, accuracy did not significantly change over time, nor depending on group. The number of trials executed within the course of a session changed

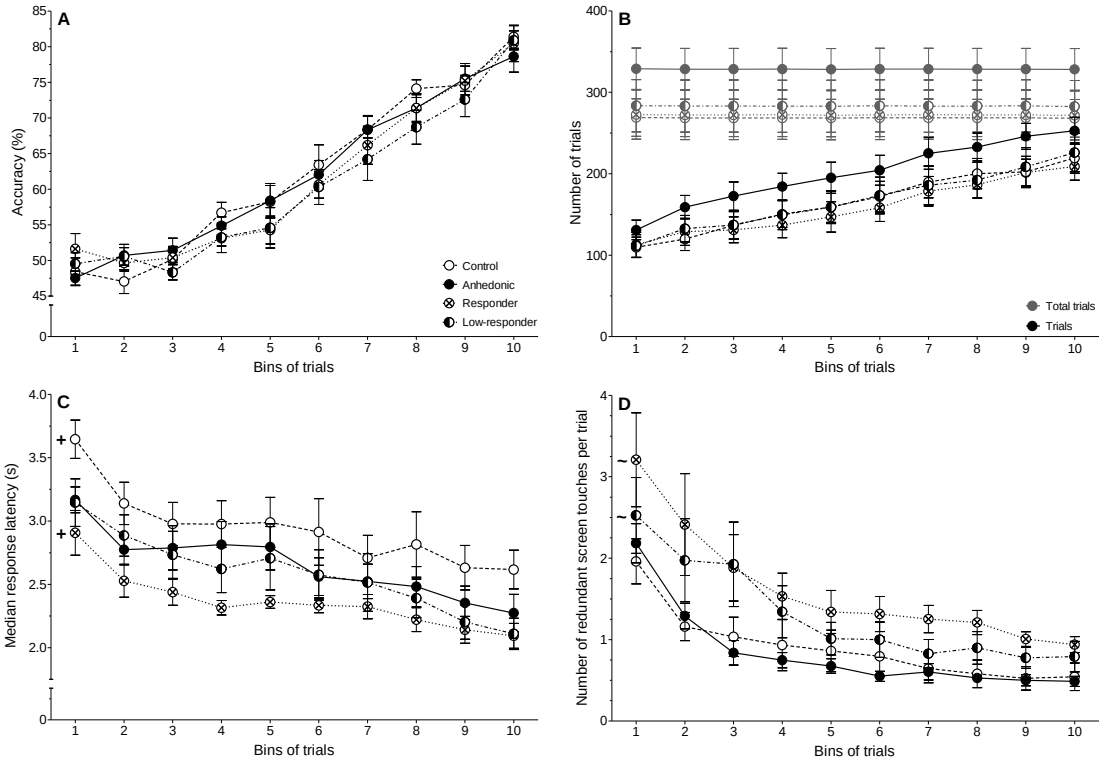

Figure 6. Behavioural parameters over the course of dPAL task acquisition. (A) Percent of correct choices. **(B)** Number of trials (black) and number of total trials (trials plus correction trials, grey). **(C)** Median response latency. **(D)** Number of redundant screen touches per trial. Group means (± SEM) are shown with '+' indicating a significant difference of the respective group to the three other groups and '~' a significant difference to controls and anhedonic-like rats (Bonferroni post-hoc comparisons).

depending on session block (main effect of session block: $F(5,180) = 3.38$, $p = 0.006$; Figure 7A).

Non-stressed controls needed significantly less correction trials within a session compared to responders (Bonferroni $p < 0.0001$) and low-responders (Bonferroni $p < 0.0001$) and a trend to anhedonic-like rats (Bonferroni $p = 0.054$). Anhedonic-like rats needed significantly less correction trials than vortioxetine responders (Bonferroni $p = 0.015$) and showed a trend to low-responders (Bonferroni $p = 0.063$; main effect of group: $F(3,36) = 3.05$, $p = 0.041$; Figure 7A). The number of correction trials significantly decreased by session block (main effect of session block: $F(5,180) = 3.71$, $p = 0.003$) indicating learning within the course of a session.

Vortioxetine responders executed more redundant touches per trial (correction or non-correction trial) than the control group (Bonferroni $p < 0.0001$) or anhedonic-like rats (Bonferroni $p < 0.0001$), but not significantly more than low-responders. Low-responders carried out more redundant screen touches than control (Bonferroni $p = 0.020$) or

anhedonic-like rats (p = 0.005; main effect of group: $F(3,36) = 3.12$, p = 0.038; Figure 7B). The number of redundant touches decreased significantly over the course of a session (main effect of session block: $F(1.73,62.22) = 9.65$, $p < 0.0001$).

Non-stressed controls took longer to execute their choice, as observed by median response latency, than anhedonic-like rats (Bonferroni p = 0.010), responders (Bonferroni p < 0.0001) or low-responders (Bonferroni $p < 0.0001$; main effect of group: $F(3,36) = 4.15$, p = 0.013; Figure 7C). Median response latency increased within a session (main effect of session block: $F(1.68,60.44) = 9.57$, $p < 0.0001$).

Collection latency was not significantly different between groups and independent of session block.

Rats were able to perform more consecutive correct trials with increasing session block (main effect of session block: $F(4.41,158.82) = 15.85$, $p < 0.0001$; Figure 7D), but no difference in the number of maximum consecutive trials was observed between groups.

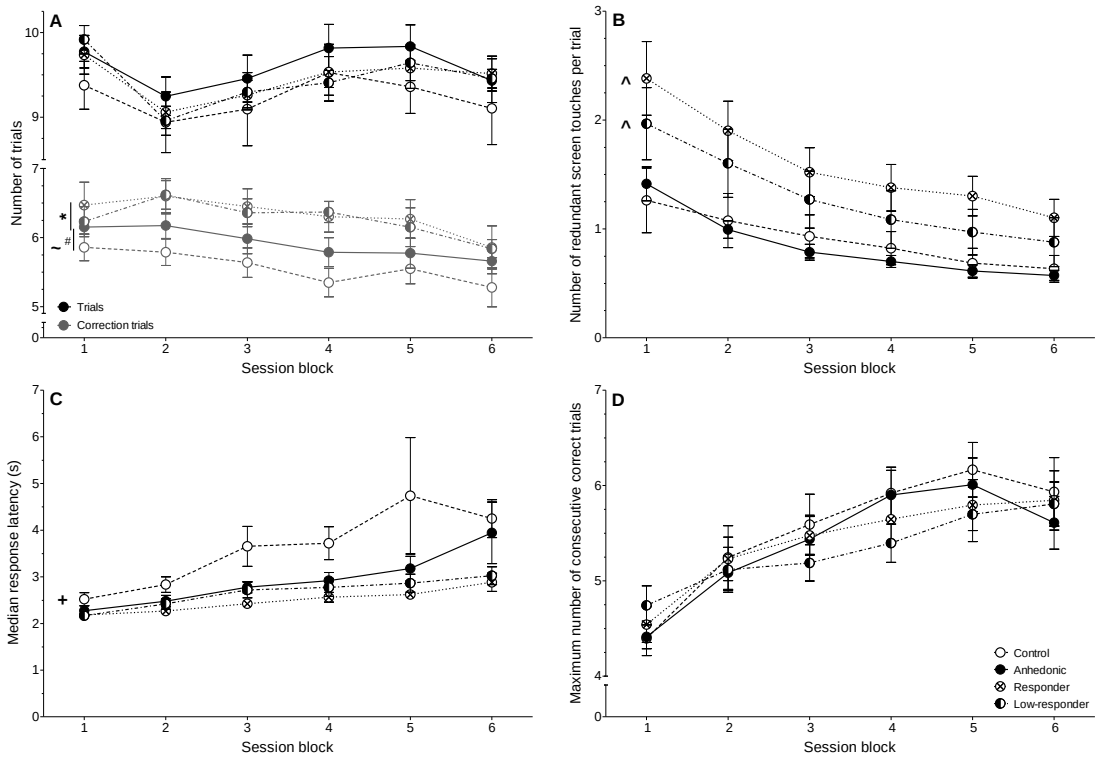

Figure 7. Touchscreen parameters within the course of an average session. (A) Number of trials (black) and correction trials (grey). **(B)** Number of redundant touches per trial (trial or correction trial). **(C)** Median response latency. **(D)** Maximum number of consecutive correct trials over the course of a session. Group means (± SEM) are displayed. Bonferroni post-hoc tests are indicated with *p < 0.05, #p < 0.06, '+' indicating a significant difference of the respective group to the three other groups, '~' a significant difference to responder and low-responder or '^' to control and anhedonic-like rats (Bonferroni post-hoc comparisons).

3.2.4 Memory of the dPAL task

Long-term memory performance was assessed by re-testing the rats in dPAL following a 10-day hiatus after dPAL acquisition. Included in the analysis was accuracy of the last session of dPAL acquisition before the break as well as the two dPAL retention sessions after the break. An interaction effect of group x session ($\chi^2(6) = 16.17$, $p = 0.013$) on accuracy performance was observed. Post-hoc comparisons revealed that on retention session one, vortioxetine responders reached a significantly lower accuracy level than controls ($p = 0.036$) and anhedonic-like rats ($p = 0.021$) and a trend to low-responders ($p = 0.055$; Figure 8A).

Additionally, individual changes in accuracy due to memory (difference in accuracy between last dPAL acquisition session and first retention session) and relearning (difference in accuracy between the two retention sessions) were evaluated. The hedonic state significantly associated with memory performance ($F(1,30) = 5.19$, $p = 0.030$; Figure 8B) and a trend in hedonic state x treatment on memory was observed ($F(1,30) = 3.06$, $p = 0.090$). Although statistically valid, the association of hedonic state and memory was unexpected examining the mean group performances (Figure 8B). Thus, a one-way ANOVA was performed to extract group difference in memory performance. Memory performance was significantly different between groups ($F(3, 30) = 3.41$, $p = 0.030$). Treatment responders (-13.56 ± 5.65%) showed a lower memory performance compared to low-responders (-6.74 ± 6.04%, Bonferroni $p = 0.046$). No effect of group was found for relearning performance with neither two-way nor one-way ANOVA (Figure 8B).

3.3 Cerebral gene expression

Expression levels of genes in the PFC as well as the dorsal and ventral HPC were investigated. We were interested in genes known to be involved in the stress response and/or altered in neuropsychiatric diseases, such as *Mr*, *Gr*, *Bdnf*, *Fkbp5*, *Disc1*, and *Gsk3b*, and, furthermore, in gene expression related to neuronal plasticity, learning and memory, such as *Nrg1*, *Cofilin 1*, *Spinophilin*, *Shank* and *Homer*. Alterations in gene expression levels were analysed in response to vortioxetine treatment and hedonic state. Furthermore, differences between dorsal and ventral HPC gene expression were examined. Supplementary Table 3 contains all gene expression levels for the four groups and all tissues.

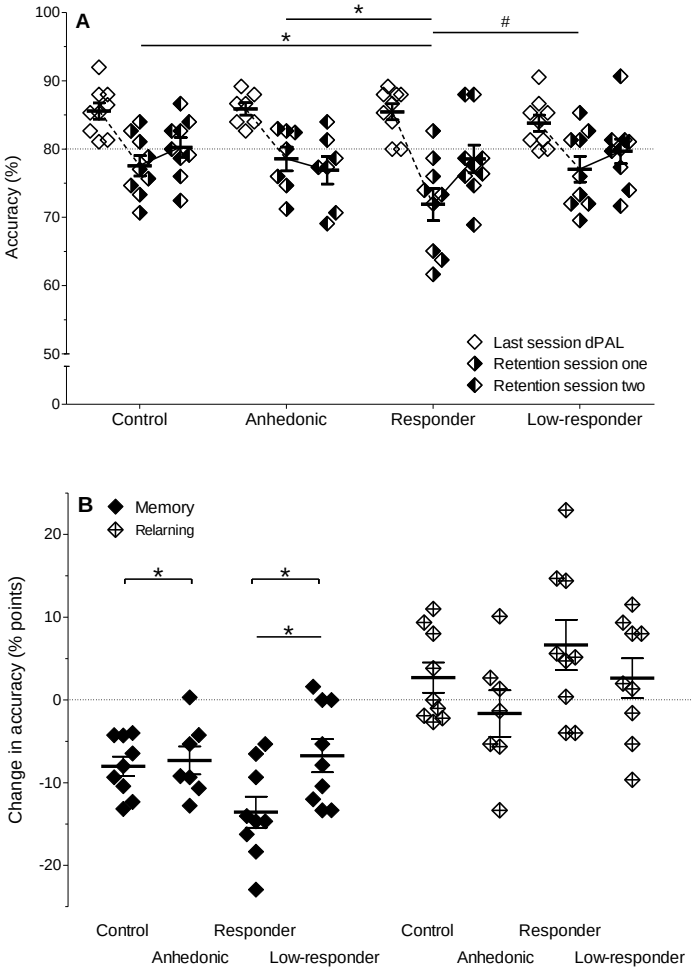

Figure 8. Retention of the dPAL task. (A) Accuracy of each rat including the last dPAL session (task acquisition) before the 10-day hiatus, the first and second retention session. The dotted line (⋯) indicates the drop in group accuracy due to the hiatus (memory performance) and the continuous line (—) presents the group relearning performance. **(B)** Individual memory and relearning performance are shown. Group means (± SEM) are displayed, main effect of hedonic state and post-hoc statistics are indicated by $^{*}p < 0.05$, $^{\#}p < 0.06$.

3.3.1 Prefrontal cortex gene expression

The expression of *Cofilin 1,* which is highly expressed in neurons and involved in growth cone dynamics, was increased in the anhedonic-like rats (Bonferroni $p = 0.022$) compared to controls (interaction effect of hedonic state x treatment: $F_{rank}(1,28) = 5.51$, $p = 0.026$). Furthermore, hedonic rats displayed a lower gene expression than anhedonic rats (main effect of hedonic state: $F_{rank}(1,28) = 6.60$, $p = 0.016$; Figure 9).

Gr signalling in the PFC is involved in an appropriate stress adaptation and mood regulation[30]. A trend of treatment reducing *Gr* gene expression was observed ($F(1,27) = 4.07$, $p = 0.054$; Figure 9).

PFC gene expression was not statistically different for *Mr*, *Fkbp5*, *Disc1*, *Gsk3b*, *Bdnf*, *Shank 1-3*, *Homer1-3*, *Nrg1* or *Spinophilin*.

3.3.2 Hippocampal gene expression

Gsk3b modulates LTP and LTD and its expression is commonly inhibited by antidepressants[31,32]. Here we found that in the dorsal HPC, *Gsk3b* expression tended to be driven by an interaction effect of hedonic state x treatment: $F(1,32) = 4.03$, $p = 0.053$). In the ventral HPC, *Gsk3b* expression tended to be decreased in rats with anhedonic phenotype (trend of hedonic state: $F(1,33) = 3.48$, $p = 0.071$). *Gsk3b* gene expression was higher in the ventral HPC compared to dorsal HPC ($t(35) = -3.13$, $p = 0.004$; Figure 9).

Disc1 is involved in neuronal development and alteration in its function are associated with psychiatric diseases. Here, *Disc1* gene expression was increased in the ventral to dorsal HPC ($t(34) = -4.72$, $p < 0.0001$). In the ventral HPC, a trend of hedonic state x treatment interaction was found ($F(1,31) = 3.46$, $p = 0.072$; Figure 9).

Homer and *Shank* are scaffolding proteins, which form a complex and are abundantly found at the post-synaptic density. Both proteins are involved in maturation of dendritic spines[33]. *Homer3* expression appears decreased in vortioxetine treated compared to non-treated rats in the ventral HPC (trend of treatment: $F(1,33) = 4.03$, $p = 0.053$; Figure 9). No statistical difference in gene expression was observed in the dorsal HPC or between the dorsal and ventral HPC.

In the dorsal HPC, *Homer2* gene expression was decreased in groups with anhedonic-like phenotype (main effect of hedonic state: $F(1,33) = 5.63$, $p = 0.024$; Figure 9), and no effect of treatment or hedonic state was observed in the ventral HPC. No difference in gene expression was revealed between dorsal and ventral HPC.

Ventral HPC gene expression was decreased compared to dorsal for *Homer1* ($t(35) = 3.01$, $p = 0.005$; Figure 9), but otherwise increased for *Shank1* ($t(34) = -3.99$, $p = 0.0003$), *Shank2* ($t(32) = -3.58$, $p = 0.001$), the neuronal growth factor *Nrg1* ($t(32) = -5.84$, $p < 0.0001$), and tentatively for *Shank3* ($t(34) = -1.88$, $p = 0.068$).

Important in the (cognitive) adaptation to stress[34], *Mr* expression tended to be decreased in groups with anhedonic-like phenotype in the dorsal HPC (trend of hedonic state: $F(1,33) = 3.47$, $p = 0.072$; Figure 9) with no other effect on *Mr* expression due to hedonic state, treatment nor tissue.

In the dorsal HPC, treatment tended to increase *Bdnf* gene expression compared to non-treated animals (trend of treatment: $F(1,30) = 3.87$, $p = 0.058$; Figure 9). No other alterations in *Bdnf* gene expression were observed.

Gene expression was not statistically significant altered depending on tissue, hedonic state or treatment for *Fkbp5*, *Gr*, *Spinophilin*, or *Cofilin 1* in the HPC.

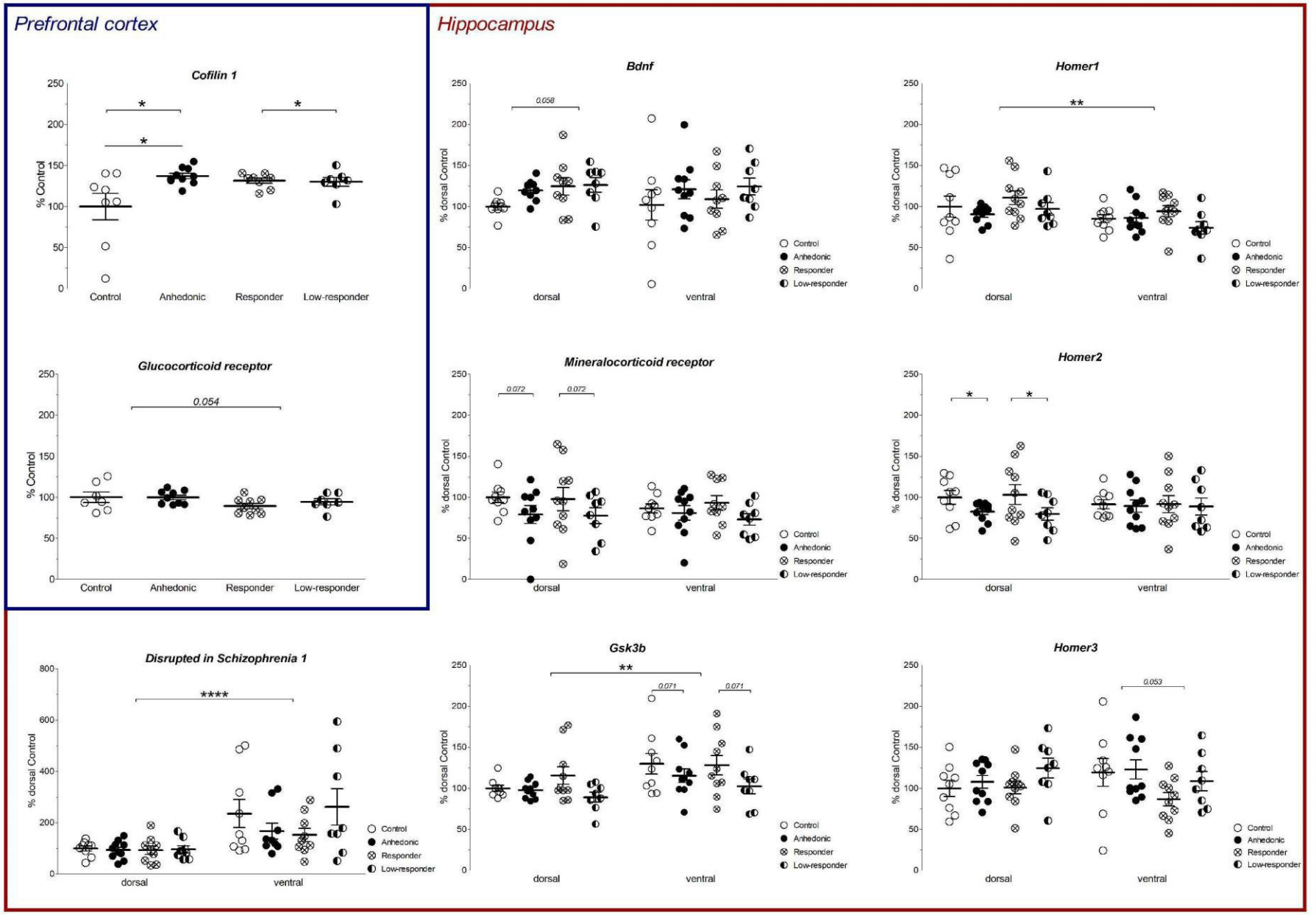
Prefrontal cortex
Hippocampus
Cofilin 1
% Control
Control
Anhedonic
Responder
Low-responder
Bdnf
% dorsal Control
dorsal
ventral
0.058
Homer1
**
Glucocorticoid receptor
0.054
Mineralocorticoid receptor
0.072
0.072
Homer2
*
*
Disrupted in Schizophrenia 1
****
Gsk3b
**
0.071
0.071
Homer3
0.053

Figure 9. Prefrontal cortex (PFC) and hippocampal (HPC) gene expression levels. Genes of interest are normalised to reference genes and displayed as percent of control mean for the PFC or as percent of the dorsal HPC of the control group for the ventral and dorsal HPC. Individual data points as well as group means (± SEM) are displayed. Statistical significance is indicated for main effects and between tissue differences (angular brackets) and Bonferroni corrected post-hoc comparisons by ****$p < 0.0001$, **$p < 0.01$, *$p < 0.05$ and trends by the respective number.
Bdnf – Brain-derived neurotrophic factor; *Gsk3b* – Glyceraldehyde-3-phosphate dehydrogenase; *Homer* – Homer scaffolding protein.

4 Discussion

In the present CMS study, non-stressed controls, anhedonic-like rats and vortioxetine treated anhedonic-like rats were evaluated with respect to their hedonic state, their cognitive performance in the dPAL touchscreen task and by cerebral gene expression profiling. Vortioxetine has a strong antidepressant effect and recovered the hedonic state in a major proportion of the treated rats. All groups were able to acquire the dPAL task. No differences in primary touchscreen task readouts were observed between groups, however, for secondary readouts, such as redundant screen touches, median response latency or number of correction trials significant group differences were observed. PFC and HPC gene expression was associated with the hedonic state or showed trends of treatment. Differences between ventral and dorsal HPC expression levels were observed.

4.1 Vortioxetine recovers the hedonic state

CMS exposed rats decreased sucrose intake over time indicating a reduced reward sensitivity and, hence, mirroring the MDD core symptom anhedonia. Administration of the antidepressant vortioxetine recovered the hedonic state in a major fraction of anhedonic-like rats (65%), whereas the remaining rats responded poorly and remained in an anhedonic-like state. Hence, vortioxetine was effective in recovering the hedonic state in anhedonic-like CMS exposed rats. Previous research found vortioxetine to be ineffective in the CMS model[14], however, vortioxetine was administered by intraperitoneal injections once daily (Papp, personal communication). The relatively short half-life of vortioxetine in rodents[14] may have made this drug schedule ineffective. In the present study vortioxetine was mixed into the diet and, hence, this route of drug administration ensured a continuous drug exposure. To achieve full SERT occupancy we used a dosing of 1.8g/kg food chow[35].

4.2 Vortioxetine affects cognition

We investigated whether the vortioxetine-induced alleviation of the hedonic state is associated with alterations in cognitive performance. Vortioxetine is known to augment cognitive functions targeting 5-HT_{1A}, 5-HT_{1B}, 5-HT_3 and 5-HT_7 receptors[14]. This pro-cognitive efficacy was considered to be due to direct actions on cognitive processes rather than to be secondary to remission from affective symptoms[20]. However, in this study vortioxetine did not alter primary touchscreen parameters (accuracy, number of trials) in either responder or low-responder groups compared to non-stressed controls or anhedonic-like rats. Evaluating cognitive performance within an average dPAL session revealed that non-stressed controls needed less correction trials than the vortioxetine groups and a tendency to anhedonic-like animals indicating inferior cognitive performance in the latter groups compared to controls. Thus, vortioxetine treatment seemingly did not improve cognitive performance in anhedonic-like rats. However, it should be noted that an increased number of untreated anhedonic-like rats (n = 3) did not pass the dPAL task within 46 sessions whereas only one animal failed to pass in any of the other groups. The inability to acquire the dPAL task may suggest cognitive impairment in the anhedonic-like group only and hence a pro-cognitive effect of vortioxetine treatment.

Importantly, latency for collecting reward pellets did not differ between groups suggesting no difference in motivation to participate in the touchscreen task. Possible motivational differences between anhedonic-like and hedonic animals are likely masked by food deprivation accompanying touchscreen testing. Thus, cognitive impairments were not cofounded by lack of motivation.

Consistently, median response latency was reduced in untreated anhedonic-like rats and both treatment groups compared to controls. During task acquisition, vortioxetine responders displayed the shortest median response latency and controls the longest latency. Similarly within an average session, control animals had the longest response time and untreated anhedonic-like rats took longer to respond than vortioxetine responders. Prolonged median response latency in the control group is consistent with a previous touchscreen studies in which control animals displayed a longer median response latency compared to CMS exposed rats[25], suggesting increased cognitive appraisal, before executing a choice in control animals. Consecutively, reduced response latency in the anhedonic and vortioxetine treated groups can be considered as impulsive behaviour, executing a less evaluated, spontaneous choice. Reduced response latency may indicate impaired HPC functioning since inactivation of the dorsal hippocampus with lidocaine and scopolamine significantly shortened reaction time in the rat dPAL task as well[36] and is in line with the well-known

important role of HPC in visuospatial learning tasks[37,38]. An alternative explanation could comprise frontostriatal reorganization resulting in a shift from effortful goal-directed to habitual behaviour. Such changes were previously observed after stress exposure[39] and might resemble the reduced response latency observed in the present study. Noticeably, responders to vortioxetine treatment displayed the shortest response latency of all groups suggesting a relationship of treatment response and decreased appraisal.

A shift to habit-like or impulsive behaviour is further supported by the number of redundant screen touches per trial. Consistently, vortioxetine treated rats executed more redundant touches than any other group. Thus, vortioxetine administration, and in particular treatment response, seems to coincide with increased impulsive behaviour. This may resemble a lack of control of executive function, which is part of PFC functions.

In order to address long-term memory trial accuracy was re-tested after a 10-day hiatus subsequent to passing dPAL. Statistic revealed an effect of hedonic state on memory performance, although group mean of hedonic control rats was comparable to anhedonic-like treated and untreated rats and thus, relevance of this finding is debatable. Vortioxetine responders showed the greatest decrease in accuracy (around twice as much) after the 10-day hiatus than any of the three other groups and performed significantly worse than low-responders. Hence, a high response to vortioxetine treatment was associated with poor memory performance. Interestingly, non-stressed controls were the only group able to restore performance to dPAL passing criterion level (≥ 80% accuracy) on the second day of retention. All other groups still performed below 80% accuracy and the untreated anhedonic-like group even decreased in accuracy on the second day of retention.

4.3 Hedonic state and vortioxetine treatment affect cerebral gene expression

mRNA levels of genes known to be altered in neuropsychiatric diseases and associated with neuronal plasticity were measured in the PFC, dorsal and ventral HPC. For example, Disc1 is a scaffolding protein involved in neurodevelopmental signalling and suggested as candidate gene in mental illnesses, such as bipolar disorder and schizophrenia[40,41]. In our study, *Disc1* mRNA levels were higher in the ventral compared to the dorsal HPC. In the ventral HPC, an interaction trend may indicate a regulatory association of the hedonic state and vortioxetine treatment on *Disc1* gene expression. These changes correspond to the literature reporting DISC1 alterations to be involved in the pathology of mental illnesses, cognitive deficits and dendritic arborisation, all of which are also known to be often a consequence of stress exposure[42,43]

DISC1 regulates downstream GSK3B expression. In the present study, *Gsk3b* mRNA was also upregulated in the ventral compared to the dorsal HPC, which could be linked to the increased *Disc1* gene expression. *Gsk3b* expression is known to be inhibited by most antidepressant treatments, e.g. SSRIs, and its dysregulation is implicated in depression[32,44–46]. Therefore, an increased expression of *Gsk3b* in the untreated anhedonic-like and possibly the low-responder group was anticipated. In the ventral HPC, an unexpected trend of decreased *Gsk3b* mRNA in dependence of the anhedonic-like phenotype (untreated and treated) compared to the hedonic phenotype (controls and responder) was observed. A possible explanation for this converse finding might be downregulated *Gsk3b* mRNA expression in the anhedonic phenotype as response to elevated *Gsk3b* protein levels. However, this theory needs to be supported by further experiments assessing protein concentrations. Lower *Gsk3b* levels were also found in a juvenile stress model, which models predisposition to neuropsychiatric diseases[47] and thus supports the finding of lower *Gsk3b* expression in the stressed, anhedonic groups. In the dorsal HPC, and interaction trend of hedonic state and treatment was found with vortioxetine responders potentially displaying increased *Gsk3b* levels compared to all other groups, particularly the low-responders. *Gsk3b* upregulation is associated with impairments in spatial memory, object recognition and long-term potentiation, which are all components of the dPAL task[48–52]. Consequently, increased *Gsk3b* gene expression levels in the dorsal HPC may underlie the observed memory impairments during dPAL retention in the vortioxetine responder group compared to low-responders. It remains unresolved how vortioxetine recovered the hedonic state, while *Gsk3b* levels are elevated. However, it should be noted that *Gsk3b* levels were only measured at the experimental endpoint and not during remission from the depressive-like phenotype and, hence, a temporal dynamic increase in *Gsk3b* levels after chronic vortioxetine treatment might explain this discrepancy.

Homer proteins, which are scaffolding proteins facilitating post-synaptic signalling, are vital for learning and memory functions[53]. Moreover, decreased scaffolding *Homer1* expression is associated with an enhanced stress response and susceptibility to psychiatric diseases such as MDD[54,55]. In this study, *Homer1* was higher expressed in the dorsal than in the ventral HPC possibly in response to spatial learning required for dPAL acquisition, a function of the dorsal HPC[56]. In the dorsal HPC, *Homer2* expression was decreased for anhedonic rats (treated and untreated). *Homer2* is required for alcohol-seeking[57] and, thus, reduced pleasure seeking in anhedonic rats (treated and untreated) may be reflected by their decreased *Homer2* levels. In contrast to the present study finding decreased *Homer3* expression in the ventral HPC of treated rats, a single dose of vortioxetine, has recently been

reported to induce a transient increase (at 8 h post-treatment) of *Homer3* in the frontal cortex[58]. Currently, we do not know the mechanisms responsible for regulation of *Homer3*.

Cofilin 1 is a key regulator in growth cone dynamics and, thus, in neuronal plasticity important for learning and memory[71,72]. In the PFC, *Cofilin 1* expression was upregulated in anhedonic-like rats compared to controls and in general upregulated in the anhedonic-like compared to the hedonic phenotype. Excessive up- or down-regulation of *Cofilin 1* was associated with impaired synaptic plasticity and learning deficits[72]. Thus, altered *Cofilin 1* gene expression might suggest subthreshold cognitive impairments associated with anhedonia, especially in untreated rats.

Bdnf is involved in neuronal plasticity[59]. Its expression levels are reduced following stress exposure as well as in PFC and HPC *post-mortem* tissue of MDD suicide victims[60,61]. Furthermore, antidepressant treatment elevates *Bdnf* levels and, in turn, treatment efficacy appears dependent on *Bdnf* levels[62–64]. Consequently, the trend of higher *Bdnf* levels in the dorsal HPC of vortioxetine treated animals is in accordance with the literature.

Mr expression is an important player in the stress response, HPA axis activity and MDD. Increased MR function is associated with resilience whereas decreased *Mr* levels suggest stress-susceptibility for developing depression[65]. Stimulation of *Mr* expression improved memory and executive function in MDD patients[66]. Thus, *Mr* expression may be a target for relieving MDD symptoms and cognitive impairments[67]. In the present study, a trend for increased *Mr* expression was found in the hedonic rats (controls and responders) compared to anhedonic rats (treated and untreated). Hence, susceptibility to CMS including a low treatment response could be associated with reduced *Mr* expression in the HPC.

Likewise, *Gr* expression plays an important role in the HPA axis' negative feedback loop and an appropriate stress response[68,69]. In this study, no differences were found for *Gr* expression in the HPC. In the PFC, *Gr* expression was downregulated by vortioxetine treatment. This was unexpected since knockdown of GR in rat PFC resulted in an altered stress response and consequent depressive-like behaviour[30]. Furthermore, *Gr* levels were found decreased in the PFC of depressed and schizophrenic patients[70].

Expression levels of *Nrg1*, *Shank* and *Spinophilin*, genes involved in neuronal plasticity, were not statistically different between groups and possibly reflecting on the small cognitive differences observed in this study. Furthermore, *Fkbp5* mRNA levels, a modulator of the HPA axis' negative feedback loop[73], were similar across groups indicating no effect of stress, hedonic state or vortioxetine on *Fkbp5* expression.

4.4 Touchscreen testing

The present touchscreen study was the first to monitor the hedonic state throughout touchscreen testing and food restriction. Operant conditioning is based on appetitive learning and hence assumed to be only mildly straining[23,74]. However, the non-stressed control group decreased their sucrose consumption in response to food restriction and touchscreen testing (Figure 4). This decline in the hedonic state of control rats illustrates how stressful touchscreen testing is experienced by the animals. Nevertheless, the sucrose consumption of control rats never decreased below the anhedonia threshold and finally rats seemed to habituate and restore sucrose consumption. Consequently, dPAL results are likely unaffected since the initial mildly stressful response to touchscreen testing occurred mostly during pre-training. However, effect size between non-stressed controls and CMS animals might have been reduced. Moreover, it was demonstrated that vortioxetine treated groups, in particular responders, maintained their phenotype during touchscreen testing although food reduction entailed a lower treatment dose. Furthermore, continued SCTs revealed that the modified, milder CMS protocol was sufficient to maintain the anhedonic-like phenotype during touchscreen testing. Continuous SCTs were possible by replacing the sugar with bacon reward pellets and, thus, avoiding desensitization to the lower concentrated sucrose solution. To our knowledge, this was the first touchscreen study to show that not only sweet rewards, such as sugar pellets or milkshakes entail for successful operant conditioning. This might become crucial, in addiction, diabetes or reward studies and expands the applicability of touchscreen testing.

4.5 Conclusion

Our study expands on the relatively new drug treatment approach of antidepressants targeting depression-associated cognitive impairments. Hence, the effect of vortioxetine on the hedonic state as well as on cognition was assessed. In contrast to a previous report (reviewed in Sanchez *et al.*[14]), we have shown that vortioxetine recovers the hedonic state in anhedonic-like rats and hence demonstrated its efficacy in a well validated preclinical model of depression. This is an important finding since vortioxetine is approved for clinical application and since the CMS model is recognized for high predictive validity[26,75,76]. Moreover, cognitive performance was assessed with the touchscreen operant platform, which was developed with focus on its translational value. Hence, clinical relevance and additional useful advantages, such as objective readout, standardization and minimal experimenter's bias, suggest the platform as an optimal tool in cognitive research. In the present study

beneficial cognitive effects of vortioxetine treatment were not prominent in the translational touchscreen dPAL task involving object-in-place learning and memory. Vortioxetine responders displayed inferior long-term memory. Vortioxetine's potential effect on cognition requires more detailed evaluation since the observed effects, such as shortened reaction time and a shift to habitual behaviour might be beneficial in a different context than dPAL touchscreen testing or stress-induced depression.

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Declaration of interest

No conflict of interest.

Supplementary

Supplementary Table 1. Modified chronic mild stress (CMS) protocol during touchscreen pre-training and acquisition. Stressors were applied during the night phase since touchscreen testing took place during the day. On Fridays, the SCT (including all animals) followed by grouping (only CMS animals) were carried out during the light phase.

Protocol	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
A	Strobe light (6 h)	Wet bedding (14 h)	Light on (3 x 2 h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (2 x 2 h) and light on (1 h)	Cage tilted 45° (14 h)	Wet bedding (14 h)
B	Cage tilted 45° (14 h)	Light on (2 h), strobe light (5 h)	Light on (3 x 2 h, 1h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (2 x 3 h) and light on (1 h)	Cage tilted 45° (14 h)	Wet bedding (14 h)
C	Cage tilted 45° (14 h)	Light on (3 h, 1h, 2 h), Strobe light (2 x 1h)	Wet bedding (14 h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (3 x 2 h) and light on (1 h)	Light on (3 h, 2 x 2 h)	Wet bedding (14 h)
A	Strobe light (6 h)	Wet bedding (14 h)	Light on (3 x 2 h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (2 x 2 h) and light on (1 h)	Cage tilted 45° (14 h)	Wet bedding (14 h)
D	Cage tilted 45° (14 h)	Light on (3 h), strobe light (5 h)	Wet bedding (14 h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (2 x 2 3) and light on (2 h)	Cage tilted 45° (14 h)	Wet bedding (14 h)
C	Cage tilted 45° (14 h)	Light on (3 h, 1h, 2 h), Strobe light (2 x 1h)	Wet bedding (14 h)	Remove food and water (15 h)*	SCT (1 h)*, Grouping (4 h), Strobe light (3 x 2 h) and light on (1 h)	Light on (3 h, 2 x 2 h)	Wet bedding (14 h)

* For CMS and non-stressed controls.

Supplementary Table 2. Characteristics of gene-specific real-time qPCR primers

Gene Symbol	Gene Name	Accession No.[1]	Primer Sequence	Amplicon size[2]
Reference genes				
18s rRNA	18s subunit ribosomal RNA	M11188	(+) acggaccagagcgaaagcat (-) tgtcaatcctgtccgtgtcc	310
ActB	Beta-actin	NM_031144	(+) tgtcaccaactgggacgata (-) ggggtgttgaaggtctcaaa	165
Ppia	Cyclophilin A	XM_345810	(+) agcactggggagaaaggatt (-) agccactcagtcttggcagt	248
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008	(+) tcaccaccatggagaaggc (-) gctaagcagttggtggtgca	168
Hmbs	Hydroxy-methylbilane synthase	NM_013168	(+) tcctggctttaccattggag (-) tgaattccaggtgagggaac	176
Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	NM_012583	(+) gcagactttgctttccttgg (-) cgagaggtccttttcaccag	81
Rpl13A	Ribosomal protein L13A	NM_173340	(+) acaagaaaaagcggatggtg (-) ttccggtaatggatctttgc	167
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	BC094305	(+) ttgagcagaagacggaaggt (-) gaagcattggggatcaagaa	136
Target genes				
Bdnf	Brain-derived neurotrophic factor	NM_001270630.1	(+) gaaagtcccggtatcaaaag (-) cgccagccaattctctttttg	183
Disc1		NM_175596.2	(+) agagagtgtggaaagcggac (-) atgagattcctgcaaaggggga	197
Spinophilin	protein phosphatase 1, regulatory subunit 9B (Ppp1r9b)	NM_053474	(+) tcctgtggagttggagaagg (-) tgcctttggtgttcctaagc	239
Homer1	Homer scaffolding protein 1	NM_031707	(+) cacccgatgtgacacagaac (-) tgttgcttccactgcttcac	220
Homer2	Homer scaffolding protein 2	NM_053309	(+) ctgccaggttagccagagac (-) tcttcacattggcagcactc	219
Homer3	Homer scaffolding protein 3	NM_053310	(+) ggtcaaagaagctgccagac (-) tgcggaacagcttctcttct	143
Shank 1		NM_031751	(+) cactctcagcacctggaaca (-) gaagggtgtctgtccgttgt	177
Shank 2		NM_201350	(+) cctccaggactgcagagaac (-) atttctgccttcgcatcgta	236
Shank 3		NM_021676	(+) ctgtgtggaggaagtgcaga (-) gaacaaagccaaaaccctca	188
Cofilin 1		NM_017147	(+) gatgctgccagacaaggact (-) cgggggcccagaaaatgaat	101
Gr	Glucocorticoid receptor	NM_012576.2	(+) ggccggtcagtgttttctaa (-) caatcgtttcttccagcaca	233
Mr	Mineralocorticoid receptor	NM_013131.1	(+) tgagttccttcccacctgtc (-) aagcctcatctccacacacc	192
Fkbp5	FK506 binding protein 5	XM_006256222.3	(+) gcactgaggaagcagaggttt (-) gtctcctcactagtccccact	175
Nrg1	Neuroregulin 1	NM_001271118	(+) agcgaaaggtatgtatcagcca (-) ggacacgggtggagacattt	111
Gsk3b	Glycogen synthase kinase 3 beta	NM_032080.1	(+) ccactcaagaactgtcaagtaacc (-) tccacggtctccagcattag	131

[1] Genbank accession number of cDNA and corresponding gene, available at http://www.ncbi.nlm.nih.gov/.
[2] Amplicon length in base pairs.

Supplementary Table 3. Target gene expression levels. Target genes were normalised to reference genes and calculated as % of the control group mean (for prefrontal cortex, PFC) or the control group mean of the dorsal hippocampus (HPC; for ventral (v) and dorsal (d) HPC). For each gene, group mean (± SD) and sample size *n* (in brackets) are displayed.

Tissue	Target gene	Control	Anhedonic	Responder	Low-responder
PFC	*Mr*	100.0 ± 16.7 (7)	90.6 ± 11.2 (9)	95.3 ± 10.5 (9)	90.9 ± 7.7 (7)
	Gr	100.0 ± 16.9 (7)	99.9 ± 8.3 (9)	89.3 ± 9.6 (9)	94.4 ± 9.9 (7)
	Fkbp5	100.0 ± 73.0 (8)	140.8 ± 69.8 (10)	104.4 ± 55.9 (10)	108.1 ± 62.0 (8)
	Disc1	100.0 ± 49.3 (8)	111.9 ± 39.2 (10)	94.5 ± 67.5 (10)	108.7 ± 47.3 (8)
	Gsk3b	100.0 ± 10.4 (7)	108.8 ± 26.2 (9)	108.5 ± 13.8 (9)	98.6 ± 9.5 (7)
	Bdnf	100.0 ± 13.3 (7)	106.9 ± 18.4 (10)	96.7 ± 23.1 (9)	101.7 ± 8.4 (7)
	Nrg1	100.0 ± 9.7 (7)	113.6 ± 16.3 (10)	102.2 ± 11.8 (9)	102.9 ± 6.0 (7)
	Homer1	100.0 ± 43.5 (8)	121.1 ± 30.9 (10)	96.8 ± 36.5 (10)	104.0 ± 27.7 (8)
	Homer2	100.0 ± 14.9 (7)	103.6 ± 26.7 (10)	91.1 ± 9.5 (9)	96.9 ± 12.1 (7)
	Homer3	100.0 ± 16.5 (7)	111.1 ± 28.1 (9)	101.0 ± 10.7 (9)	96.0 ± 37.1 (8)
	Shank 1	100.0 ± 16.0 (7)	104.4 ± 10.4 (9)	95.1 ± 7.3 (9)	98.4 ± 13.2 (7)
	Shank 2	100.0 ± 15.1 (7)	93.4 ± 11.9 (9)	93.3 ± 9.0 (9)	87.6 ± 30.0 (8)
	Shank 3	100.0 ± 16.6 (7)	92.3 ± 8.3 (9)	95.7 ± 15.3 (9)	81.4 ± 26.9 (8)
	Spinophilin	100.0 ± 46.2 (8)	109.6 ± 25.5 (9)	99.3 ± 43.7 (10)	100.4 ± 42.1 (8)
	Cofilin 1	100.0 ± 45.2 (8)	137.1 ± 10.3 (10)	131.4 ± 9.1 (8)	130.1 ± 14.1 (7)
dHPC	*Mr*	100.0 ± 19.4 (9)	79.2 ± 34.6 (10)	97.9 ± 44.7 (10)	77.6 ± 26.9 (8)
	Gr	100.0 ± 39.9 (9)	90.0 ± 40.8 (10)	126.7 ± 42.5 (9)	98.3 ± 23.7 (7)
	Fkbp5	100.0 ± 34.6 (8)	97.1 ± 34.8 (10)	112.5 ± 34.4 (9)	91.2 ± 32.8 (8)
	Disc1	100.0 ± 29.4 (9)	93.7 ± 35.1 (10)	93.2 ± 50.1 (9)	96.7 ± 39.7 (8)
	Gsk3b	100.0 ± 6.3 (8)	100.9 ± 10.5 (10)	119.4 ± 34.5 (10)	92.0 ± 17.3 (8)
	Bdnf	100.0 ± 11.7 (8)	119.8 ± 12.9 (9)	124.7 ± 32.1 (9)	126.5 ± 25.0 (8)
	Nrg1	100.0 ± 36.5 (9)	79.0 ± 12.6 (8)	90.2 ± 18.1 (10)	98.1 ± 21.1 (8)
	Homer1	100.0 ± 38.3 (9)	90.6 ± 10.9 (9)	110.8 ± 25.9 (10)	97.2 ± 21.9 (8)
	Homer2	100.0 ± 24.7 (9)	82.6 11.8 (10)	103.3 ± 38.3 (10)	79.8 ± 21.0 (8)
	Homer3	100.0 ± 29.6 (9)	108.0 ± 24.6 (10)	101.0 ± 24.3 (10)	124.6 ± 34.1 (8)
	Shank 1	100.0 ± 47.2 (9)	108.9 ± 19.0 (9)	100.3 ± 42.3 (10)	94.9 ± 34.0 (8)
	Shank 2	100.0 ± 31.6 (9)	95.0 ± 25.1 (10)	92.2 ± 41.4 (10)	83.7 ± 25.2 (7)
	Shank 3	100.0 ± 14.7 (9)	86.6 ± 23.2 (10)	104.4 ± 19.0 (9)	95.5 ± 14.4 (7)
	Spinophilin	100.0 ± 34.0 (9)	117.9 ± 21.8 (9)	105.9 ± 22.7 (9)	109.7 ± 37.1 (8)
	Cofilin 1	100.0 ± 7.8 (8)	86.9 ± 25.3 (10)	88.5 ± 31.0 (10)	100.7 ± 6.5 (7)
vHPC	*Mr*	86.7 ± 16.3 (9)	81.0 ± 27.7 (10)	93.3 ± 26.2 (9)	73.1 ± 19.7 (8)
	Gr	108.9 ± 13.8 (8)	101.0 ± 26.6 (9)	101.4 ± 28.9 (10)	93.5 ± 47.0 (8)
	Fkbp5	106.6 ± 19.1 (6)	108.5 ± 54.1 (9)	105.1 ± 43.6 (8)	112.2 ± 46.1 (8)
	Disc1	236.0 ± 163.7 (9)	167.5 ± 92.3 (9)	153.1 ± 78.2 (3)	261.8 ± 200.8 (8)
	Gsk3b	134.0 ± 39.0 (9)	119.1 ± 26.8 (10)	132.3 ± 38.6 (10)	105.7 ± 26.5 (8)
	Bdnf	102.0 ± 55.5 (9)	121.1 ± 36.0 (10)	109.1 ± 33.9 (10)	124.5 ± 28.7 (8)
	Nrg1	196.4 ± 110.0 (9)	162.1 ± 74.0 (9)	164.2 ± 73.7 (9)	187.6 ± 88.0 (8)
	Homer1	85.3 ± 14.3 (9)	86.0 ± 18.4 (10)	99.9 ± 12.7 (9)	74.2 ± 21.0 (8)
	Homer2	91.6 ± 16.4 (9)	89.3 ± 23.6 (10)	91.7 ± 32.8 (10)	89.0 ± 29.0 (8)
	Homer3	119.4 ± 50.7 (9)	123.0 ± 37.0 (10)	86.7 ± 25.4 (10)	108.8 ± 33.3 (8)
	Shank 1	155.9 ± 80.5 (9)	161.8 ± 90.9 (10)	145.0 ± 68.4 (9)	130.4 ± 65.7 (8)
	Shank 2	130.2 ± 49.8 (9)	115.9 ± 40.1 (9)	116.3 ± 20.7 (8)	132.9 ± 55.3 (8)
	Shank 3	133.6 ± 72.7 (9)	123.5 ± 66.4 (10)	93.2 ± 70.2 (10)	101.0 ± 37.8 (8)
	Spinophilin	129.4 ± 42.8 (9)	121.5 ± 38.7 (10)	110.8 ± 32.3 (10)	112.7 ± 26.6 (8)
	Cofilin 1	105.2 ± 16.7 (8)	95.5 ± 27.9 (10)	92.7 ± 34.6 (9)	105.8 ± 18.3 (8)

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Manuscript IV

BDNF$^{+/-}$ rats show depressive phenotype and altered expression of genes relevant in mood disorders

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Abstract

Major depressive disorder (MDD) is a leading contributor to the global burden of disease. However, the causal relationship of risk factors, such as genetic predisposition or experience of augmented stress and subsequent pathology of MDD, remain unknown. Numerous studies in humans and rodents have implicated brain-derived neurotrophic factor (BDNF) in MDD pathology, as a genetic risk factor and a factor regulated by stress. Until now, the majority of preclinical studies have employed genetically modified mice as their model of choice. However, mice display a limited behavioural repertoire and lack expression of circulating BDNF, which is present in rats and humans. Furthermore, few studies have investigated the relationship between altered BDNF levels and expression of genes associated with affective disorders. We found that heterozygous BDNF rats ($BDNF^{+/-}$), which have reduced BDNF levels in the brain and plasma, displayed anhedonia, a core symptom of MDD, and mild signs of anxiety but no behavioural despair or cognitive impairments. This was accompanied by changes in the expression of genes that are implicated in modulation of the stress response and affective disorders. Hence, glucocorticoid receptor, neuregulin 1 and disrupted-in-schizophrenia 1 gene expression were upregulated in the prefrontal cortex of $BDFN^{+/-}$ rats, whereas FK506 binding protein 5 levels were decreased in the hippocampus. We conclude that a reduction in BDNF levels alters expression of genes associated with affective disorders, which may contribute to the development of depressive-like symptoms.

1 Introduction

Major depressive disorder (MDD) is the leading cause for disability worldwide affecting 300 million people and their socio-economic environment.[1] Stressful life events, such as constant stress at work or family disharmony, can trigger MDD development, especially in predisposed individuals.[2–5] By implication, MDD pathology emerges from a gene x environment interaction eliciting a heterogeneity of symptoms.[6] Heritability of MDD is presumed to be 30-40%.[7] However, many genes which exert small effects on their own may interact to contribute to the overall pathogenesis of MDD;[8] confounding the identification of MDD-specific candidate genes. Furthermore, MDD patients display high comorbidities with other neuropsychiatric diseases, such as anxiety disorders, which additionally enhances the complexity of MDD.[9,10] Hence, the aetiology of MDD pathogenesis is still insufficiently understood, which precludes the tailoring of antidepressant treatment and reduces drug efficacy.

Brain-derived neurotrophic factor (BNDF), involved in neural circuit function and plasticity,[11] was identified as a possible contributor to MDD pathogenesis and drug efficacy.[12,13] The BDNF polymorphism Val66Met, linked to reduced BDNF activity,[14,15] was shown to have a strong interaction with stressful life events in MDD pathogenesis.[15] Moreover, stress, which is known as major environmental risk factor for MDD development,[4,5,16] reduces BDNF levels in the hippocampus (HPC).[17] The HPC is a brain region that also shows reduced plasticity in environmentally-induced preclinical MDD models[18] and atrophy in humans with MDD.[19] Furthermore, reduced BDNF levels in prefrontal cortex (PFC) and HPC were found in post-mortem tissue of MDD patients;[20] whereas antidepressant treatment elevated BDNF levels in the HPC.[21] Similarly, serum BDNF was decreased in depressed patients and elevated following medication.[13,22] Moreover, infusion of BDNF in the midbrain induced an antidepressant-like effect[23] and antidepressant drug efficacy was shown to be BDNF level dependent.[24–26] Hence, these findings promoted preclinical research into MDD using mice with genetically reduced BDNF expression.

However, these preclinical studies resulted in inconsistent findings with genetically-reduced levels of BDNF provoking depressive-like phenotypes in only a limited proportion of mouse studies.[26–28] One possible explanation may be that mice, unlike humans and rats, do not express peripheral BDNF.[29] Peripheral administration of BDNF in mice altered gene expression in the brain and produced an antidepressant-like and anxiolytic behavioural response.[30] Thus, peripheral BDNF levels might contribute to the pathogenesis and treatment of depression and highlight that rats may be a more appropriate species to investigate the relationship between BDNF and MDD. Furthermore, rats exhibit a more extensive behavioural repertoire than mice and are considered translationally more relevant to humans. Finally, most behavioural tests are designed to characterize rat behaviour,[31] making the interpretation of BDNF mouse studies difficult. Rats heterozygous for the BDNF gene ($BDNF^{+/-}$) express lower BDNF levels in the brain and periphery[32] and may be a more relevant preclinical model, overcoming the inconsistent findings in mice and generating more translational results.

Therefore, the present study aimed to determine the direct effect of reduced BDNF levels on behavioural alterations and disease-related gene expression levels in the brain. Rats heterozygous for the BDNF gene were behaviourally phenotyped for anxiety and depressive-like behaviours. Reduced plasticity and altered release of hypothalamic neuropeptides in response to lower BDNF levels might impair the homeostasis of the hypothalamic-pituitary-adrenal (HPA) axis, which is an important regulator of the stress response, and often altered

in patients with affective disorders.[2,33,34] Thus, we measured the expression of genes involved in regulating the stress response (the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), corticotropin releasing hormone (*CRH*), and FK506 binding protein 5 (*FKBP5*)) and expression of genes relevant in neuropsychiatric diseases (disrupted in schizophrenia-1 (*Disc1*), glycogen synthase kinase 3 beta (*GSK3B*) and neuregulin 1 (*NRG1*)). We hypothesized these genes would be differentially regulated in $BDNF^{+/-}$ rats compared to controls and be associated with depressive- and/or anxiety-like behaviours.

2 Material and Methods

2.1 Animals

All animal experiments were approved by the University of Edinburgh Ethical Review Committee and studies were carried out in strict accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive of 22 September 2010 (Directive 2010/63/EU).

Animals were generated by crossing male Sprague-Dawley (Hsd:SD) rats that were heterozygous for a BDNF knockdown mutation (HET, SD-$BDNF^{tm1sage}$; generated using zinc finger nuclease technology, SAGE®Labs, St Louis, MO, USA) with control female SD rats (SAGE®Labs, St Louis, MO, USA). Litters comprised of $BDNF^{+/+}$ wild type rats (WT) and BDNF heterozygous rats ($BDNF^{+/-}$). $BDNF^{+/-}$ (n = 13) and WT rats (n = 14) were 11-12 weeks old and weighed an average of 384 ± 49 g at the beginning of behavioural testing (except for Morris water maze (MWM) test). For the MWM test, a separate cohort of 5 WT and 10 $BDNF^{+/-}$ rats were employed (30 weeks of age). Another group of 10 $BDNF^{+/-}$ and 9 WT rats at 11-13 weeks of age were used for testing brain gene expression levels. Animals were housed in mixed genotype groups of 3-4. Rats had free access to food and water and were kept on a 12 h light/dark cycle (lights on at 7:00 am). All of the following behavioural tests are listed in the order that they were conducted and took place in the first half of the light cycle (except Sucrose preference test (SPT) which was assessed over 48 h).

The behavioural test battery was conducted in the following order: sucrose preference test, elevated-plus-maze, novelty induced hypophagia, spontaneous alternation behaviour, open field and forced swim test. This order was chosen to minimize the interference of stressful tests with other tasks.

2.2 Sucrose preference test

The sucrose preference test (SPT) measures the hedonic state of each animal. Rats were habituated to drink a palatable sucrose solution (1.5%) for two days. A bottle of water and a bottle of the sucrose solution were made available in the animals' home cage. One and a half days after habituation, animals were single-housed and exposed to two bottles, one with water and one with the sucrose solution, for 48 h. Bottle position (left/right) was counterbalanced across cages and switched after 24 h during habituation and test phase. Water and sucrose solution consumption, body weight and food intake were measured after 48 h.

2.3 Elevated plus-maze

Anxiety-related behaviour was assessed in the elevated plus-maze (EPM) 3–5 days after the first SPT. Rats were habituated in their home cage to the experimental room 1 h prior to testing. The EPM consisted of a maze shaped like a plus sign (arm size: 10 cm width, 45 cm length) and elevated 66 cm from the floor. Two opposing arms, the closed arms, were enclosed with high walls (50 cm height), the two other arms were open, leaving a central area (10 x 10 cm) in the middle of the EPM. Illumination in the closed arms was 2.5 lux and 45 lux in the open arms. In a randomized order, rats were positioned in the centre of the EPM facing a closed arm. Each rat could explore the maze for 5 min. EPM was cleaned with 70% ethanol between animals. Distance travelled and time spent in open or closed arms as well as head dips and rearing were recorded using ANY-maze automatic tracking software (ANY-maze, Stoelting Co.,Wood Dale, IL, USA).

2.4 Novelty induced hypophagia

The novelty induced hypophagia (NIH) task tested anhedonic-like behaviour (decreased motivation to consume a reward) and anxiety-related behaviour (fear of eating in a novel and open environment). Eight days after the EPM test, rats were habituated to eat a chocolate chip (280 mg; Milk chocolate chips, Wm Morrison Supermarket PLC, Bradford, UK) in their home cage on four consecutive days. On the following day, the animals were moved to the experimental room 30 min prior to testing. The room illumination was adjusted to approximately 65–70 lux. After acclimation to the experimental room, a chocolate chip was positioned at the one end (35 lux) of an experimental box (66 cm length, 28 cm width, 40 cm height, non-transparent) and the rat was placed at the other end of the box (24–25 lux). Latency to consume the chocolate chip was manually scored from recorded videos. Rats

which did not consume the chocolate chip within the time limit were listed with the full experimental duration of 15 min. Two animals were tested in parallel and boxes were cleaned with 70% ethanol between animals.

2.5 Spontaneous alternation behaviour test

The spontaneous alternation behaviour (SAB) task assesses working memory in rodents. The SAB test was carried out 5 days after the NIH task and according to Henningsen et al..[35] Animals were acclimatized to the testing room 45 min prior to testing. Light intensity was 10–13 lux in the arms (49 cm length, 17 cm width, 32 cm height) and 17 lux in the triangular centre of the Y-shaped maze (120° angles). Each rat was placed at the end of the same arm facing the back wall and allowed to explore the Y-maze for 10 min. Arm entries (all four paws in arm) were recorded using ANY-maze automatic tracking software (ANY-maze, Stoelting Co.,Wood Dale, IL, USA). The primary readout was alternation ratio, which was calculated by the number of alternations (visiting all three arms consecutively) divided by the maximum possible alternation score (number of arm entries minus two). A high alternation ratio shows that the rat is not re-entering an arm that was previously visited, indicating good working memory. The apparatus was cleaned with 70% ethanol between animals.

2.6 Forced swim test and open field

The forced swim test (FST) is primarily used to investigate immobility behaviour (floating with minimum movements to keep the head above water) indicating a rat's propensity to surrender to a seemingly hopeless situation. On the contrary, swimming (horizontal movements throughout the cylinder) or struggling (vertical movements with the forepaws, usually against to cylinder wall) are counted as active escape attempts of the situation. Increased immobility is associated with a depressive-like phenotype.[36] On day one of FST, 4 weeks after the SAB test, rats were acclimatized to the testing room (150 lux) 1 h prior to testing. In parallel, two rats were immersed in transparent cylinders (20 cm diameter, 50 cm height) filled with water (38 cm depth; 24 ± 1 °C) for 15 min. The water was renewed between rats. On the following day, the rats' locomotor activity was assessed in the open field (OF). Rats were acclimatized to an adjacent room with dim illumination containing an OF (97 x 97 cm) for 1 h. The OF was divided into a centre area (31 cm from the edge, 30 lux), an outer area (12 cm from edge, 25 lux) and a middle area in between the centre and the outer area. Each rat was placed in the centre of the OF and tracked using ANY-maze automatic tracking software (ANY-maze, Stoelting Co.,Wood Dale, IL, USA) for 10 min.

Time spent and distance travelled in each zone as well as number of fecal boli were measured. After the OF test, the rat was transferred to the room of the FST and exposed to 7 min of forced swimming. Predominant behaviour (immobility, swimming or struggling) was scored manually from recorded videos with time-sampling technique (5 sec) by an examiner blindfolded to group identity until score reliability was within 10%. Diving was scored as struggling.

2.7 Morris Water Maze

Spatial learning and memory, as well as reversal learning was examined in the MWM (2 m diameter, 0.5 m height) with a naïve cohort of rats (5 WT, 10 $BDNF^{+/-}$). Rats were trained to find a hidden Atlantis escape platform (12 cm diameter; Ugo Basile, Italy) for five days with two trials per day. Each rat was released from one of four release points in a pre-determined random order. Rats had up to 120 s to find and mount the platform and were guided there if they failed to find it. Latency, swim speed and distance were recorded using Actimetrics Watermaze software (Actimetrics, IL, USA). On day six (24 h after the last test), each rat received a 60 s probe trial in which the escape platform was absent, but rose up after 60 s (to prevent extinction of the location). During the probe trial, % time spent in quadrant, % thigmotaxis (swimming within 15 cm of the pool edge), swim speed and number of platform crossings were analysed. On day seven and eight, perseverant behaviour was assessed using a reversal protocol in which the platform was moved to a randomly determined location (middle of one of the four quadrants). Rats received two swims on day seven and one swim on day eight.

To assess visual ability, a trial in which the platform was visible (i.e. the water level was lowered) was conducted two weeks after the probe trial with curtains pulled around the maze to hide spatial cues (both groups reached the platform in under 12 ± 2 s; data not shown).

2.8 Tissue collection and RT-qPCR

Quantitative PCR (qPCR) was used to investigate the cerebral expression of genes associated with neuropsychiatric disease and hypothalamic-pituitary-adrenal (HPA) axis regulation. Naïve WT and $BDNF^{+/-}$ rats were culled by decapitation. The HPC and PFC were quickly removed from the right hemisphere, immediately frozen on dry ice and stored at -80 °C. Approximately 70–80 mg (PFC) and 50-60 mg (HPC) of tissue was homogenised in Qiazol Lysis Reagent (Qiagen GmbH, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. RNA was then isolated from the supernatant using the RNase

MiniKit (250) (Qiagen GmbH) with an on column DNAse step to reduce possible sample contamination with DNA (Qiagen GmbH). RNA quantification and integrity (absorbance at 280/260 nm > 2.10, 260/230 nm > 1.45) was analysed by Nanodrop (Spectrophotometer ND-1000, Software ND-1000 V3.8.1; NanoDrop Technologies Inc., Delaware, USA). High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, ThermoFisher, Massachusetts, USA) was used for reverse transcription PCR, by adding 500 ng of RNA to 10 µl of reverse transcription reagent, resulting in a final volume of 20 µl. Samples were incubated for 10 min at 25 °C, 120 min at 37°C, 5 min at 85 °C, cooled down to 4 °C and stored at -20 °C. For the qPCR, cDNA was diluted 1:20 with diethyl pyrocarbonate treated water and triplicates of 2 µl of the sample and 8 µl of master mix (Roche, LightCycler 480 Probes Master, Baden-Württemberg, Germany) with TaqMan Gene expression Assays (Applied Biosystems, ThermoFisher) were used. Reference genes and target genes are listed in Table 1. The thermal conditions for the qPCR were 5 min at 95 °C, followed by 50 cycles of 10 s denaturation at 95 °C and 30 s annealing at 60 °C. Finally, qPCR was completed with 30 s extension at 40 °C.

A standard curve of eight two-fold dilutions was run for each target and reference gene on the same plate as the respective samples. PCR efficiency was calculated from the standard curve's slope and outliers within the triplicate were excluded if standard deviation (SD) of the triplicates was > 0.4. A combination of reference genes were chosen based on good correlation with other reference genes and low variance within the reference gene across groups and tissue. Hence, an average of the relative concentration of Actb and Hprt1[37] were used to normalise target genes.

2.9 Statistical analysis

Behavioural and qPCR data (normalised to WT group mean) were analysed with Student's t-test or Welch's unequal variance t-test if the assumption of homogeneity of variance was violated. Normality was assessed with QQ-plots and Shapiro Wilk test, and if violated, data was log-transformed or non-parametric Mann-Whitney *U* test was applied. Two $BDNF^{+/-}$ animals were excluded from the SPT because their total fluid intake was < 5 g within 48 h, which could be due to a measurement error. Repeated measurement data of the MWM was analysed with multivariate repeated measures ANOVA. Outliers were removed according to Grubb's ($\alpha = 0.05$, two-sided) or ROUT test (Q = 1%; GraphPad Prism 6, GraphPad Software Inc., California, USA). Data analysis was carried out in Stata (Stata 14.0, StataCorp, Texas, USA). Graphs were created with GraphPad Prism 5.

Table 1 Details of genes and primers used for qPCR.

Used gene abbreviation	Gene name	Probe	Amplicon length	Application
GR (*Nr3c1*)	Nuclear receptor subfamily 3, group C, member 1 coding for glucocorticoid receptor	Rn00561369_m1	73	Target gene
MR (*Nr3c2*)	Nuclear receptor subfamily 3, group C, member2 coding for mineralocorticoid receptor	Rn00565562_m1	79	Target gene
Crh	Corticotropin releasing hormone	Rn01462137_m1	112	Target gene
Fkbp5	FK506 binding protein 5	Rn01768371_m1	74	Target gene
Disc1	Disrupted in schizophrenia 1	Rn00598264_m1	73	Target gene
Nrg1	Neuregulin 1	Rn01482168_m1	86	Target gene
Gsk3b	Glycogen synthase kinase 3 beta	Rn01444108_m1	96	Target gene
Actb	Beta Actin	Rn00667869_m1	91	Reference gene
Hprt1	Hypoxanthine phosphoribosyltransferase 1	Rn01527840_m1	64	Reference gene
45S	45S pre-ribosomal RNA	Rn03928990_g1 RN45s	61	(Reference gene)
Tbp	Tata box binding protein	Rn01455646_m1	75	(Reference gene)

3 Results

3.1 BDNF$^{+/-}$ rats exhibit anhedonia but not behavioural despair

3.1.1 Forced swim test reveals no difference in behavioural despair

The FST assesses behavioural despair indicated by a longer time spend passively coping, i.e. immobile, relative to actively coping behaviours, i.e. swimming or struggling, in an unescapable situation. No difference between genotypes was observed for time spent immobile, engaged in swimming or struggling behaviour. Salient is the bimodal distribution in the WT group for struggling behaviour and the low immobility scores of the WT group (nearly 40% of WT animals have a lower immobility score than the minimum score for BDNF$^{+/-}$; Figure 1).

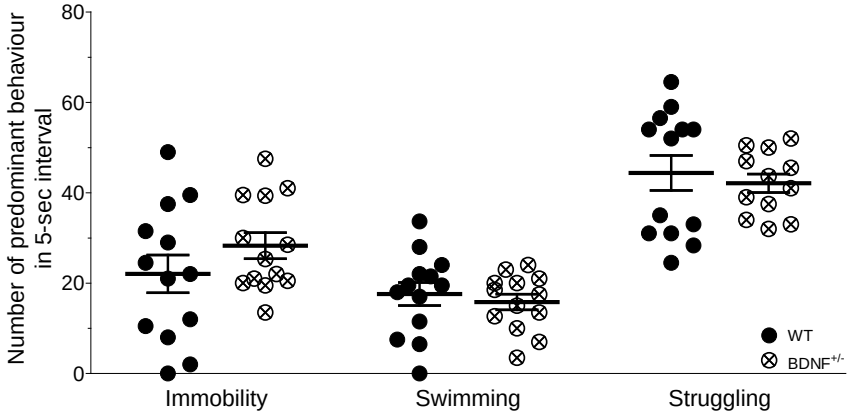

Figure 1. Behavioural parameters during the forced swim test. Time spent engaged in immobility, swimming and struggling during 5-s time intervals during 7 min FST. Group mean (± SEM) as well as individual scores are shown.

3.1.2 BDNF$^{+/-}$ rats exhibit anhedonic behaviour in the sucrose preference test

The SPT assesses the hedonic state of the rats by measuring the preference for a sucrose solution over water intake. No significant difference was observed in total fluid intake between the WT and BDNF$^{+/-}$ rats (Figure 2A). Sucrose preference, i.e. sucrose solution consumption normalised to total fluid intake, was significantly different between groups ($t(14.61) = 2.82$, $p = 0.013$) with BDNF$^{+/-}$ animals exhibiting a lower sucrose preference than WT animals (Figure 2B), thus indicating increased anhedonic-like behaviour in the BDNF$^{+/-}$ rats compared to WT controls. Food intake and percent change in body weight (both normalised to body weight) were not significantly different between the genotypes during single-housing.

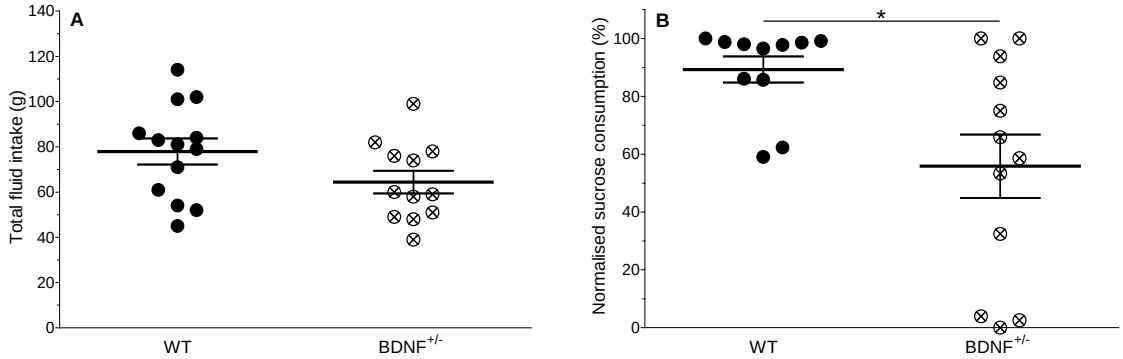

Figure 2. Sucrose preference test. (A) Total fluid consumption. **(B)** Percent normalised sucrose consumption, i.e. sucrose preference. The individual results are plotted with mean (± SEM) for each group. Statistical significance between groups is indicated with *$p < 0.05$.

3.1.3 Novelty induced hypophagia is similar between genotypes

In the NIH test, the drive to consume a palatable chocolate reward competes with the fear of a novel environment and thus assesses anxiety- as well as anhedonic-like behaviour[38]. No significant difference was found for reward collection latency between WT (254.69 ± 183.40 s, *Median* = 220) and BDNF$^{+/-}$ rats (344.00 ± 334.54 s, *Median* = 202). This data support no differences in a combined readout on anhedonic-like behaviour and anxiety-related behaviour between WT and BDNF$^{+/-}$ rats. However, three BDNF$^{+/-}$ rats did not consume the chocolate reward within the time limit (900 s) and hence the time limit was used as their collection latency although the true value could have been much higher.

3.2 BDNF$^{+/-}$ rats display mild anxiety-like behaviour

3.2.1 BDNF$^{+/-}$ rats display normal anxiety-like behaviour in the elevated plus-maze

Anxiety-like behaviour was evaluated in BDNF$^{+/-}$ and WT rats since MDD is often accompanied by anxiety. The % distance travelled in the open arms, % time spent in the open arms and % number of open arm entries was similar across genotypes (Figure 3).

However, WT rats (10.37 ± 3.13 m) travelled a significantly greater total distance ($t(25) = 2.37$, $p = 0.026$) in the EPM than BDNF$^{+/-}$ animals (7.56 ± 3.04 m). This effect is likely due to WT rats (7.32 m ± 2.61 m) travelling a greater distance compared to BDNF$^{+/-}$ rats (5.17 ± 2.55 m) in the closed ($t(25) = 2.16$, $p = 0.041$), but not in the open arms. A trend in number of entries to the closed ($U = 1.779$, $p = 0.075$), but not open arms, was observed between WT (14.46 ± 7.11 entries, *Median* = 11) and BDNF$^{+/-}$ animals (9.08 ± 5.25 entries, *Median* = 8). A trend ($t(25) = 1.98$, $p = 0.059$, data log-transformed) in rearing behaviour was observed with WT animals (6.42 ± 10.12 s, *Median* = 11.8) spending more time rearing than their BDNF$^{+/-}$ littermates (10.79 ± 8.25 s, *Median* = 9.05). Time spent head dipping into open arms was similar between groups. WT rats (2.77 ± 3.00 boli, *Median* = 2) produced significantly more faecal boli ($U = 2.371$, $p = 0.018$) than BDNF$^{+/-}$ rats (0.31 ± 0.63 boli, *Median* = 0) during testing on the EPM. Body weight of the animals, which can influence behaviour in the EPM, was not significantly different between genotypes at the time of testing. In sum, the primary readouts of the EPM suggest equal anxiety-like behaviour between genotypes, but decreased locomotor activity in the BDNF$^{+/-}$ rats.

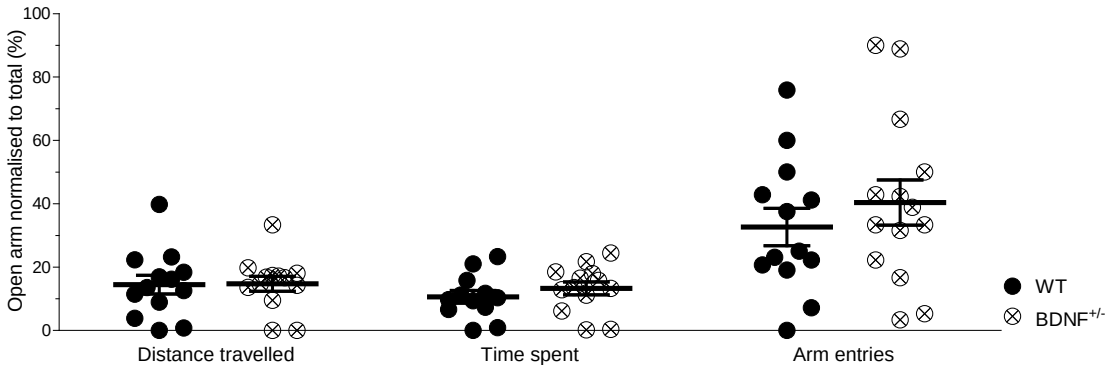

Figure 3. Behavioural parameters in the EPM. Distanced travelled in the open arm, time spend in the open arm and number of open arm entries normalised to total distance travelled, duration of experiment and number of arm entries to closed and open arm respectively. Group mean (± SEM) and individual scores are shown, $^{*}p < 0.05$, $^{\#}p < 0.06$ indicate significance level.

3.2.2 BDNF$^{+/-}$ rats show increased anxiety-like behaviour in open field test

The open field (OF) test assesses the conflict between anxiety-related behaviour (fear of open and lit areas) and a desire to explore. Furthermore, the OF allows assessment of locomotor activity, which could be a cofounder, for example in the FST.[36] BDNF$^{+/-}$ rats (*Median* = 2.10 s) spent less time in the centre than their WT littermates (*Median* = 9.70 s; $t(12.13) = 3.31$, $p = 0.006$; Figure 4A). BDNF$^{+/-}$ rats (21.29 ± 16.97 s) also spent less time in the middle zone ($t(25) = 2.28$, $p = 0.031$) than WT rats (41.29 ± 27.70 s). Accordingly, BDNF$^{+/-}$ rats (574.59 s ± 21.14, *Median* = 574.85) spent more time in the outer zone ($t(16.39) = -2.38$, $p = 0.030$) than WT animals (540.76 ± 47.05 s, *Median* = 564.80). However, WT animals travelled a greater total distance ($t(25) = 3.21$, $p = 0.004$) than BDNF$^{+/-}$ animals in the OF test (Figure 4B) suggesting decreased locomotor activity in BDNF$^{+/-}$ animals than in WT rats. Since locomotor activity could interfere with the time spent in a zone, % distance travelled in each zone was analysed. Similarly to time spent in a zone, BDNF$^{+/-}$ rats (*Median* = 2.60%) travelled less in the centre zone ($U = 1.99$, $p = 0.047$; Figure 4C) than their WT littermates (*Median* = 4.55%); and less distance in the middle zone ($t(25) = 2.36$, $p = 0.027$; BDNF$^{+/-}$: 10.31 ± 6.68%; WT: 16.19 ± 6.27%). Subsequently, BDNF$^{+/-}$ rats (86.95 ± 8.90%) travelled more in the peripheral zone ($t(25) = -2.47$, $p = 0.021$) compared to WT rats (78.38 ± 9.10%). These data support greater anxiety-like behaviour in the BDNF$^{+/-}$ rats than in their WT littermates. No significant difference between genotypes was found for number of faecal boli in the OF.

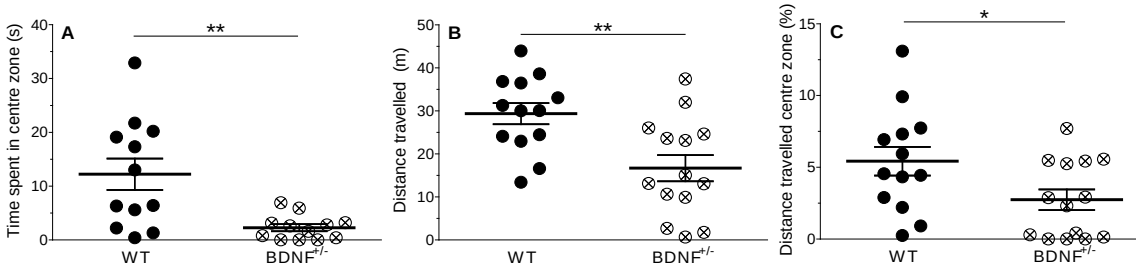

Figure 4. Open field behaviour. (A) Time spent in centre zone of the OF. (B) Total distance travelled in the OF. (C) Percentage distance travelled in centre zone. Significant differences between genotypes are indicated with *$p < 0.05$, **$p < 0.01$. Individual results and group mean (± SEM) are displayed.

3.3 BDNF$^{+/-}$ rats exhibit normal cognitive performance

3.3.1 BDNF$^{+/-}$ rats exhibit normal working memory

The alternation ratio (number of sequentially alternating arm entries normalised to the total number of arm entries), a readout of spatial working memory, did not differ between the genotypes (WT: 70.97 ± 7.54%; BDNF$^{+/-}$: 67.13 ± 16.89%). Results were not cofounded by a difference in total number of arm entries between WT (21.31 ± 7.02 entries) and BDNF$^{+/-}$ (15.57 ± 8.68 entries) rats. No difference in distance travelled in the Y-maze was observed between the two groups (WT: 19.32 ± 4.84 m; BDNF$^{+/-}$: 17.42 ± 7.60 m). Hence, this data suggest no difference in working memory or locomotor activity between BDNF$^{+/-}$ and WT rats.

3.3.2 BDNF$^{+/-}$ rats show normal performance in the Morris water maze

Spatial learning and memory performance, as well as perseverance behaviour was assessed in the MWM task because cognitive deficits have been frequently reported in depression. BDNF$^{+/-}$ rats showed a similar learning curve as WT controls. Both groups improved performance over time by decreasing their latency to find the platform in the water ($F(4,55.27) = 13.55$, $p < 0.0001$; Figure 5A). When the platform was removed the day after acquisition, BDNF$^{+/-}$ and WT controls showed comparable performance for % time in target quadrant (WT: 34.33 ± 10.51%; BDNF$^{+/-}$: 37.00 ± 7.53%), number of platform crossings (WT: 2.4 ± 2.07 crossings; BDNF$^{+/-}$: 1.3 ± 1.16 crossings), thigmotaxis (WT: 15.4 ± 8.99%; BDNF$^{+/-}$: 21 ± 12.58%), and swim speed (WT: 21.60 ± 3.85 cm/sec; BDNF$^{+/-}$: 22.63 ± 1.92 cm/sec). Both groups improved performance during reversal learning ($F(2,27) = 18.68$, $p <$ 0.0001), but no difference in reversal learning latency was found between groups (Figure 5B). WT (39.00 ± 7.93%) and BDNF$^{+/-}$ (39.20 ± 13.03%) rats spent similar time in the

original target quadrant during reversal learning. Thus, BDNF$^{+/-}$ rats display intact spatial learning and memory as well as normal perseverative behaviour.

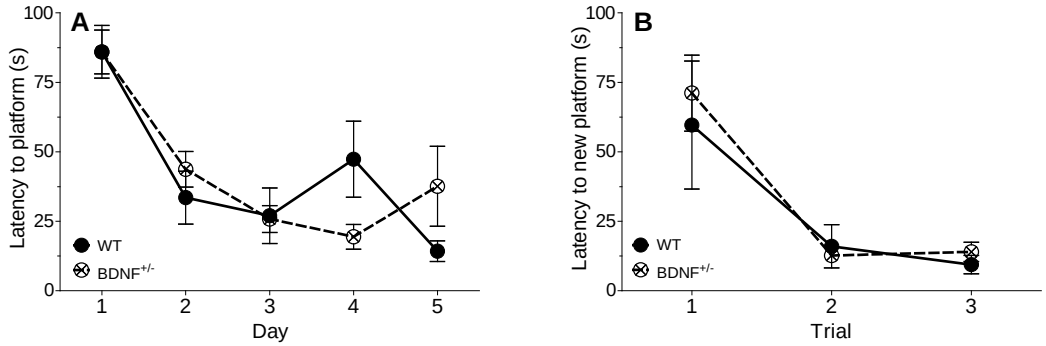

Figure 5. Morris water maze test. (A) Learning phase of finding the hidden platform (mean of two trials per day). (B) Reversal learning of finding the platform in a new location.

3.4 Locomotor activity

As many of the tests in behavioural mazes can be confounded by the general locomotor activity of the rats, it is important to determine if there is an alteration in general activity between genotypes. There was no difference in the total distance travelled in the Y-maze (section 3.3.1) or swim speed in the water maze (section 3.3.2) suggesting similar locomotor activity across genotypes. However, BDNF$^{+/-}$ rats moved less in the OF (Figure 4B) and in the EPM (section 3.2.1) compared to the WT controls. Thus, when altered locomotor activity between genotypes was present in tasks, it was accounted for in the respective analysis.

3.5 Gene expression

To test whether reduced BDNF levels alter expression patterns of a selection of genes thought to underpin the depressive phenotype, we investigated mRNA expression of genes involved in affective disorders and relevant for an appropriate stress response in the PFC and HPC of naïve WT and BDNF$^{+/-}$ rats. Gene expression was upregulated in the PFC of BDNF$^{+/-}$ rats compared to WT animals for GR ($t(17) = -2.30$, $p = 0.035$), *Nrg1* ($t(17) = -2.25$, $p = 0.038$) and *Disc1* ($t(17) = -4.71$, $p = 0.0002$) displayed in Figure 6. There were no significant differences between the mRNA levels of MR, *Crh*, *Fkbp5* and *Gsk3b* in the PFC of WT and BDNF$^{+/-}$ rats (Supplementary Table 1). In the HPC, *Fkbp5* mRNA expression was reduced in BDNF$^{+/-}$ animals compared to WT animals ($t(9.45) = 3.09$, $p = 0.012$; Figure 6). However, no significant difference in HPC mRNA expression was identified for GR, MR, *Crh*, *Nrg1*, *Disc1* and *Gsk3b* between WT and BDNF$^{+/-}$ rats (Supplementary Table 1).

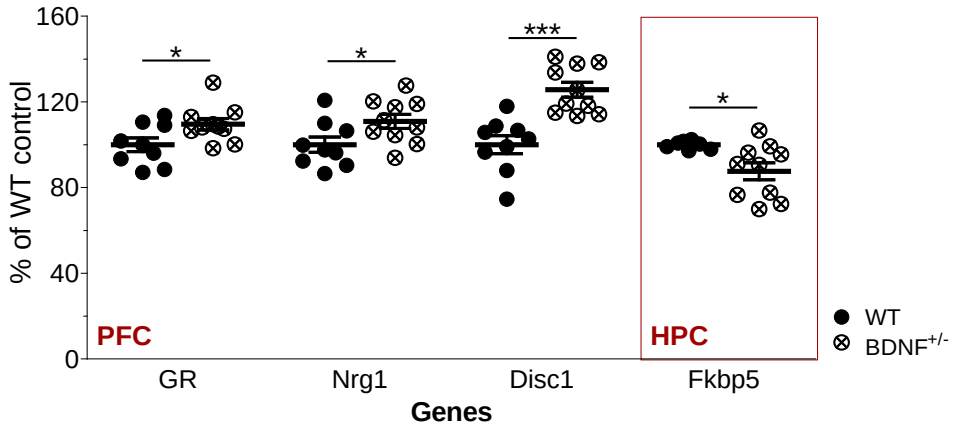

Figure 6. Gene expression. Presented are the individual and group (± SEM) gene expression levels (as % of WT group mean) from the prefrontal cortex (PFC) and hippocampus (HPC). Statistical significance is indicted with $*p < 0.05$, $***p < 0.001$.

4 Discussion

In the present study, we showed that BDNF$^{+/-}$ rats display depressive-like behaviour, decreased locomotor activity and altered mRNA expression of genes in the PFC and HPC compared to WT animals. Anxiety-like behaviour, behavioural despair and cognition was comparable between BDNF$^{+/-}$ and WT littermates.

Depressive-like behaviour was assessed by testing for anhedonia, a core symptom of MDD. Sucrose consumption is frequently used to evaluate the hedonic state of an animal.[39] In the present study, BDNF$^{+/-}$ rats consumed less sucrose solution in favour of a higher water intake compared to WT controls. Hence, reduced BDNF levels resulted in an anhedonic-like state and, thus, BDNF$^{+/-}$ rats exhibit a depressive-like phenotype.

The NIH test evaluates the conflict between avoidance of open and lit areas and the desire for consuming a food reward.[38] No difference for NIH was observed between genotypes. However, all WT rats consumed the reward whereas 20% of the BDNF$^{+/-}$ group did not, which might indicate increased anxiety or decreased motivation or sensitivity for the reward in BDNF$^{+/-}$ rats, consistent with an anhedonic-like phenotype that we found in the SPT. Interestingly, Schmidt and Duman[30] observed a shortened collection latency in the NIH test in WT mice after peripherally administering BDNF, but unaltered behaviour in the sucrose consumption test in these mice, suggesting an anxiolytic effect of BDNF administration with no effect on the hedonic state. These opposite effects compared to the present study might be due to differences in methodology, such as the nature of the reward or

the duration of testing, as well as peripheral administration versus knockdown (KD) of BDNF. Also the model, mouse versus rat, could have an essential effect of BDNF on the behaviour, since mice do not naturally express peripheral BDNF, which can influence brain gene expression and affective behaviour[30]. The importance of the model is further emphasized by the findings that temporal KD of BDNF in the dentate gyrus of rats[40] but not mice[41] during adulthood resulted in a decrease of sucrose consumption. Finally, reduced reward sensitivity in BDNF$^{+/-}$ rats was also shown in a test of cocaine seeking behaviour,[42] strengthening the results of the present study.

In this study, no difference in anxiety-related behaviour was found in the EPM. Anxiety disorders are frequently observed as comorbidity in MDD patients and, hence, the depressive-like behaviour in BDNF$^{+/-}$ rats may have been accompanied by increased anxiety. Peripheral BDNF administered to mice was shown to have an anxiolytic effect in the EPM,[30] however, another study, also with BDNF$^{+/-}$ rats, failed to observe altered anxiety behaviour in the EPM[43] and therefore substantiating our results. Nevertheless, in the present study, BDNF$^{+/-}$ rats spent less time in the centre or middle area of the OF, and significantly more time in the periphery than their WT littermates. Although this behaviour was accompanied with decreased locomotor activity in BDNF$^{+/-}$ rats, the % distance travelled in each zone reflected the findings of time spent in a specific zone. Our OF results are supported by another study,[43] in which BDNF$^{+/-}$ rats spent less time in the centre of the OF and showed decreased locomotor activity. Thus, findings in BDNF$^{+/-}$ rats support anxiety-like behaviour in the OF but not in the EPM.

Cognitive impairments are often seen in MDD[44] and reduced BDNF levels have been implicated with impaired spatial memory.[45] In the SAB test, cognitive impairments were linked to the anhedonic-like phenotype in a preclinical MDD stress model.[35,46] Conversely, we found that working memory was intact in the BDNF$^{+/-}$ group compared to WT rats as examined in the SAB test. These results are supported by BDNF$^{+/-}$ rats displaying intact spatial learning and memory as well as normal perseveration in the MWM test. Thus, BDNF$^{+/-}$ rats displayed intact cognition although reduced BDNF might attenuate neuronal plasticity.[25]

In the present study, BDNF$^{+/-}$ rats did not show behavioural alterations compared to controls in the FST, which is a common test used in preclinical depression research. However, the FST was developed to assess antidepressant drug efficacy rather than depression symptomatology and is sensitive to acute antidepressant treatment while only chronic treatment is efficacious in MDD patients.[36] Therefore the FST may not be the best test for assessing the enduring effects of genetic manipulations.

A selection of cerebral genes were chosen to investigate the neurobiological underpinnings of the behavioural alterations observed in the BDNF$^{+/-}$ rats. Firstly, genes involved in regulating the stress response by modulating the HPA axis were examined since it was shown that BDNF affects HPA axis activity in healthy individuals[47]. MR expression is associated with a predisposition to show resilience or susceptibility to stress and depression[48,49]. MR and GR regulate HPA axis activity. Increased expression of GR is associated with increased sensitivity of the HPA axis and thus a healthy stress response[49,50]. Both genes are transcription factors and therefore are important regulators of gene expression. *Fkbp5* competes with glucocorticoids to bind to GR and, thus, modulates the negative feedback sensitivity of the HPA axis as well.[51] *Crh* mRNA expression can be taken as activity readout of the HPA axis since it is part of the activation cascade resulting in the downstream release of the corticosteroid stress hormones. BDNF expression modulates CRH levels and, hence, HPA axis function.[52] Increased levels of *Crh* can induce a depressive-like phenotype and, thus, *Crh* might be involved in MDD aetiology.[50] Secondly, genes associated with affective disorders were investigated. In schizophrenia, bipolar disorder and MDD, translocation and loss of function of *Disc1* was shown[53–55]. Overexpression of *Disc1* in the mouse ventral HPC resulted in a depressive-like phenotype[56]. *Disc1* downstream regulates *Gsk3b* and altered expression pattern in the latter one was associated with neuropsychiatric diseases, such as MDD and anxiety disorder[57]. Finally, *NRG1* drives neuronal plasticity and was identified as a susceptibility gene in neuropsychiatric diseases and associated with cognitive impairments[58]. In the present study, gene mRNA expression was upregulated in BDNF$^{+/-}$ rats compared to WT controls in the PFC for GR, *Nrg1* and *Disc1*, whereas no differences were observed on MR, *Crh*, *Fkbp5* or *Gsk3b* mRNA levels between the groups. The mRNA levels of the same genes were also assessed in the HPC. Here, only *Fkbp5* expression was reduced in BDNF$^{+/-}$ compared to WT rats. MDD patients show a downregulation of GR mRNA in the frontal cortex[59] and, hence, we would have expected a down- rather than an upregulation of GR in BDNF$^{+/-}$ rats. Furthermore, polymorphisms in *FKBP5* leading to an increased expression of the gene were found in MDD patients.[51] Increased binding of *Fkpb5* to GR reduces the sensitivity of the HPA axis and results in a prolonged stress response, such is often found in patients with depression.[51] Therefore, downregulation of *Fkbp5 mRNA* in the BDNF$^{+/-}$ group was unexpected and did not result in altered GR mRNA expression in the HPC. Furthermore, loss of function of *Disc1* is associated with mental disorders. In the present study, *Disc1* mRNA was upregulated by 26% in the PFC of BDNF$^{+/-}$ animals. Furthermore, *Nrg1* was also upregulated in the PFC of BDNF$^{+/-}$ rats. *Nrg1* is a growth factor involved in synaptic plasticity underlying cognitive

processes, such as spatial learning.[58,60] *NRG1* protein was found upregulated in the PFC of rats exposed to chronic unpredictable mild stress and these rats displayed depressive-like behaviour in the FST and SPT.[61] Thus, increased *Nrg1* gene expression in $BDNF^{+/-}$ rats is complementary to the literature and implicates the role of BDNF in association with *Nrg1* expression in the context of depression.

Although, mRNA levels for GR, *Fkbp5* and *Disc1* were regulated in a different direction than expected, we found that those genes, as well as *Nrg1*, were differently expressed as a consequence of lower BDNF levels. However, to fully understand the functional consequences of these unexpected changes, we will need to determine the corresponding protein levels.

Overall, we have shown that a genetically induced reduction of BDNF levels lead to a depressive-like phenotype as well as alterations in expression levels of genes that are relevant for psychiatric disorders. In future studies, a combination of stress and genetic manipulation might be ideal to provoke a more differentiated phenotype. However, the present study established a solid basis for future research, with the rat as a better model for preclinical studies than the abundantly studied mouse, due the similarity of BDNF expression in rats and humans. Moreover, our study suggests a link of decreased BDNF levels with the MDD core symptom of anhedonia. It is also demonstrated that BDNF expression levels regulate expression of *Disc1*, *Nrg1*, GR and *Fkbp5* genes, relevant in affective disorders and a healthy stress response. Thus, the present study adds to the complex field of entangling the role of BDNF in the development and pathology of MDD.

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Conflict of interest

The authors declare no conflict of interests in relation to the current study.

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Supplementary

Supplementary Table 1 Gene expression. Displayed are the normalised gene expression levels as % WT control mean (group mean ± standard deviation) in the prefrontal cortex (PFC) and hippocampus (HPC). *indicates statistically significant differences between groups.

Brain region	*Gene*	*WT*	*BDNF$^{+/-}$*
PFC	GR*	100 ± 9.6	109.6 ± 8.5
	MR	100 ± 5.2	106.6 ± 13.6
	CRH	100 ± 17.1	111.0 ± 16.2
	FKBP5	100 ± 10.2	92.9 ± 11.9
	*NRG1**	100 ± 10.7	110.9 ± 10.3
	*DISC1**	100 ± 12.7	125.7 ± 11.1
	GSK3B	100 ± 11.0	111.7 ± 8.2
HPC	GR	100 ± 6.6	105.5 ± 12.5
	MR	100 ± 9.7	101.6 ± 9.3
	CRH	100 ± 12.9	91.8 ± 14.0
	*FKBP5**	100 ± 1.8	87.6 ± 12.6
	NRG1	100 ± 11.3	93.8 ± 11.3
	DISC1	100 ± 9.4	99.7 ± 15.6
	GSK3B	100 ± 5.0	104.5 ± 6.0

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Declaration of co-authorship

Full name of the PhD student: Lena-Sophie Martis

This declaration concerns the following article/manuscript:

Title:	The effect of rat strain and stress exposure on performance in touchscreen tasks
Authors:	Lena-Sophie Martis, Simone Krog, Thao Phuong Tran, Elena Bouzinova, Sofie L. Christiansen, Arne Møller, Megan C. Holmes, Ove Wiborg

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If accepted or submitted, state journal: Physiology & Behavior

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

A. Has essentially done all the work
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C. Has contributed considerably (34-66 %)
D. Has contributed (10-33 %)
E. No or little contribution
F. N/A

Element	Extent (A-F)
1. Formulation/identification of the scientific problem	C
2. Development of the method	N/A
3. Planning of the experiments and methodology design and development	C
4. Involvement in the experimental work/clinical studies/data collection/obtaining access to data	C
5. Development of analysis plan and preparation of data for analysis	A
6. Planning and conducting the analysis of data	A
7. Interpretation of the results	A
8. Writing of the first draft of the manuscript	A
9. Finalization of the manuscript and submission	A

Signatures of first- and last author, and main supervisor

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This declaration concerns the following article/manuscript:

Title:	Resilient and depressive-like rats show distinct cognitive impairments in the touchscreen paired-associates learning (PAL) task
Authors:	Lena-Sophie Martis, Claudia Brision, Megan C. Holmes, Ove Wiborg

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

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D. Has contributed (10-33 %)
E. No or little contribution
F. N/A

Element	Extent (A-F)
1. Formulation/identification of the scientific problem	A
2. Development of the method	A
3. Planning of the experiments and methodology design and development	A
4. Involvement in the experimental work/clinical studies/data collection/obtaining access to data	B
5. Development of analysis plan and preparation of data for analysis	A
6. Planning and conducting the analysis of data	B
7. Interpretation of the results	A
8. Writing of the first draft of the manuscript	A
9. Finalization of the manuscript and submission	A

Signatures of first- and last author, and main supervisor

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This declaration concerns the following article/manuscript:

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Authors:	Lena-Sophie Martis, Kristoffer Højgaard, Megan C. Holmes, Betina Elfving, Ove Wiborg

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C. Has contributed considerably (34-66 %)
D. Has contributed (10-33 %)
E. No or little contribution
F. N/A

Element	Extent (A-F)
1. Formulation/identification of the scientific problem	A
2. Development of the method	A
3. Planning of the experiments and methodology design and development	A
4. Involvement in the experimental work/clinical studies/data collection/obtaining access to data	B
5. Development of analysis plan and preparation of data for analysis	A
6. Planning and conducting the analysis of data	A
7. Interpretation of the results	A
8. Writing of the first draft of the manuscript	A
9. Finalization of the manuscript and submission	A

Signatures of first- and last author, and main supervisor

Date	Name	Signature
16.02.18	Ove Wiborg	
16.02.18	Megan Holmes	

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Date: 17.02.2018

Signature of the PhD student

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Declaration of co-authorship

Full name of the PhD student: Lena-Sophie Martis

This declaration concerns the following article/manuscript:

Title:	BDNF$^{+/-}$ rats show depressive phenotype and altered expression of genes relevant in mood disorders
Authors:	Lena-Sophie Martis, Ove Wiborg, Megan C. Holmes, Anjanette P. Harris

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Genes, Brain and Behavior

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

A. Has essentially done all the work
B. Has done most of the work (67-90 %)
C. Has contributed considerably (34-66 %)
D. Has contributed (10-33 %)
E. No or little contribution
F. N/A

Element	Extent (A-F)
1. Formulation/identification of the scientific problem	B
2. Development of the method	A
3. Planning of the experiments and methodology design and development	A
4. Involvement in the experimental work/clinical studies/data collection/obtaining access to data	B
5. Development of analysis plan and preparation of data for analysis	A
6. Planning and conducting the analysis of data	A
7. Interpretation of the results	A
8. Writing of the first draft of the manuscript	A
9. Finalization of the manuscript and submission	B

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16.02.18	Megan Holmes	
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