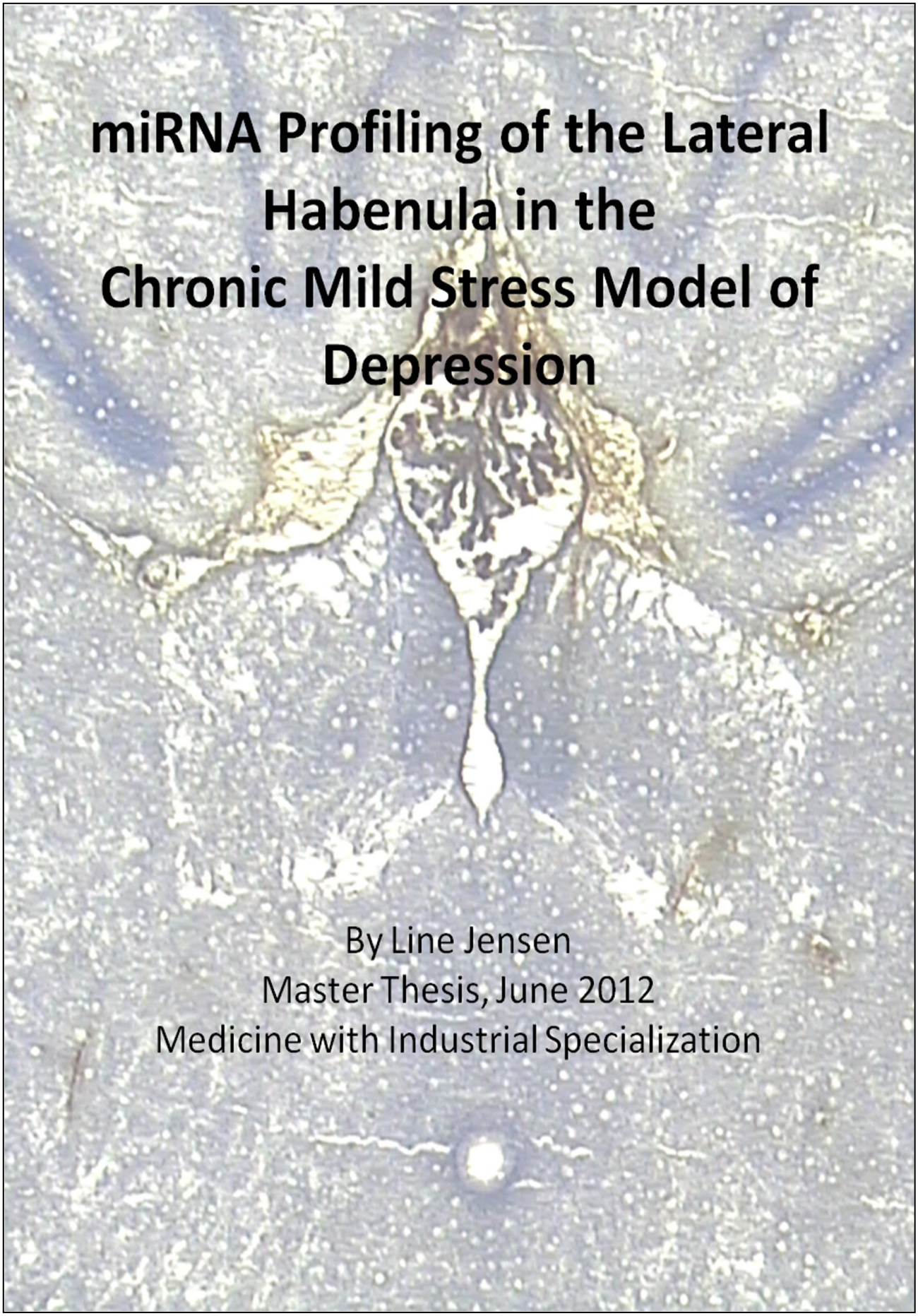

miRNA Profiling of the Lateral Habenula in the Chronic Mild Stress Model of Depression

By Line Jensen

Master Thesis, June 2012
Medicine with Industrial Specialization

Abstract

Depression is a major cause of disability worldwide affecting both the society and patients and, if untreated, the disease carries a high mortality rate due suicide. The disease is vastly comlex and involves disturbances of several neurobiological mechanisms. The therapeutic intervention of depressive-like symptoms has evolved rapidly over the past two decades. Nevertheless, treatment refraction continues to represent a frequent problem in the clinic and up to 40 % fails to respond to the first line of treatment. Despite extensive research the etiology of depression is still unclear. However, emerging evidence suggests that the expression of microRNAs (miRNAs) is altered during stress in brains of behaviourally depressed animals, and in post-mortem brains of depressed patients. Hence, it is suggested that altered miRNA levels may contribute to the progression of depression. Research in the pathophysiology of depression has mainly involved the hippocampus. However, increased neuronal activity of the lateral habenula (LHb) has been observed in depressive patients and in animal models of depression. Additionally, an inhibition of the LHb shows antidepressive effects. The LHb has efferent projections to the monoaminergic system, and hyperactivity of the LHb is associated with an inhibition of this system, a condition observed in depressive patients. These findings indicate a role for LHb in the etiology of major depressive disorder (MDD).

The aim of the present study was to investigate the function of LHb in the development and treatment of MDD at the miRNA level in order to find novel disease targets and treatment regimens. Additionally, a search for biomarkers involved in treatment resistance and stress-resilience was included in the present study.

Depression-related miRNA changes were investigated in the highly validated Chronic Mild Stress Model of depression which generate the phenotypes reflecting depressive-like behavior, stress resilience, and drug response (after chronic treatment with escitalopram). Laser capture microdissection was used for a homogeneous isolation of the LHb and TaqMan® Low Density Arrays were used for large scale miRNA profiling. According to predetermined expression patterns relevant for depression miR-130b, miR-205, miR-327, miR-331-5p, and miR- 336 were associated with the induction and/or recovery from depression, miR-546 were found to be implicated in treatment resistance, and miR-331-5p and miR-409 were involved in stress resilience. Since miR-331-5p is involved in both recovery and stress resilience, it seems likely that the level of miR-331-5p is essential for the healthy state of rats exposed to the CMS model of depression.

Based on the present findings it is difficult to determine the exact role of the LHb in depression-related behaviour. However, several of the predicted mRNA targets, identified by TargetScan Version 6.1, are involved in mechanisms already associated with depression, including neuronal plasticity, neurogenesis, synaptic release, and demethylation suggesting an involvement of LHb in depression. To clarify the exact role of the LHb in the pathophysiology of depression, further investigations are needed.

Preface

This master thesis was written by Line Jensen during the 3rd and 4th semester of the Master of Science in Medicine with Industrial Specialisation at the Department of Health Science and Technology, Aalborg University, Denmark. The experimental work was performed at Centre for Psychiatric Research at Aarhus University Hospital, Risskov, and the supervisors of the project was Jacek Lichota and Ove Wiborg.

The reference list of publications cited can be found at the end of the thesis. References are cited in square brackets, with author´s last name and publication year. A list of the protocols and chemicals used in the present study is found in appendix I. Appendix II contain the weekly sucrose consumption measurements of the different groups investigated in the present study.

I would like to thank Ove Wiborg for giving me the opportunity to perform my master thesis in the Laboratory for Molecular Neurobiology, for his supervision, helpful comments, and guidance during the period. Additionally, I would like to thank Trine Christensen for great supervision, our many discussions, and for your patience. Furthermore, I would like to thank Stine Aistrup Eriksen for great support during the period. At least, I would like to thank all at Centre for Psychiatric Research, Skejby, for having a great time!

Aarhus, 1st of June 2012

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Line Jensen

Abbreviations

5-HT	Serotonin
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ApoER2	Apolipoprotein e receptor 2
BDNF	Brain-derived neurotrophic factor
CBP	CREB binding protein
CMS	Chronic mild stress
CMS-Esc-NR	CMS escitalopram non-responders
CMS-Esc-R	CMS escitalopram responders
CMS-Res	CMS resilience
CMS-V	CMS vehicle
CRH	Corticotropin releasing hormone
CREB	cAMP Response Element Binding protein
CV	Cresyl violet
DA	Dopamine
DEPC	Diethylpyrocarbonate
DRP-2	Dihydropyrimidinase-like 2
DSM-IV	Diagnostic and Statistical Manual IV
ECT	Electroconvulsive treatment
ESC	Electroconvulsive seizures
GR	Glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
ICD-10	International Classification of Disorders 10^{th} revision
IDO	Indoleamine 2,3 dioxygenase
IL	Interleukin
LCM	Laser captured microdissection
LHb	Lateral habenula
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MBD2	Methyl-CpG binding domain protein 2
miRNA	microRNA
MR	Mineralocorticoid receptor
NE	Norephinephrine
PEN	Polyethylene napthalate
PET	Emission tomography
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RIN	RNA integrity number
RISC	RNA-induced silencing complex
SAB	Spontaneous alteration behaviour
SNc	Substantia nigra pars compacta
SRCAP	Snf2-related CBP activator protein
SYN1	Synapsin 1
TB	Toluidine blue
TNF	Tumour necrosis factor
U-V	Unchallenged vehicle
VTA	Ventral tegmental area

Contents

1 | Introduction

Depression, also known as major depressive disorder (MDD), affects approximately 121 million people worldwide, and has been estimated to become the second largest contributor to the global burden of disease and disability by the year 2020 (WHO). MDD affects the patient's emotions, behaviour, and motivation in the everyday life which interferes significantly with the quality of life and general functioning of the patient. MDD affects both sexes, all ages, and all ethnic backgrounds. However, women are more frequently diagnosed with MDD compared to men; rates of depression among women and men are highest in those aged 25-44 years; and finally, the incidence is less common in the black population. MDD can be triggered by daily occurring events such as loss of job, illness, or owing to bereavement (Medscape, Chopra et al. 2011).

Most depressive episodes last for approximately three to four months. About half of the first time suffers will recover and never experience a recurrence, however, approximately 20 % of depressive episodes become chronic (Wager-Smith & Markou 2011). Due to the complexity of MDD and the various pathological mechanisms contributing to the development only 60-70 % of the patients receiving antidepressants gain an effect (Lanni et al. 2009). In severe cases of MDD, patients have an increased risk of suicide resulting in about 850.000 MDD-associated deaths every year globally (WHO). The severity and proportions of MDD motivate scientists all around the world to investigate this multifaceted disorder. Despite extensive research in the field the underlying molecular mechanisms are yet to be discovered, and it remains essential to obtain more knowledge about the disease and to optimize antidepressant treatment options.

1.1 | Major Depressive Disorder

Depression is a heterogeneous disease and no objective diagnostic tests are developed, hence, the diagnosis of MDD is based on a highly variable set of symptoms. The two frequently used diagnostic systems of MDD are the International Classification of Disorders 10th revision, ICD-10 and the Diagnostic and statistical Manual of Mental Disorders Manual IV, DSM-IV. Both diagnostic systems comprise two core symptoms of MDD: (1) Anhedonia; the inability to experience pleasure from activities previously found enjoyable and (2) depressed mood. Several other minor symptoms are associated with MDD, including appetite disturbance, insomnia or hypersomnia, psychomotor retardation or agitation, fatigue or loss of energy, decreased ability to think and concentrate, cognitive difficulties, and suicidal thoughts. One core symptom accompanying four secondary symptoms (or both core symptoms and three secondary symptoms) must be present every day for at least two weeks for the diagnosis of MDD (Vestergaard et al. 2008, Chopra et al. 2011).

As mentioned above the underlying pathology of MDD has not yet been clearly defined. However, increasing evidence suggests that MDD is caused by an interaction between genetic predisposition and environmental factors. Epidemiologic studies have shown that roughly 40-50 % of the vulnerability to MDD is genetic (Nestler et al. 2002), thus making it a highly heritable disorder. Nevertheless, vulnerability genes for MDD have not yet been identified (Krishnan & Nestler 2008). Concerning environmental factors the involvement of stress-full life-events regarding the development of depressive episodes has long been recognised. In 60-70 % of depression cases the first episode is associated with severe life-events. Hence, it is suggested that stress can initiate a cascade of biological events leading to MDD (Wager-Smith & Markou 2011). The following sections focus on current knowledge and different hypotheses of the underlying mechanisms of MDD.

Several hypotheses of depression have been proposed during the last decades. The most accepted theories are the monoamine hypothesis, the hypothalamic-pituitary-adrenal (HPA) axis dysregulation hypothesis, the neurotrophic hypothesis, and the neuroinflammatory hypothesis. However, none of these theories are able to fully clarify the complexity of the disease. Instead, it is suggested that the different hypotheses interact to induce the development of MDD.

1.1.1 | The Monoamine Hypothesis

The monoamine hypothesis was the first molecular theory of MDD (Schildkraut 1965) and has been the central topic of depression research for the last 40 years. The hypothesis is based on the fact that the level of several synaptic monoamines (serotonin (5-HT), norepinephrine (NE), and dopamine (DA)) is reduced in the synaptic cleft in patients suffering from MDD. Additionally, the deficiency in monoamines is acutely reversed by the use of antidepressants, acting as either inhibitors of monoamine reuptake (SSRI and SNRI); antagonists of monoamine receptors and transporters (TCA); or inhibitors of monoamine degradation (MAOI), and thus improve the symptoms of depression. Therefore, a decreased level of monoamines in key structures of the brain could be a contributory cause of MDD. Clinically approved antidepressants acutely modulate the monoaminergic system have a latency period up to 6 weeks, and only 60-70 % of the patients gain an effect. This indicates that the monoamine hypothesis itself does not fully cover the action of antidepressants and the underlying pathophysiology of MDD (Lanni et al. 2009, Lee et al. 2010).

1.1.2 | Involvement of the Hypothalamic-Pituitary-Adrenal axis

As mentioned in section 1.1, stress is a causal factor in the development of MDD. Both physical and psychological stress trigger the paraventricular nucleus in hypothalamus to secrete corticotropin releasing hormone (CRH) which activates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH stimulates the adrenal cortex to enhance the secretion of glucocorticoids (cortisol in humans and corticosterone in rodents), and under normal physiological circumstances the glucocorticoids exert a negative feedback effect on the pituitary and hypothalamus. This pathway is known as the HPA axis and is illustrated in figure 1 (Pariante & Lightman 2008, Nestler et al. 2002, Pariante 2006). Hyperactivity of the HPA axis, observed in approximately half of the patients suffering from MDD, is thought to be related to an inhibition of the feedback mechanism. Under normal physiological circumstances endogenous glucocorticoids bind to either mineralocorticoid receptors (MR) or glucocorticoid receptors (GR) and trigger a potent negative feedback mechanism which influences the synthesis and release of CRH. During prolonged and severe stress hypersecretion of glucocorticoids disrupt the negative feedback regulation of the HPA axis (Pariante 2006). This may reduce neurogenesis in the hippocampal dentate gyrus and cause neuronal apoptosis in hippocampus (Chopra et al. 2011). To overcome the increased activity of the HPA axis, an increased volume of both the pituitary gland and of the adrenal glands is present in most depressed patients (Pariante 2006).

The impaired glucocorticoid-mediated negative feedback mechanism is demonstrated in studies where administration of the synthetic glucocorticoid dexamethasone does not result in a decreased cortisol secretion in depressed patients (Pariante & Miller 2001). To further support the HPA axis hypothesis of depression, successful treatment with antidepressants is associated with resolution of the impaired HPA axis, whereas persistence of an impaired HPA axis is associated with high risk of relapse (Pariante 2006).

Due to the fact that only a fraction of depressed patients display HPA axis abnormalities, dysregulation of this system may only be secondary to other initiating causes.

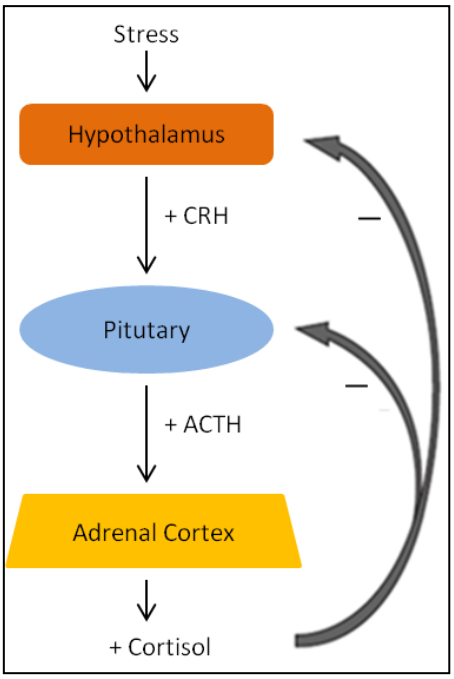

Figure 1 | Regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress triggers the hypothalamus to increase the secretion of corticotropin releasing hormone (CRH). CRH acts on the pituitary and adrenocorticotrophic hormone (ACTH) are released, which stimulates the adrenal cortex to increase the release of glucocorticoids. Under normal physiological circumstances the glucocorticoids suppress CRH and ACTH release. Sustained elevations of glucocorticoids, seen under conditions of prolonged and severe stress, reduce the inhibitory effect of the HPA-axis, which further increases the circulating glucocorticoids levels, leading to hippocampal damage (Modified from (Chopra et al. 2011)).

1.1.3 | The Neurotrophic Hypothesis

Dysfunctions of neuroplasticity or remodelling play a significant role in the pathogenesis of MDD. This is supported by an observed volume loss in hippocampus and other forebrain structures in depressed patients. In the neurotrophic hypothesis of depression a reduced level of neurotrophic factors is suggested to be a causal factor of MDD. Until now, brain-derived neutrophic factor (BDNF) has been in focus because it is one of the most prevalent neurotrophic factors in limbic structures. BDNF-mediated signalling is reduced in hippocampus during acute and chronic stress, whereas chronic treatment with antidepressants reverses this reduction (Duman & Monteggia 2006, Krishnan & Nestler 2008). Additionally, BDNF has several important functions, including inhibition of cell death cascades, enhancement of cell survival proteins responsible for proliferation and maintenance of neurons in the central nervous system, and modulation of neuronal plasticity (Chopra et al. 2011). BDNF is regulated by the cAMP response element binding protein (CREB) which is further regulated through the cAMP cascade. The cAMP cascade is regulated by 5-HT and NE which leads to generation of cAMP and subsequently activation of the cAMP-dependent protein kinase (PKA). A substrate of PKA is CREB, a transcription factor regulating the expression of genes containing a functional cAMP response element (CRE); one of these genes is BDNF (Vaidya & Duman 2001, Duman et al. 1999).

As mentioned above, chronic treatment with antidepressants increases the level of BDNF, caused by an upregulation of the cAMP cascade (Vaidya & Duman 2001). This upregulation could help to repair the stress-induced damage of hippocampal neurons and protect vulnerable neurons. In addition, upregulation of BDNF has been suggested to increase hippocampal neurogenesis in the subgranular zone resulting in new neurons in the hippocampal dentate gyrus. The neurotrophic hypothesis could explain the delayed antidepressant response, because it requires some time for BDNF to reach a sufficient level and exert neurotrophic effects (Nestler et al. 2002, Krishnan & Nestler 2008). However, the complete link between BDNF and MDD is still limited. A study by Rios et al. (2001) has shown that BDNF knock-out mice exhibit anxiety-like behaviour, but no obvious depression-like symptoms (Rios et al. 2001). Furthermore, several studies have not been able to confirm the neurotrophic changes induced by stress and antidepressants, and this

emphasises that the neurotrophic hypothesis alone cannot completely explain the pathophysiology of MDD and the effects of antidepressants.

1.1.4 | Neuroinflammatory hypothesis

The first suggestions of the involvement of cytokines in the pathophysiology of MDD arose when patients with either infectious diseases or cancer were treated with interferon and interleukin (IL)-2, and displayed nonspecific neuropsychiatric symptoms. Some of these symptoms are characteristic for MDD. Cytokines are known to be involved in many aspects associated with MDD, including neurotransmitter metabolism, neuroendocrine function, neural plasticity, and neurotrophic support (Miller et al. 2009).

Activation of the immune system is associated with increased plasma and cerebrospinal fluid concentrations of IL-1, IL-2, IL-6, and tumour necrosis factor (TNF)-α and their receptors. In fact, IL-1, IL-6, and TNF- α have been shown to induce "sickness behaviour" in both animals and humans. This syndrome has many features in common with MDD including anhedonia, alterations in mood, neurovegetative function and cognition (Chopra et al. 2011). These behavioural changes are associated with an altered metabolism of 5-HT, NE, and DA in the limbic system and the basal ganglia (Raison et al. 2006). Cytokines within the brain have the ability to influence the metabolism of monoamines through an activation of the enzyme indoleamine 2,3 dioxygenase (IDO). Activated IDO break down tryptophan, the precursor of 5-HT, into kynurenine. This is believed to contribute to the reduced level of 5-HT observed in depressed patients (Miller et al. 2009).

In addition to the effects on neurotransmitter metabolism, the inflammatory cytokines have a stimulatory effect on CRH, ACTH, and cortisol; all involved in the HPA axis. One possible explanation of this phenomenon is that cytokines may affect the negative feedback regulation of the HPA axis. Cytokines disrupt the GR translocation from the cytoplasm to the nucleus, or influence GR expression leading to a decrease in the active form of GR (GR-α). Both mechanisms decrease the levels of active GR. In addition, cytokines have the ability to diminish the neurotrophic support and decrease the level of hippocampal neurogenesis (Miller et al. 2009, Pace et al. 2007). The neuroinflammatory hypothesis could be primary to the HPA dysregulation and the decreased level of monoamines.

The complexity of MDD becomes evident from diverse hypotheses described above. The different hypotheses demonstrate dynamic interactions between various molecules, pathways, and systems. However, none of the above mentioned hypotheses are able to clarify the complete neurobiological alterations in the different brain regions suggested to be involved in MDD.

1.2 | Biomarkers of Depression

To understand the pathology of depression and to develop antidepressants with a higher efficacy, novel biomarkers would be favorable. A biomarker is a measurable feature of an individual that represents indicators of a disease, or outcome of treatment. Biomarkers often have a biological feature, e.g., genome variation, protein variation etc (Schmidt et al. 2011). The search for novel biomarkers is performed on different molecular levels, including alterations in gene, protein and microRNA (miRNA) levels. Even though extensive research is performed all over the world with the aims – to discover novel biomarkers of MDD as well as treatment response, no single biomarkers have yet been identified.

Altered gene and protein expression are observed in all of the hypotheses of depression described in section 1.1. In addition, many regions associated with MDD have been investigated in different animal models

of depression, including the cerebral cortex (Liu et al. 2010), hippocampus (Henningsen et al. 2012), subregions of the hippocampus (Christensen et al. 2011), and the prefrontal cortex (Hill et al. 2011). The experimental design of the various studies investigating novel biomarkers differs significantly. However, numerous studies use a stressor to induce a depressive-like behaviour. Some studies segregate the animals exposed to stressors into different groups, based on behaviour: Anhedonic-like, stress-resilient, and an intermediate group (Christensen et al. 2011, Henningsen et al. 2012). Others defined the whole group exposed to stressors as anhedonic-like (Yazir et al. 2012, Zadrożna et al. 2011). Additionally, investigation of antidepressant efficacy is performed in different ways. Some studies test the effect of treatment on healthy mice (Miller et al. 2007), whereas others test the efficacy of antidepressants on anhedonic-like rats. (Christensen et al. 2011). Thus, a complete comparison between the individual biomarker studies is difficult due to stress, treatment and/or region-specific differences. In order to find novel biomarkers of MDD it is important to mirror the disease and the treatment outcomes, hence, highly validated animal models of depression must be applied for investigation of the pathology of MDD and antidepressant efficacy.

Since the discovery of novel biomarkers is still lacking at a gene or protein level, miRNAs are widely investigated.

1.2.1 | miRNA as a Biomarker of Major Depressive Disorder

Since the discovery of miRNAs, these small non-coding RNA transcripts have been widely investigated in order to find novel markers of different diseases. miRNAs are involved in embryonic development, cancer, neuronal differentiation, and neuronal plasticity. Since emerging evidence indicates that MDD is associated with altered synaptic and structural plasticity and neurogenesis, miRNAs could be a key feature of MDD (Dwivedi 2011).

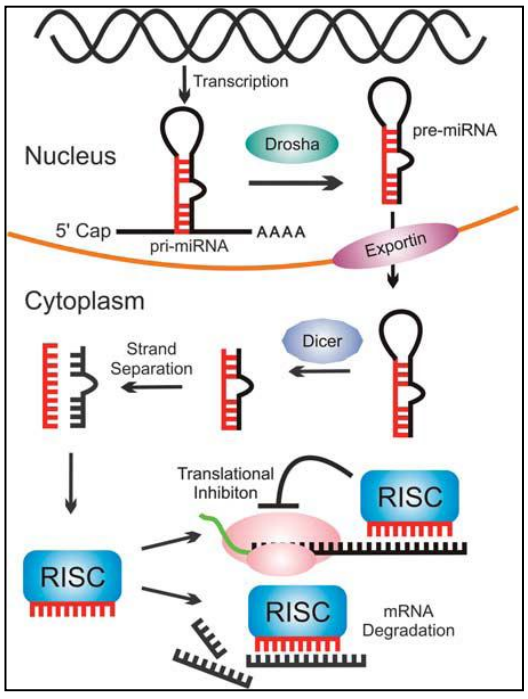

Figure 2 | Biogenesis of microRNAs (miRNAs). miRNAs are transcribed by RNA polymerase II to generate primary miRNA (pri-miRNA). Drosha then catalyze the formations of the hairpin structures called precursor miRNA (pre-miRNA). Exportin-5 then transports the pre-miRNA to the cytoplasm, where it is cleaved into mature miRNAs. These mature miRNAs are then incorporated into the RNA-induced silencing complex (RISC), which is able to inhibit the translation, either by degradation of the target mRNA or by blocking access of the cells´ translational machinery to the mRNA (O'Connor et al. 2011).

miRNAs are small (21-22 nucleotides) non-coding RNA transcripts regulating posttranscriptional mechanisms on mRNA level. It is estimated, that miRNA regulates the translation of 50% of the protein-coding genes (Mouillet-Richard et al. 2011). miRNAs are encoded in the genome as segments of longer transcripts.

They are transcribed by RNA polymerase II, which produces primary miRNA (pri-miRNA) that carries a hair-pin-shaped structure of ~60-100 nucleotides. pri-miRNAs are cleaved by the type-III endonuclease Drosha and produce precursor miRNA (pre-miRNAs), which are exported to the cytoplasm through Exportin-5 and further processed by Dicer, which converts pre-miRNA to mature miRNA containing the mature miRNA guide strand and the passenger miRNA strand. The guide strand is then incorporated into the RNA-induced silencing complex (RISC). This complex allows the miRNA to exert the regulatory activity, see figure 2 (O'Connor et al. 2011, Dwivedi 2011). Selection of miRNA targets are mediated by imperfect interaction of miRNAs with their target mRNAs through the 5´ seed region of the miRNA and one or more binding sites in the 3´UTR region of the mRNA. This imperfect interaction means that a single miRNA has the potential to target ten to hundred mRNAs (Davis-Dusenbery & Hata 2010). The miRNA-RISC complex is able to induce translational repression or degradation of the mRNA involved, depending on the level of complementarity between the miRNA and the target mRNA. A high level of complementarity will results in degradation, whereas lesser complementarity will results in translational inhibition (O'Connor et al. 2011, Dwivedi 2011).

1.2.1.1 | miRNA and Depression

In the central nervous system miRNAs have a central role in virtually all governing processes, making it probable that altering the expression levels will produce many downstream changes at a physiological and behavioural level (O'Connor et al. 2011). An altered expression of miRNAs has been demonstrated during stressful conditions, both in brains of behaviorally depressed animals, and in post-mortem studies of depressed patients (Dwivedi 2011). However, the knowledge about the involvement of miRNA in MDD is still limited, though several miRNAs are suggested to be involved in the pathology of MDD. The most canonical hypotheses of MDD are all influenced by miRNAs, including the monoamine system, the HPA axis, and the neurotrophic system. The 5-HT transporter is shown to be a target of microRNA-16 (miR-16). In addition, chronic treatment with the antidepressant fluoxetine increases the miR-16 level, which reduces 5-HT transporter expression (Baudry et al. 2010). miR-18a has been shown upregulated in rats exposed to 14 days of repeated restraint stress, and results in an inhibition of GR translation (Uchida et al. 2008). Furthermore, miR-30a and miR-195 are shown to regulate the expression levels of BDNF (Mellios et al. 2008).

The involvement of miRNA in MDD described above is based on inhibiting expression of individual mRNA targets. Another possibility is that miRNAs regulate the homeostasis of neural pathways involved in MDD, such as the CREB-BDNF pathway (Dwivedi 2011).

Thus, any dysregulation of miRNAs involved in neuronal pathways or mRNA levels associated with the vulnerability to MDD may precipitate or aggravate the progression of MDD.

1.3 | Habenula – a Region Potentially Involved in the Pathology of Major Depressive Disorder

During the last decades, the main focus in the research of depression has involved hippocampus and the prefrontal cortex. However, a structure called habenula has recently been recognised as a region suggested to be involved in MDD. Habenula is a pair of small nuclei located above the thalamus at its posterior end. It is regarded as a part of the epithalamus, including the pineal body. Habenula is divided into a lateral and a medial part. However, a detailed examination of these regions suggests that the lateral part should be further divided into ten distinct subnuclei and the medial part into five distinct subnuclei (Lecourtier & Kelly 2007). Figure 3 shows habenula from a horizontal view. Habenula is suggested to be involved in the behavioural response to pain, stress, anxiety, sleep and reward. In addition, lesions of the habenula cause cogni-

tive and motor dysfunctions (Hikosaka 2010). The following detailed description of the habenular complex is focused on the lateral part due to its suggested association with depression.

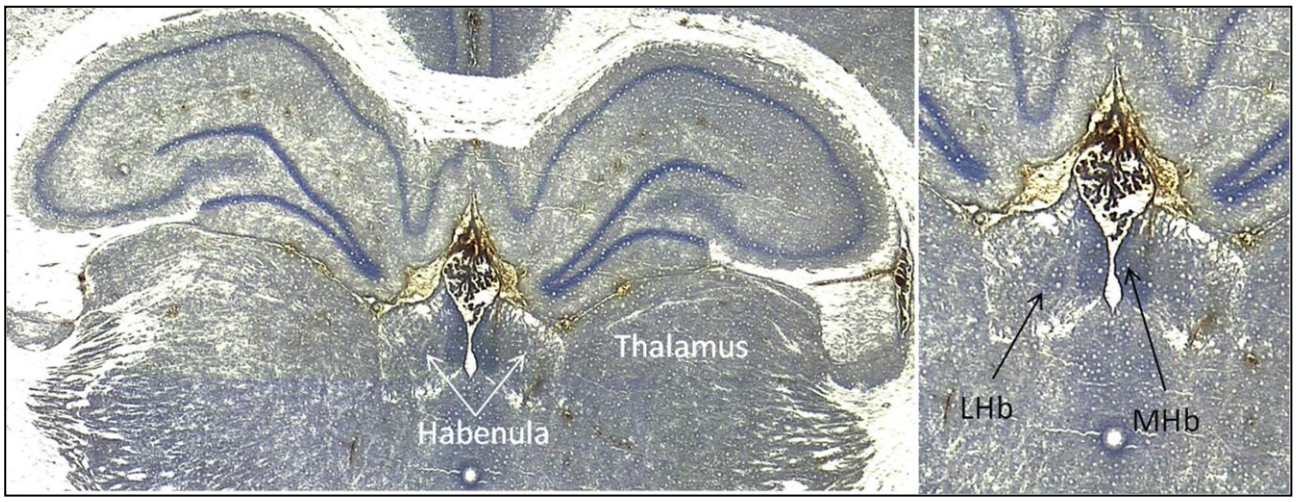

Figure 3 | The habenular complex in a rat. MHb = The medial part of the habenula, LHb = The lateral part of the habenula (Pictures are from the present study)

The lateral habenula (LHb) is mainly innervated by the basal ganglia, in particular globus pallidus; limbic brain regions, including the lateral hypothalamic and lateral preoptic area; and basal forebrain structures, including the ventral pallidum and parts of the extended amygdala. Stria medullaris forms the neuronal input to the LHb. The efferents of the LHb, formed by fasciculus retroflexus, primarily descend to brainstem structures, mainly targeting the nuclei containing monoamine neurons: the dopaminergic ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), and serotonergic dorsal and median raphe nuclei, see figure 4 (Hikosaka 2010, Hikosaka et al. 2008). Hence, the LHb has a relay function, conveying limbic forebrain inputs to the brainstem (Yang et al. 2008).

The LHb neurons are heterogeneous in their neurochemical expression pattern although the majority appear to have a glutamatergic phenotype (Hikosaka et al. 2008). The results of physiological experiments indicate a probable disynaptic circuit involving LHb projections to the rostromedial tegmental nucleus (RMTg), containing short-range GABA cells that ultimately inhibit 5-HT and DA cell firing (Hikosaka et al. 2008, Hong et al. 2011).

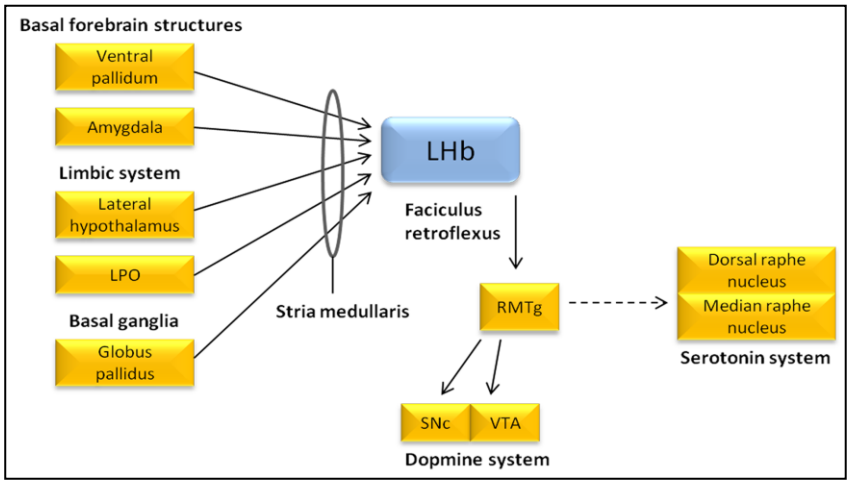

Figure 4 | Afferent and efferent connections of the lateral habenula (LHb). LHb receives input from the basal ganglia, the lateral hypothalamus, the lateral preoptic area (LPO), and basal forebrain structures. LHb sends output to substantia nigra pars compacta (SNc), the ventral tegmental area (VTA), and the dorsal and median raphe nucleus. The efferents are interconnected in the rostromedial tegmental nucleus (RMTg) (Modified from (Hikosaka 2010)).

Various studies have shown that the activity of the entire habenular complex or specifically the LHb is altered in depressed patients or animals, thus suggesting an important role in MDD. A study by Morris et al. (1999) induced depressive relapse by depleting plasma tryptophan, the precursor of 5-HT, in earlier diagnosed MDD patients. Emission tomography (PET) was used to measure the neural activity in different brain areas. They observed an increased synaptic activity in the habenular complex in depressed patients (Morris et al. 1999). Additionally, a study by Caldecott-Hazard et el. (1988) used C-2-Deoxyglucose (2DG) to clarify the rate of cerebral metabolism in three rat models of depression. The depressed behaviour was induced by either injections of α-methylpara-tyrosine, withdrawal from chronic amphetamine, or chronic stress. An elevation in the neural activity was observed in LHb for all three rat models of depression. Since the changes in neural activity were observed in all of the three animal models, there may be a correlation to depressed behaviour (Caldecott-Hazard et al. 1988). Furthermore, in a study by Shumake et al. (2002) congenitally helpless rats were used to determine differences in regional brain metabolism between congenitally helpless and non-helpless rats. The metabolism was measured using quantitative cytochrome oxidase histochemistry. The results demonstrated a 64-71 % elevated metabolism in the habenular complex in congenitally helpless rats when compared to non-helpless rats (Shumake et al. 2003).

To support the above mentioned findings associating a hyperactive habenula to depression, recent studies have performed stimulation of the LHb on both animals and humans. Stereotaxic application of the GABA agonist muscimol into the LHb is demonstrated to inhibit the LHb and exert antidepressive effects in treatment resistant congenital learned helpless rats (Winter et al. 2011). Furthermore, a study by Meng et al. (2011) demonstrates improved depression-like symptoms 28 days after deep brain stimulation of the LHb in the chronic mild stress model of depression. These improvements were manifested as an increased concentration of NE, DA, and 5-HT in blood serum and brain tissues (Meng et al. 2011).

In line with the preclinical studies, a clinical case has shown that deep brain stimulation of LHb exerts profound effects in a patient suffering from treatment resistant MDD. A woman with treatment resistant MDD suffered from severe MDD for 46 years. After 12 weeks of deep brain stimulation of the LHb the woman reached remission (Sartorius et al. 2010). The time course of the experiment is illustrated in figure 5.

All these findings support the hypothesis that elevated activity of LHb may be a common factor in the development of MDD and could be a novel target for future antidepressants. However, further research is needed.

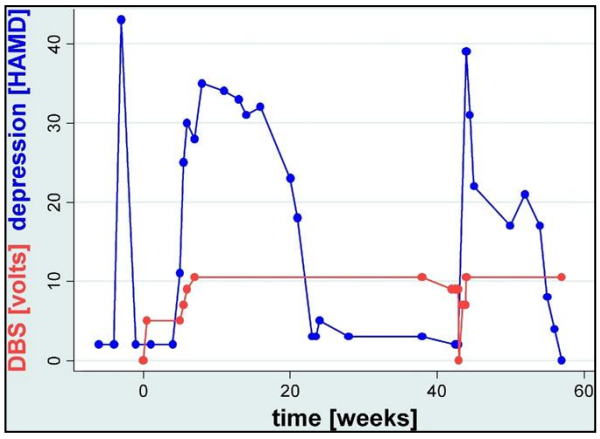

Figure 5 | Deep brain stimulation of the lateral habenula. The severity of major depressive disorder (MDD) and the relapses are quantified using the Hamilton depression scale ($HAMD_{21}$) (Shown in blue). The relapse prior to deep brain stimulation was treated with electroconvulsive therapy sessions. At week 0 the surgery was performed. During the first weeks the stimulation (shown in red) was performed at 5 volt, and the patient relapse. Afterwards, the stimulation was stepwise increased to 10.5 volt, and the patient reaches full remission. The third relapse was caused by a bicycle accident, where the deep brain stimulation was swift of. But after 12 week the patient, again, reach full remission.

1.4 | The Chronic Mild Stress Model of Depression

Modelling depression in animals is a complicated challenge. Some symptoms of MDD, such as depressed mood, negative thinking, suicidality, and decreased self-esteem are believed to be unique to the human cognition and not present in animals. However, animal models of depression offer the possibility to model certain features of the disease. One of the core symptoms of MDD, anhedonia, is mimicked in the Chronic Mild Stress (CMS) Model of depression. The development of anhedonic-like behaviour is monitored by measuring sucrose consumption during periods of stress and chronic treatment with antidepressants. Since palatable sweet solutions are considered to have rewarding properties and CMS is known to suppress consumption, a reduction in sucrose intake is indicative of a stress-induced decrease in sensitivity to reward. This diminished sensitivity to reward is suggested to model human anhedonia (Willner 2005).

An animal model of depression must exhibit behavioural features of depression and should model a core symptom of the disorder, employ realistic inducing conditions, and respond to antidepressive drugs. This has been translated into three major criteria:

- **Face validity**: How well the model resembles the disease/condition.
- **Construct validity**: How well the model is consistent with theoretical rationale.
- **Predictive validity**: How well the model responds favourably to clinically established drugs.

The CMS model fulfils all the three criteria (Willner 1997).

1.4.1 | Face Validity

The primary behavioural readout from the CMS model is anhedonia, a symptom present in all patients suffering from MDD. In addition, the CMS model has shown to cause the appearance of other symptoms of depression, including decreased sexual and aggressive behaviours (D´Aquila et al. 1994) decreased locomotor activity (Gorka et al. 1996), disturbances of diurnal (D´Aquila et al. 1997) and circardian rhythms (Moreau et al. 1995), increased activity of the HPA axis (Muscat et al. 1992), and immunological dysfunction (Grippo et al. 2005). In addition, rats exposed to CMS gain weight more slowly. The only symptoms not demonstrated are the unique human symptoms, symptoms that are only accessible to verbal inquiry (Willner 1997).

1.4.2 | Construct Validity

The rationale is that the stress paradigm induces anhedonia. This rationale rests on two assumptions: (1) The sucrose intake is a valid measure of reward sensitivity, and (2) the CMS causes a generalised decrease in reward sensitivity and not only a specific effect on responses to palatable solutions (Willner 1997). CMS does not induce non-specific changes in fluid consumptions (e.g. decreased thirst), since the water intake is unaffected by the CMS regime (Muscat & Willner 1992). A two-bottled preference test confirmed that the level of sucrose consumption is a preference phenomenon rather than an issue of thirst (Willner et al. 1987). Moreover, the calorie content appears to be unimportant since similar effects are seen in animals consuming calorie-free saccharine solutions. Additionally, decreases in sucrose consumption were observed in both food-deprived and non-deprived animals (Willner et al. 1992).

1.4.3 | Predictive Validity

A wide range of antidepressants are tested in the CMS model. Drugs shown to be effective in reversing CMS-induced anhedonia include tricyclic antidepressants, monoamine oxidase inhibitors, SSRI´s, and NA reuptake inhibitors (Willner 1997). In addition, electroconvulsive treatment (ECT) has been shown to re-

store normal responsiveness to reward (Moreau et al. 1995) Additionally, lesser conventional, but clinically affective drugs are shown to exert an antidepressive function, including litium and carbamazepine (Papp et al. 1996). To gain full recovery of CMS-induced anhedonic-like rats, 3-5 weeks of treatment are required. This delayed time of action is comparable with the clinical time course observed in patients suffering from MDD. In addition, the antidepressive effects observed in animal exposed to the CMS regime are not observed in non-stressed control rats (Willner 1997).

Together, these findings suggest that the CMS model of depression is an appropriate model for investigating the pathophysiology of MDD as well as to investigate potential new antidepressants.

2 | Aim of the Project

The molecular mechanisms leading to MDD have not yet been clarified and it is important to search for potential biomarkers whose function is involved in the pathophysiology of MDD, and which may be useful as potential therapeutic targets. Until now, the main focus in the search for potential biomarkers according to MDD, has involved hippocampus and the prefrontal cortex. More recently, the LHb has been recognised as a potential area associated with MDD. Hyperactivation of this area is observed in both animal models of depression and depressed patients. Additionally, inhibition of the neural activity in the LHb exerts profound effects in depressed individuals.

To investigate the function of LHb in the development and treatment of MDD, the validated CMS model of depression is used. The molecular investigation of LHb is performed on a miRNA level. miRNAs are known to be involved in various disease processes. However, the knowledge about miRNAs involvement in psychiatric diseases is still limited.

The CMS paradigm generates different stress-response phenotypes, including anhedonic-like, stress-resilience, treatment responders, and treatment non-responders. This allows investigation of different aspects of MDD. To test miRNA expression changes in the LHb in relation to MDD, the effect of stress and chronic treatment with escitalopram will be examined by analysing miRNA profiling using TaqMan® Low Density Arrays on laser capture microdissected LHb tissue.

3 | Methods and Materials

3.1 | Animals

Male Wistar rats were purchased from Taconic, Denmark. The rats were singly housed, food and water were available ad libitum, and the animals were kept on a standard 12 hour light/dark cycle except when one of these parameters was applied as a stress parameter. All the procedures involving animals were accepted by the Danish National Committee for Ethics in Animal Experimentation (2008/561-447).

3.2 | Sucrose Consumption Test

During the first five weeks the rats were trained to consume a palatable sucrose solution (1.5 %). In this period, sucrose test was performed twice a week during the first three weeks and once a week during the last two weeks. Baseline sucrose consumption was measured in the last three sucrose tests conducted before stress initiation and the baseline sucrose intake was defined as the mean sucrose consumption during the last two sucrose measurements. During the stress period the sucrose consumption test was performed once a week. The test consisted of a one hour period with free access to a bottle of the sucrose solution. 14 hours prior to the test, food and water were removed. Sucrose consumption was measured by weighing the bottles at the beginning and at the end of the test.

3.3 | The Chronic Mild Stress Model

According to baseline sucrose intake the animals were divided into two matched groups – control and stress. Initially, the stress group was exposed to four weeks of chronic mild stressors and the control group was left undisturbed. The weekly stress schedule is presented in table 1. All the stressors lasted from 10 to 14 hours.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Day	Intermittent illumination	Water deprivation	Strobe	No stress	Sucrose test	Food deprivation	Box tilting
Night	No stress	Box tilting	Wetting	Food/water deprivation	Grouping	Box tilting	Wetting

Tabel 1 | The Chronic Mild Stress Schedule. The stressors were applied due to this weekly schedule.

Following exposure to stress, rats were characterised as anhedonic (defined as a >25 % within-subject decrease in sucrose intake) or resilient (defined as a <10 % within-subject decrease in sucrose intake). Rats not included in the two groups were excluded from the experiment.

After the initial four weeks of stress, the unchallenged control and the anhedonic group were divided into two matched subgroups and subjected to chronic escitalopram (n=18) or vehicle (n=9) administration for four weeks. Stress was continued during the entire period of treatment. Escitalopram or vehicle were administrated intraperitoneally once every morning. Escitalopram was dissolved in physiological saline and were given at a dosage of 5 mg/kg/day. During treatment with chronic escitalopram, the antidepressant treated rats segregated into drug responders (defined as ≥ 20 % within-subject increase in sucrose intake) and drug non-responders (defined as < 20 % within-subject increase in sucrose intake). The study design of the present study is illustrated in figure 6.

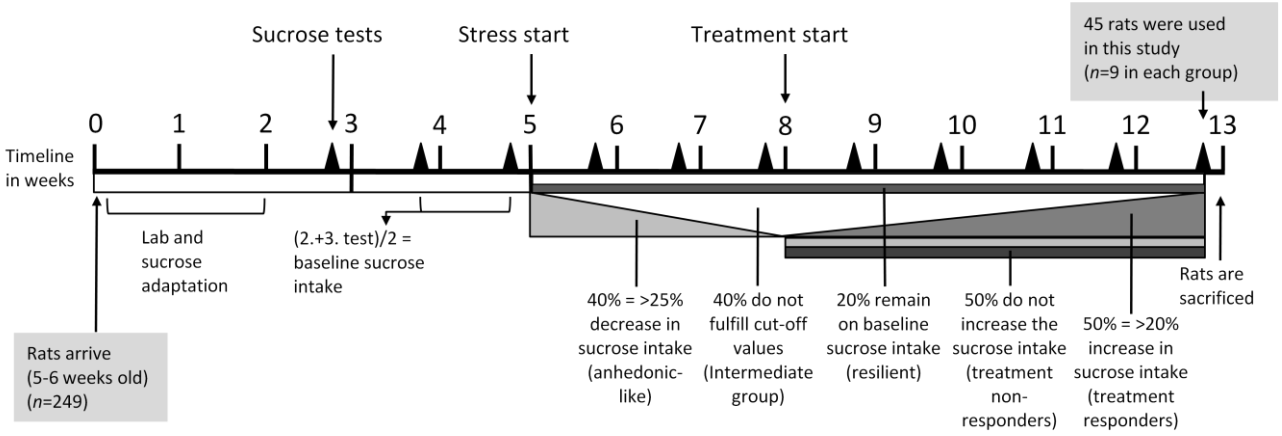

Figure 6 | The study design of the present study showing the experiment lineout and time course. (Modified from (Henningsen et al. 2012))

For miRNA analysis individual CMS groups (n=9) were divided and pooled into 3 subgroups (n=3), see table 2.

Animal groups	n	Abbreviation	No. of subgroups
Unchallenged control vehicle	9	**U-V**	**3**
CMS vehicle	9	**CMS-V**	**3**
CMS resilience	9	**CMS-Res**	**3**
CMS escitalopram responders	9	**CMS-Esc-R**	**3**
CMS escitalopram non-responders	9	**CMS-Esc-NR**	**3**

Tabel 2 | Animal groups. Based on the sucrose measurements and escitalopram treatment, five groups were investigated in the present study. n = number of rats.

3.4 | Tissue Processing

Animals were decapitated and the brains were removed and stored at -80 °C until further processing. Frozen brains were sectioned horizontally on a cryostat (CM3050S, Leica Microsystems, GmbH, Germany). In the present study, investigations were performed on the LHb defined as -2.12 to -4.16 mm relative to bregma. The 50 µm sections were mounted on polyethylene napthalate (PEN) glass slides (Molecular Devices, USA) and stored at -80°C until further processing.

3.5 | Tissue Staining

In order to find the most appropriate tissue staining procedure, in proportion to visualisation, RNA quality, and laser capture microdissection (LCM), different staining protocols were investigated.

3.5.1 | Pilot Study 1

0.1% toluidine blue (TB) (Sigma-Aldrich®) diluted in diethylpyrocarbonate (DEPC) water displays good visualisation properties. However, 0.75% EtOH diluted cresyl violet (CV) (Sigma-Aldrich®) (1% CV stock solution dissolved in absolute EtOH and further diluted to 0.75% CV in DEPC water) is an already known staining method with beneficial results according to both LCM and RNA quality (Christensen et al. 2011, Christensen et al. 2010), hence, a comparison of these staining procedures was performed. The slides were taken directly from -80 °C storage and placed in 96% EtOH for one minute followed by 30 seconds in 75% EtOH. Tissue sections were then stained in 0.75% EtOH diluted CV or 0.1% DEPC diluted TB for 30 seconds and 2 minutes, respectively. Slides were then dehydrated in alcohol – 30 seconds in 75% EtOH, 30 seconds in 96%

EtOH, 30 seconds in absolute EtOH, and further 1 minute in absolute EtOH. The slides were air-dried prior to LCM.

The 0.1% TB DEPC diluted staining procedure results in degraded RNA whereas the 0.75% CV EtOH diluted staining procedure displays poor visualisation properties of the LHb. Further details are described in section 4.2.1.

3.5.2 | Pilot Study 2

To overcome the difficulties experienced in pilot study 1, CV and TB were dissolved in absolute EtOH (1% CV and 1% TB). The dehydration procedure for 1% TB staining was the same as described in pilot study 1 whereas the 75% EtOH steps were excluded from the dehydration procedure for 1% CV staining. The visualisation properties were improved using the 1% CV staining protocol and the RNA quality was high. Despite of an altered TB protocol the RNA was still degraded. Hence, 1% CV was used for the present study. Further details are described in section 4.2.2.

3.6 | Laser Capture Microdissection

The LCM procedure was performed using the Veritas Microdissection Instrument model 704 (Molecular Devices, USA) with CapSure Macro caps (Molecular devices, USA). This procedure isolates the LHb without contamination from surrounding tissue. The LHb was visualised in the microscope of the LCM instrument and captured by the "cut and capture" feature (figure 7). The settings were 80 mW pulse power, 3500 µs pulse duration, 45 µm laser spot diameter, and a UV laser power at 15. The area of the LHb were selected for capture using the 2X objective, whereas capture was performed using the 20X objective. Captured LHb was removed from the caps and placed in 0.5 ml tubes containing 20 µL QIAzol (Qiagen). The sample was centrifuged at 14,500 rpm for 30 seconds and stored at -80°C until RNA isolation.

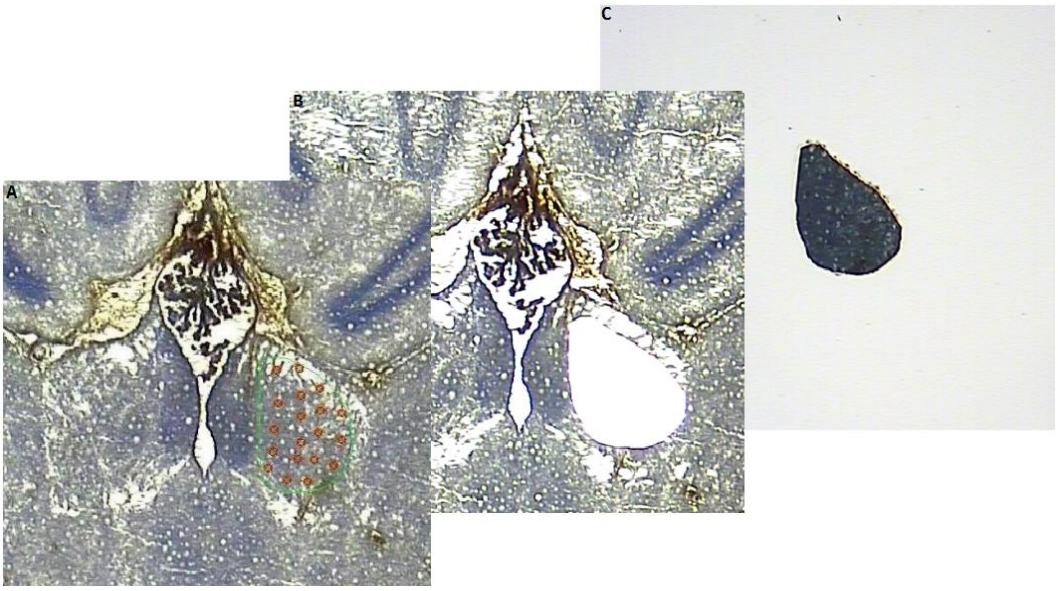

Figure 7 | Illustration of the laser capture microdissection (LCM) procedure. Horizontal section at 2X magnification of the rat lateral habenula (LHb). (A) Area marked for LCM. (B) The cap was placed on the tissue and the UV laser was activated to cut the LHb from beneath the membrane slide. An infrared laser then pulses through the top of the cap and interacts with a transfer film, which then melts and binds to the LHb. (C) Subsequently, the cap was removed from the tissue, having separated the LHb from the remaining habenular tissue.

3.7 | RNA Isolation and Quality Control

After tissue homogenisation, total RNA was isolated from each subgroup by use of miRNeasy mini Kit (Qiagen, USA) according to the manufacture´s protocol. Before miRNA analysis, the quality of the isolated RNA was assessed with respect to integrity and purity. The RIN (RNA integrity number) value of the RNA, was measured by the 2100 BioAnalyzer (Agilent Technologies, USA), and the quantity of RNA samples was measured by use of NanoDrop ND-1000 (Thermo Scientific, USA).

3.8 | miRNA Analysis

In the present study the miRNA expression profiles of rats exposed to CMS and chronic escitalopram treatment were determined using the TaqMan® Low Density Arrays. This study investigates 5 different groups, listed in table 2, and all the groups contain three replicates, hence, 15 arrays were analysed. Tissue from three rats was included in each replicate.

Briefly, 100 ng total RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and the Megaplex™ RT Primers (Applied Biosystems) according to manufacture´s protocol. The thermal-cycle profile was as follows: 40 cycles of 16°C for 2 minutes, 42°C for 1 minute and 50°C for 1 second, followed by reverse transcriptase inactivation at 85°C for 5 minutes. 2.5 µL of the Megaplex RT product was pre-amplified using TaqMan® PreAmp Master Mix (Applied Biosystems) and Megaplex™ PreAmp Primers (Applied Biosystems) according to manufacture's protocol. Thermal-cycling conditions were as follows: 95°C for 10 minutes, 55°C for 2 minutes and 72°C for 2 minutes followed by 12 cycles of 95°C for 15 seconds and 60°C for 4 minutes followed by enzyme inactivation at 99.9°C for 10 minutes. The PreAmp product was diluted 4-fold using 0.1XTE pH 8.0. miRNA expression was profiled with TaqMan® array Rodent MicroRNA card A (Applied Biosystems), containing 384 TaqMan® miRNA assays. Each well was loaded with 100 µL PCR reaction mix containing 50 µL TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X (Applied Biosystems), 1 µL diluted PreAmp product and 49 µL nuclease-free water. The thermal-cycling conditions were as follows: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60 °C in 60 seconds. Real-time PCR was performed using the Applied Biosystems 7900HT Real-Time PCR system. Data were processed and exported with Applied Biosystems SDSv2.2 software. All samples were normalized to the miRNA mammalian endogenous control gene mammU6 on the first running array to correct for technological differences. The miRNA analyses were performed by Aros Applied Biotechnology, Skejby.

3.9 | Cluster Analysis

The cluster analysis was performed with Cluster software version 2.11 and based on all 384 miRNAs being determined by the TaqMan® miRNA assays. Data were adjusted by log2 transformation, normalization, and mean center of miRNAs and arrays. The hierarchical clustering was performed by clustering of arrays followed by average linkage clustering. Subsequently, the cluster analysis was visualized by TreeView software version 1.60.

3.10 | Data Analysis

3.10.1 | Sucrose Consumption Test

A two-tailed student´s T-test was used to analyse the overall stress-effects and treatment-effects. Stress-effects were estimated by comparing all stress rats in the trial to all non-stress rats. Treatment effects were estimated by comparing all drug treated rats (both responders and non-responders) to non-treated rats.

Data obtained from weekly sucrose consumption tests were analysed using multivariate analysis of variance (MANOVA). Stress and antidepressant effects were investigated separately, with stress and treatment, respectively, as between-subject factors and time (weeks) as within-subject factor. According to the statistically significant interactions revealed by MANOVA, additional analyses were performed by one-way analysis of variance (ANOVA) to reveal time-specific differences caused by stress or treatment. Group-wise comparisons were performed by Bonferroni post hoc tests in order to adjust for multiple comparisons.

3.10.2 | miRNA Analysis

The $2^{-\Delta Ct}$ method was used for relative quantification, and a ≥ 20 % change in regulation was applied as a cut-off. To identify miRNA differences according to stress response and treatment response relevant groups were compared in a two-tailed student´s t-test. Differential changes in miRNA expression between relevant groups were identified by one-way ANOVA followed by Tukey post-hoc tests. The statistical level of significance was set at $P<0.05$. SPSS Statistics Version 19 was used for the statistical analyses. To investigate the predicted functional implications of selected miRNAs TargetScan Version 6.1 was used.

4 | Results

4.1 | Sucrose Consumption

Initially, all rats were divided into two groups; one group being exposed to CMS and another group that was left undisturbed. During the first four weeks of CMS, the animals segregated into three groups; anhedonic-like (CMS-V), stress-resilient (CMS-Res), and an intermediate group, see figure 6. Figure 8A shows the presence of an overall stress-effect when all animals exposed to CMS (n=249) are compared to unchallenged controls (U-V) (n=80) (P<0.001). A subset of the U-V (n=9), CMS-V (n=9) and CMS-Res (n=9) rats were used for miRNA analysis. Figure 8B shows the sucrose intake (indexed to baseline) for the rats used for miRNA analysis.

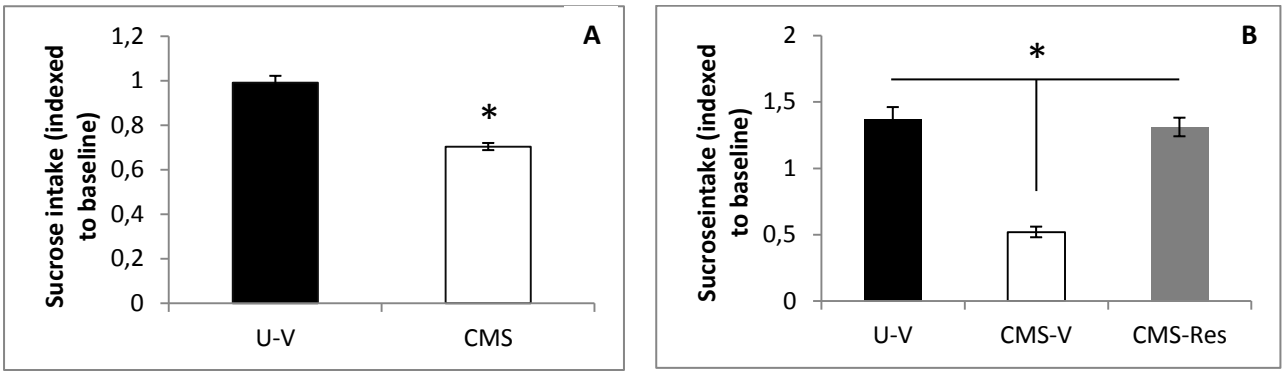

Figure 8 | (A) Four weeks exposure to chronic mild stress (CMS) resulted in a significantly decreased sucrose consumption when compared to unchallenged controls (U-V) (CMS, n=249) (U-V, n=80) (P<0.001). **(B)** Rats used for miRNA analysis. After four weeks of CMS the rats were segregated into two groups; anhedonic-like (CMS-V) and stress-resilient (CMS-Res). After 2 weeks of initial exposure to CMS, the sucrose intake was significantly diminished in CMS-V rats (n=9) when compared to U-V (n=9) ($F_{2,42}$=28.902, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) and CMS-Res (n=9) ($F_{2,42}$ =28.902, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) rats. Statistical significances were set at * P<0.05. Data are presented as the mean group sucrose intake indexed to baseline values.

MANOVA analysis on stress-effects after four weeks revealed statistical significance ($F_{10,76}$=14.56, P<0.001) when comparing CMS-V (n=27), U-V (n=9), and CMS-Res (n=9) groups. After two weeks of initial exposure to stress the intake of the sucrose solution was significantly diminished in CMS-V when compared to U-V ($F_{2,42}$=28.90, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) and CMS-Res ($F_{2,42}$=28.90, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) rats, indicating a stress-induced decrease in sensitivity to reward.

After four weeks exposure to CMS, CMS rats (n=18) were chronically treated with escitalopram. After four weeks of treatment a significantly increase in the sucrose consumption was observed in rats treated with chronic escitalopram when compared to CMS-V (n = 9) (p=0,002); results can be seen in figure 9A. 50 % of the treated rats showed a more than 10 % increase in sucrose intake and were defined as responders (CMS-Esc-R). On the other hand, 50 % of the rats treated with escitalopram did not increase their sucrose intake and were designated non-responders (CMS-Esc-NR), see figure 9B.

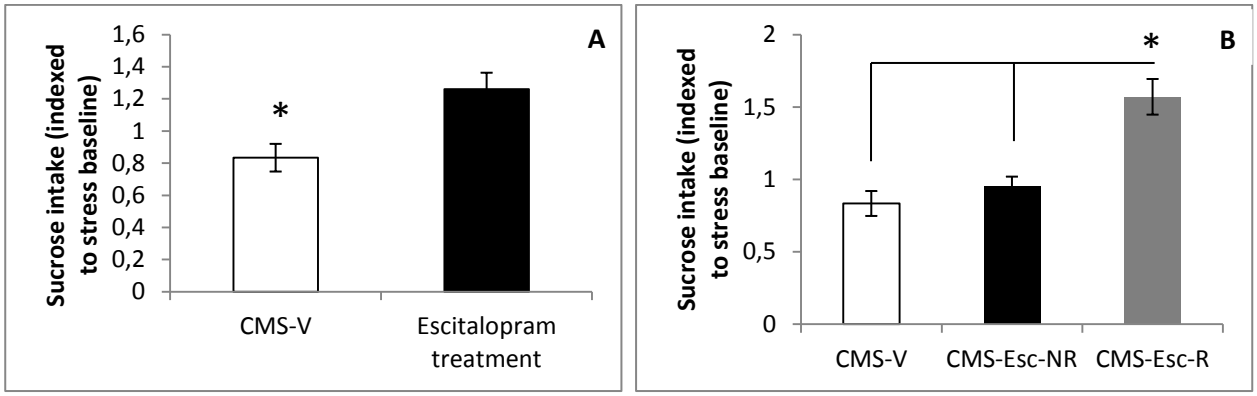

Figure 9 | (A) Four weeks of chronic escitalopram administration (n=18) resulted in a significantly increased sucrose consumption when compared to vehicle treated CMS rats (CMS-V) (n=9) (P<0.01). **(B)** After four weeks of chronic escitalopram treatment the rats were subgrouped into two categories; CMS-Esc-R (n=9) and CMS-Esc-NR (n=9). Significant effects of drug treatment were present in CMS-Esc-R after three weeks of escitalopram administration when compared to CMS-V ($F_{2,24}$=14.114, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) and CMS-Esc-NR ($F_{2,24}$=14.114, P_{ANOVA}<0.001, $P_{bonferroni}$=0.001) . Statistical significances were set at * P<0.05. Data are presented as the mean group sucrose intake indexed to stress baseline values.

MANOVA analysis on treatment effects after four weeks revealed statistical significance ($F_{8,42}$ = 3.96, P = 0.001) when comparing CMS-V (n=9), CMS-Esc-R (n=9), and CMS-Esc-NR (n=9) groups. Significant effects of treatment were present after three weeks of esitalopram administration in CMS-Esc-R rats when compared to CMS-V ($F_{2,24}$=14.114, P_{ANOVA}<0.001, $P_{bonferroni}$<0.001) and CMS-Esc-NR ($F_{2,24}$=14.114, P_{ANOVA}<0.001, $P_{bonferroni}$=0.001) rats.

4.2 | Tissue Staining and RNA Quality

4.2.1 | Pilot Study 1

The initial staining procedure investigated was 0.1 % TB dissolved in DEPC water. This procedure allows good visualisation compared to 0.75 % CV dissolved in EtOH, see figure 12A+D. However, 0.75 % CV is an already known staining procedure with good results according to LCM and RNA quality (Christensen et al. 2010, Christensen et al. 2011), hence, a comparison between 0.1 % TB and 0.75 % CV on RNA quality was performed. The results of the two staining protocols are illustrated in figure 10.

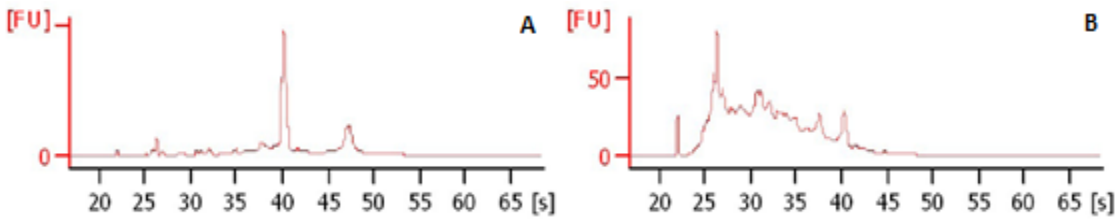

Figure 10 | Electropherograms of pilot study 1. (A) Electropherogram of the 0.75 % EtOH diluted cresyl violet (CV) staining procedure. Shows high quality RNA since clear 28s and 18s peaks are observed. Additionally, low noise between the peaks and minimal low molecular weight contamination are observed. **(B)** Electropherogram of the 0.1 % DEPC diluted toluidine blue (TB) staining procedure. Shows highly degraded RNA since 28s and 18s are lacking. In addition, the intensity of smaller RNA increases. FU, fluorescence; s, time in seconds.

The 0.1 % DEPC diluted TB staining procedure results in RNA degradation, whereas staining with 0.75 % EtOH diluted CV results in RNA of high quality. However, visualisation of LHb was difficult using 0.75 % CV.

4.2.2 | Pilot Study 2

To overcome the problems observed in pilot study 1, the protocols for 0.1 % DEPC diluted TB and 0.75 % EtOH diluted CV were modified. Both CV and TB were dissolved in absolute EtOH. To compare these staining procedures with the previous method, the ordinary 0.75 % CV was included. The results are shown in figure 11.

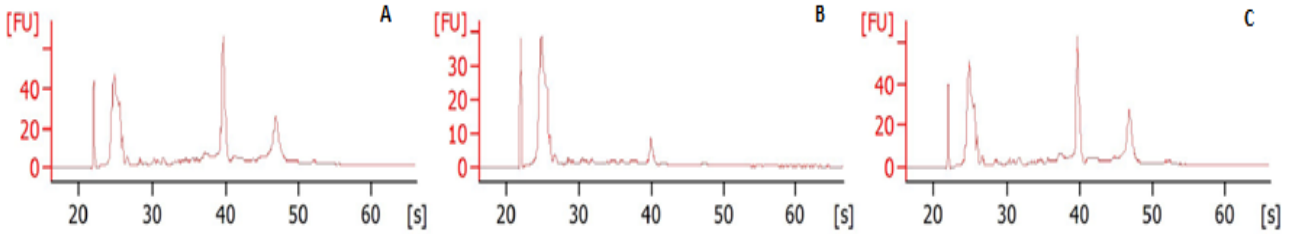

Figure 11 | Electropherograms of pilot study 2. The peaks observed after 25 seconds cannot be explained, but it is not due to degraded RNA, since the 28s and 18s peaks are well-defined. **(A)** The electropherogram of the 0.75 % EtOH diluted CV staining procedure, shows RNA of high quality because of clear 28s and 18s peaks, and low noise between the peaks. **(B)** The electropherogram of the 1 % EtOH diluted TB staining procedure, shows degraded RNA, illustrated by lack of the 28s and 18s peaks. **(C)** The electropherogram of the 1 % EtOH diluted CV staining procedure, shows RNA of high quality, demonstrated by clear 28s and 18s peaks, and low noise between the peaks. FU, fluorescence; s, time in seconds.

Despite that TB was dissolved in absolute EtOH RNA was still degraded, whereas both 1 % CV and 0.75 % CV result in high quality RNA. The 0.75 % EtOH diluted CV staining procedure (figure 11D) shows blurred borders between the medial and lateral habenula which makes it difficult to make an accurate LCM, thus risking contamination from the medial part. The 1 % EtOH diluted CV staining procedure shows well-defined borders to both the medial habenula and thalamus (figure 11C). According to these results I decided to use the 1 % CV, because of high RNA quality and good visualisation opportunities. Figure 11 shows pictures of all the used staining procedures.

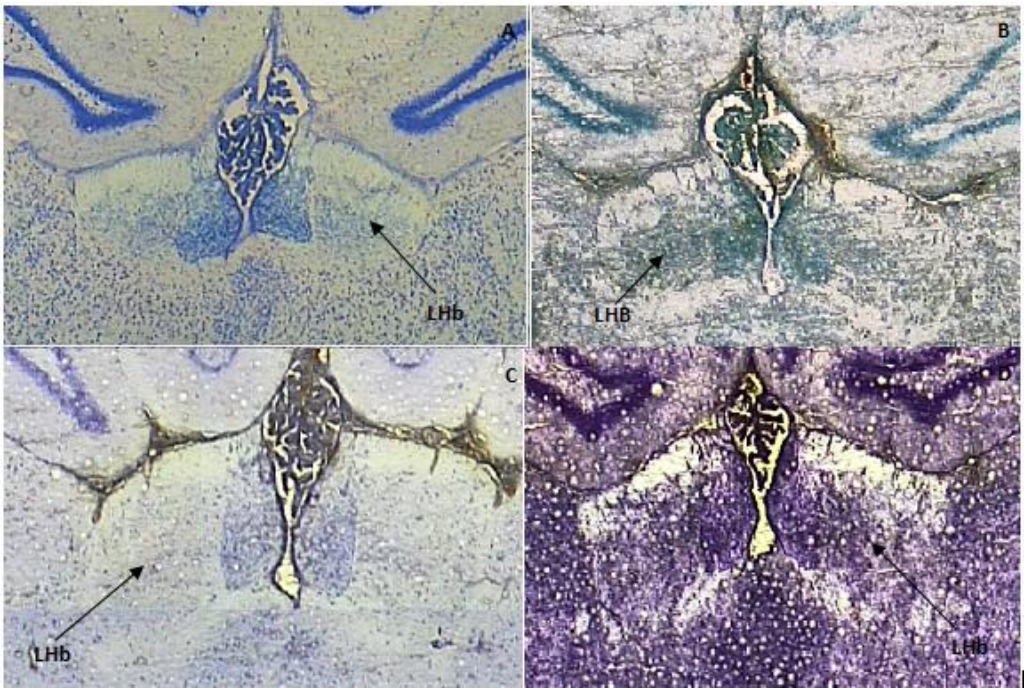

Figure 12 | The lateral habenula (LHb) stained with four different staining procedures. **(A)** LHb stained with 0.1 % DEPC diluted toluidine blue (TB). **(B)** LHb stained with 1 % EtOH diluted TB. **(C)** LHb stained with 1 % EtOH diluted cresyl violet (CV). (D) LHb stained with 0.75 % EtOH diluted CV.

4.3 | miRNA analysis

The aim of the present study was to investigate the function of LHb in the development and treatment of MDD in order to find novel miRNA targets. miRNAs were identified using qRT-PCR miRNA expression profiling arrays (TaqMan® Low Density Arrays), and the expression levels of 380 miRNAs were detected. However, 84 miRNAs were undetectable (Ct value > 40) in all the groups, because of tissue specific expression. In addition, 15 miRNAs showed different expression patterns between the groups, since these miRNAs were not expressed in all the groups. However, these miRNAs were included for further analysis, if they were expressed in two of the subgroups. To calculate p-values and fold-changes the Ct value of these miRNAs were set to 40.

Five experimental groups were analysed: Unchallenged control vehicle (U-V), CMS vehicle (CMS-V), CMS resilience (CMS-Res), CMS escitalopram responders (CMS-Esc-R), and CMS escitalopram non-responders (CMS-Esc-NR).

4.3.1 | Hierarchical Clustering Analysis

The TreeView cluster analysis, illustrated in figure 13, was based on all 384 miRNAs represented on the TaqMan® miRNA array. The cluster analysis showes clear differentiation between the CMS groups. Furthermore, the analysis shows a clear differentiation between the CMS groups only exposed to the stress paradigm, and those groups receiving chronic escitalopram treatment, which indicates a strong drug effect. Additionally, the U-V group is closer related to the CMS-V group than to the CMS-Res group.

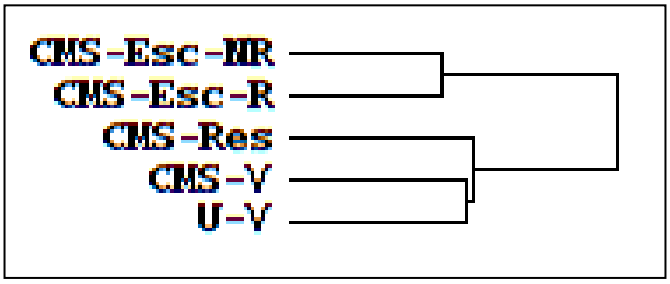

Figure 13 | Hierarchical clustering analysis. The cluster analysis indicates a clear relation among animals exposed to the stress paradigm and the animals receiving antidepressant treatment. The analysis is based on all 384 TaqMan® miRNAs assays.

4.3.2 | Anhedonia versus Resilience

According to the sucrose consumption measurements the rats exposed to CMS segregated into two groups: CMS-V and CMS-Res. This segregation demonstrates a different response to CMS. miRNA expression levels of U-V rats were compared to CMS-V and CMS-Res rats. Resilient and anhedonic groups are compared in figure 14 showing that 12 and nine miRNAs, respectively, were significantly affected by CMS. Five miRNAs show common effects to CMS. Fold changes and the direction of regulation are listed in table 3.

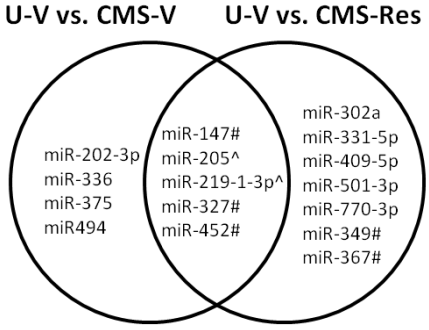

Figure 14 | Anhedonia versus resilience. A dual comparison between unchallenged controls (U-V) and anhedonic-like (CMS-V) and stress-resilient (CMS-Res) rats, respectively, were performed using a two-tailed student´s t-test (P<0.05, fold change > 20 %). ^ = A tendency in regulation with p = 0.06 in U-V vs. CMS-V. # = Ct values appointed to 40.

miRNA	Group	Fold change U-V vs. CMS-V	U-V vs. CMS-Res
miR-202-3p	CMS-V	-3.62	
miR-336	CMS-V	-1.57	
miR-375	CMS-V	1.66	
miR-494	CMS-V	1.70	
miR-302a	CMS-Res		3.11
miR-331-5p	CMS-Res		1.89
miR-409-5p	CMS-Res		1.20
miR-501-3p	CMS-Res		3.07
miR-770-3p	CMS-Res		1.36
miR-349#	CMS-Res		4.14
miR-367#	CMS-Res		12.04
miR-147#	CMS-V + CMS-Res	29.38	29.38
miR-205^	CMS-V + CMS-Res	1.60	1.69
miR-219-1-3p^	CMS-V + CMS-Res	2.83	4.54
miR-327#	CMS-V + CMS-Res	-24.99	-16.46
miR-452#	CMS-V + CMS-Res	2.75	2.75

Tabel 3 | miRNAs significantly regulated between CMS groups. # = Ct value appointed to 40. ^ = A tendency in regulation with p = 0.06 in U-V vs. CMS-V.

4.3.3 | Escitalopram Responders vs. Non-responders

According to the sucrose consumption measurements, the rats receiving chronic escitalopram treatment segregated into two groups: CMS-Esc-R and CMS-Esc-NR. This segregation demonstrates a different response to treatment. miRNA expression levels of CMS-V rats were compared to CMS-Esc-R and CMS-Esc-NR rats. CMS-Esc-R and CMS-Esc-NR are compared in figure 15 showing that 35 and one miRNAs, respectively, were significantly affected by chronic escitalopram treatment. One miRNA shows common effects to escitalopram treatment. Fold changes and the direction of regulation are listed in table 4.

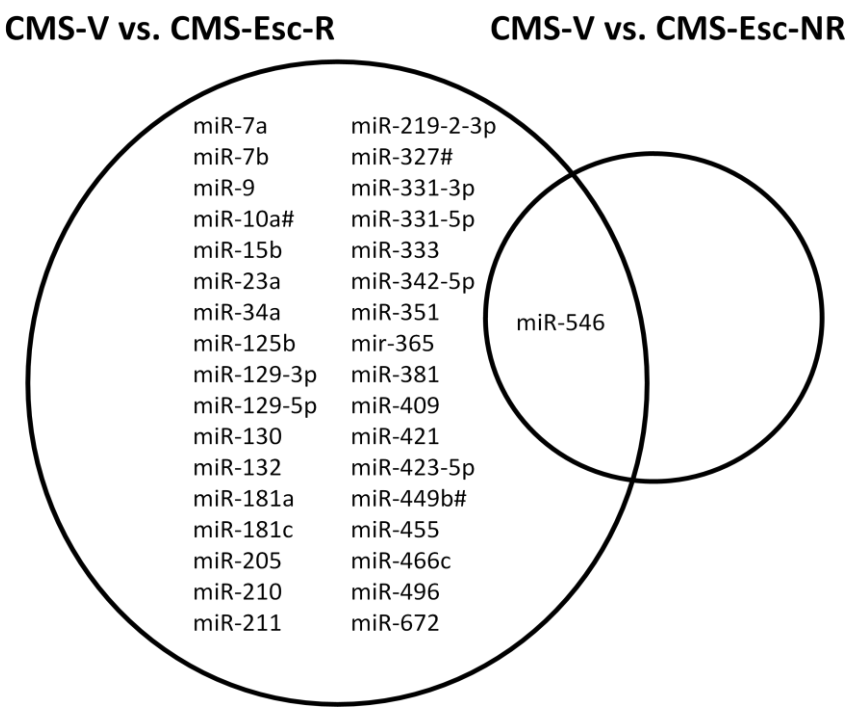

Figure 15 | Escitalopram responders versus non-responders. A dual comparison between CMS vehicle (CMS-V) and CMS escitalo-pram responders (CMS-Esc-R) and CMS escitalopram non-responders (CMS-Esc-NR) rats. were performed using a two-tailed student´s t-test (P<0.05, fold change > 20 %). # = The Ct value was appointed to 40.

miRNA	Group	Fold change	
		CMS-V vs. CMS-Esc-R	CMS-V vs. CMS-Esc-NR
miR-7a	CMS-Esc-R	2.10	
miR-7b	CMS-Esc-R	2.01	
miR-9	CMS-Esc-R	1.98	
miR-10a#	CMS-Esc-R	4.30	
miR-15b	CMS-Esc-R	1.75	
miR-23a	CMS-Esc-R	2.67	
miR-34a	CMS-Esc-R	1.69	
miR-125b-5p	CMS-Esc-R	2.02	
miR-129-3p	CMS-Esc-R	1.85	
miR-129-5p	CMS-Esc-R	1.59	
miR-130b	CMS-Esc-R	1.72	
miR-132	CMS-Esc-R	1.86	
miR-181a	CMS-Esc-R	2.02	
miR-181c	CMS-Esc-R	2.54	
miR-205	CMS-Esc-R	2.01	
miR-210	CMS-Esc-R	2.32	
miR-211	CMS-Esc-R	6.91	
miR-219-2-3p	CMS-Esc-R	1.74	
miR-327#	CMS-Esc-R	17.79	
miR-331-3p	CMS-Esc-R	1.85	
miR-331-5p	CMS-Esc-R	2.23	
miR-333	CMS-Esc-R	1.78	
miR-342-5p	CMS-Esc-R	1.96	
miR-351	CMS-Esc-R	1.68	
miR-365	CMS-Esc-R	2.06	
miR-381	CMS-Esc-R	2.83	
miR-409-5p	CMS-Esc-R	2.15	
miR-421	CMS-Esc-R	3.59	
miR-423-5p	CMS-Esc-R	1.65	
miR-449b#	CMS-Esc-R	2.89	
miR-455	CMS-Esc-R	1.54	
miR-466c	CMS-Esc-R	3.19	
miR-496	CMS-Esc-R	2.18	
miR-672	CMS-Esc-R	1.67	
miR-546	CMS-Esc-R + CMS-Esc-NR	-2.73	-6.19

Tabel 4 | miRNAs significantly regulated between escitalopram treated groups. # = Ct value was appointed to 40.

4.3.4 | Markers of Induction and Recovery from Anhedonia

To identify miRNAs involved in the induction and/or recovery of anhedonic-like behaviour, three different expression patterns were investigated. Illustrated in figure 16A-C are the expression patterns that a potential biomarker has to fulfil to be designated as a marker of induction and/or recovery from anhedonia. miRNAs belonging to these patterns are thus candidates as causal factors for the behavioural changes observed during recovery and/or induction of anhedonia. The expression patterns investigated include: (1) Significantly diverging miRNA expression in CMS-V versus U-V and CMS-Esc-R, see figure 16A. Nine miRNAs were significantly regulated after CMS (U-V versus CMS-V) whereas 35 miRNAs were significantly regulated between CMS-V and CMS-Esc-R. None miRNAs significantly fulfil the applied constrains; however, three miRNAs trended to follow the applied constraints, including miR-130b, miR-205, and miR-327. The expression level of miR-327 was increased in the CMS-V group, while chronic treatment with escitalopram reversed this effect. The miRNA expression levels of miR-130b and miR-205 were decreased in the CMS-V group, while treatment with escitalopram resulted in further miRNA decrement. Individual fold changes are listed in table 5. (2) Significantly diverging miRNA expression in CMS-V versus CMS-Esc-R and CMS-Res, respectively. None of the investigated miRNAs fulfil these constraints; however, miR-331-5p trended to follow the applied constraints. The expression level of miR-331-5p was increased in the CMS-V group whereas

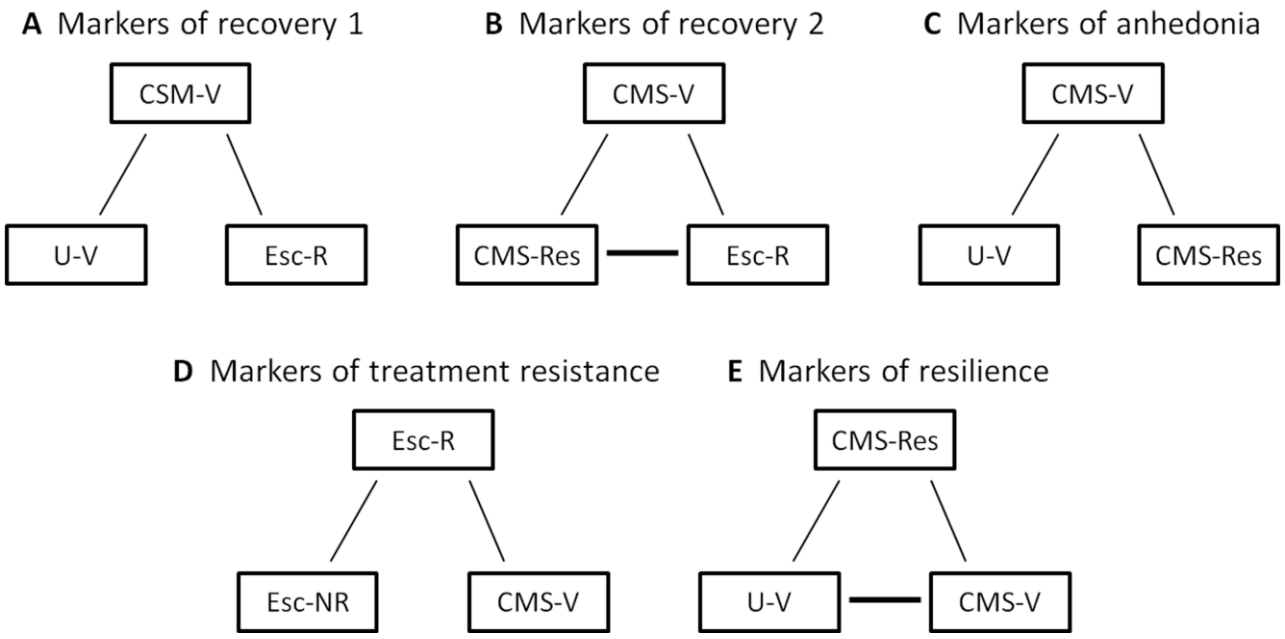

Figure 16 | Shows the different hypothesis investigated. (A-C) Induction and/or recovery from anhedonia. **(D)** Treatment resistance. **(E)** Stress resilience. The lines indicate a significant difference between the groups (P<0.05, fold change > 20 %), and the black bars indicate similar expression levels. Each of the five illustrated hypothesis could be inversely represented i.e. the miRNA level either increase or decrease. CMS-V, CMS vehicle; U-V, unchallenged controls; CMS-Res, CMS resilience; Esc-NR, escitalopram none responders; and Esc-R, escitalopram responders.

both antidepressant treatment and the resilience-phenomenon resulted in a decreased expression. Individual fold change is listed in table 5. (3) Conserved miRNA expression levels for CMS-Res and U-V, but significant diverging miRNA expression in CMS-V versus CMS-Res and U-V. None miRNAs fulfil the criteria for the hypothesis of anhedonia; however, miR-336 trended to follow the applied constraints. The expression level was increased 1.57-fold in the CMS-V group, whereas the resilience-phenomenon results in a decreased expression level at 2-fold.

4.3.5 | Markers of Treatment Resistance

In the CMS model of depression a unique feature is the segregation of drug treated rats into responders and non-responders is, a phenomenon also known from the clinic. The investigation of potential miRNAs involved in treatment resistance was performed by comparing the miRNA expression levels between CMS-V, CMS-Esc-R and CMS-Esc-NR. A potential biomarker has to fulfil these constraints: Significantly diverging miRNA expression between CMS-V and CMS-Esc-R and between CMS-Esc-R and CMS-Esc-NR, as illustrated in figure 16D. miR-546 fulfilled these constraints for treatment resistance. miR-546 is upregulated in both treatment groups. The individual fold changes for both miRNAs are listed in table 5.

4.3.6 | Markers of Stress Resilience

In order to investigate potential markers of stress resilience, the CMS-Res group was compared to the CMS-V and U-V groups. To be designated as a marker of stress resilience the following constraints have to be fulfilled: Conserved miRNA expression between U-V and CMS-V, but significant diverging miRNA expression in CMS-Res versus U-V and CMS-V, see figure 16E. miR-331-5p follows the applied constrains for stress resilience. Additionally, miR-409-5p trended to follow the applied constraints. Both miRNAs were downregulated in the CMS-Res group. The individual fold changes are listed in table 5.

miRNA	Hypothesis	Fold change U-V vs. CMS-V	CMS-V vs. CMS-Esc-R	CMS-Esc-R vs. CMS-Esc-NR	CMS-V vs. CMS-Res	U-V vs. CMS-Res
miR-130b^	Recovery 1	1.45	1.72			
miR-205^	Recovery 1	1.59	2.01			
miR-327#	Recovery 1	-24.98	17.79			
miR-331-5p^	Recovery 2		2.23		1.95	
miR-336^	Anhedonia	-1.57			2.05	
miR-546	Resistance		-2.73	-2.26		
miR-331-5p	Resilience				1.95	1.88
miR-409-5p	Resilience				1.54	1.20

Tabel 5 | Fold changes of the respective miRNA significantly represented in the different hypothesis. miRNAs potentially involved in recovery, anhedonia, treatment resistance, and stress-resilience were identified by one-way ANOVE followed by Turky post hoc analyses (P<0.05). Significant fold changes (>20%) of the relevant CMS groups are listed for each miRNA. # = The Ct value was appointed to 40. ^ = A tendency in regulation

5 | Discussion

The aim of the present study was to investigate the function of LHb in the development and treatment of MDD at the miRNA level in order to find novel disease targets and treatment regimens. miRNA alterations have been observed in MDD, however, the knowledge about miRNA involvement in the development of MDD is still limited. To my knowledge this is the first study investigating the LHb at the miRNA level in the CMS model of depression.

Rats exposed to the CMS regime segregated into different stress reactivity and treatment response phenotypes. Sucrose consumption was measured as readout on hedonic-like status and Taqman® Low Density Arrays were used to detect stress- and antidepressants-related miRNA changes in the LHb. This nonhypothesis-driven approach was chosen to explore the LHb miRNA profile in the CMS model of depression in the search for novel pathways or genes that were stimulated or impaired as a consequence of different stress and treatment responses. miRNA regulation in depression was investigated using five distinct subgroups: unchallenged vehicle (U-V), anhedonic-like (CMS-V), stress-resilient (CMS-Res), escitalopram responders (CMS-Esc-R), and escitalopram non-responders (CMS-Esc-NR). Only miRNAs fulfilling stringent constraints with respect to regulation patterns between groups, described in section 4, will be further analysed.

A single miRNA can regulate multiple different mRNAs and a single mRNA can be regulated by several miRNAs. Therefore, the stress- and treatment-associated changes in miRNA expression observed in the present study may affect several biological functions. None of the regulated miRNAs found in the present study have previously been associated to MDD. Many miRNAs are tissue specific and previous studies investigating miRNAs role in the pathophysiology of MDD have not focused on the LHb. This may explain why none of the significant miRNAs found in the present study have not previously been associated with MDD. To study the biological importance of the significantly regulated miRNAs, I focused on genes with a possible relation to MDD based on TargetScan Version 6.1 and Pubmed searches.

5.1 | The Chronic Mild Stress Model of Depression

The highly validated CMS model of depression mimics anhedonia, the core symptom of MDD. In the present study this depression model was used to investigate the effects of chronic escitalopram treatment. Rats exposed to a chronic sequence of mild stress diminished their sensitivity to rewarding stimuli, which is measured as a decrease in consumption or preference for a sweet solution, such as sucrose (Willner et al. 1987). The CMS model of depression has the unique feature to segregate rats exposed to CMS into anhedonic-like and stress resilient rats. In addition, rats treated chronically with the antidepressive drug escitalopram segregates into treatment responders and treatment non-responders (figure 6).

Segregation into the two CMS-induced phenotypes are confirmed at different levels. The anhedonic-like rats display hypercortisolism as well as disturbance of circadian rhythmicity of the HPA axis when compared to resilient rats (Christiansen et al. 2012). In addition, differences in global gene (Christensen et al. 2011) and protein expression (Henningsen et al. 2012) are observed between the two groups. Despite the described differences, the two phenotypes show similarities. The CMS regime has a negative effect on working memory assessed by the spontaneous alternation behaviour (SAB) task in both CMS-V and CMS-Res rats (Henningsen et al. 2009). Furthermore, a CMS-induced decrease in proliferation in the hippocampal den-

tate gyrus was observed in both CMS-V and CMS-Res rats (Jayatissa et al. 2010). Both of these studies suggest that CMS-Res rats are affected by stress exposure. However, their hedonic-like state remains unchanged during the stress procedure. The mechanisms responsible for the hedonic segregation are unknown. The segregation of stress-exposed animals from the CMS model of depression imitate humans in their variability of how to cope with the exposure of chronic stress. Hence, the occurrence of the resilience phenomenon improves the translation value of the CMS model of depression.

By exposing rats to the CMS regime followed by chronic escitalopram treatment, anhedonic-like rats split into treatment responders and treatment non-responders. Approximately 50 % of the CMS rats receiving antidepressant treatment recover from anhedonia whereas the remaining rats display a treatment-resistant phenotype. The proportion of treatment responders in the CMS model of depression correlate to the clinical response rates ranging from 60-70% in patients suffering from moderate or severe MDD (Lanni et al. 2009). The placebo effect observed in clinical studies is 25-35% (Quitkin et al. 2000) and may explain the higher response rates observed in clinical studies when compared to animal studies. To my knowledge, the CMS model of depression is the only animal model of depression investigating the phenomenon of treatment resistance, a feature that enhances the construct validity of the model.

5.2 | miRNA Expression Profiling Associated with Chronic Mild Stress and Antidepressant Treatment

Stringent constraints were applied for analysis of the differential miRNA regulation profiles. As described in section 4.3.4, development and/or recovery from anhedonia were investigated according to three distinct set of constraints (figure 16A-C). The first and second constrain revealed the identification of four individual miRNAs, miR-130b, miR-205, miR-327, and miR-331-5p (table 5) as possible markers of recovery. The third constraint, concerning anhedonia, revealed the identification of one miRNA, miR-336.

5.2.2 | Markers of Anhedonia

To investigate potential miRNAs involved in the induction of anhedonia, the CMS-V group was compared to the U-V and CMS-Res group, see figure 16C. These comparisons revealed the identification of one miRNA, miR-336, relevant for anhedonia. miR-336 has several predicted target mRNAs and two of them have previously been suggested to be implicated in MDD. These two are CREB binding protein (CBP) and Snf2-related CBP activator protein (SRCAP). Since miR-336 is upregulated in the CMS-V group, the mRNA targets may be downregulated in rats expressing anhedonic-like behaviour.

General information about CREB is described in section 1.1.3. Phosphorylation of CREB promotes interaction with CBP that activates the transcription of genes containing CRE mortifs in their promoters (Crisafulli et al. 2012). CBP has intrinsic histone acetyltransferase activity that acetylates the nearby histones, which loosens the chromatin allowing transcriptional activation (Tsankova et al. 2007). Histone acetyltransferase activity of CBP has a significant role in synaptic plasticity (Miller 2011), and increased levels of hippocampal CBP are highly correlated with increased neurogenesis (Li et al. 2009). In addition, it is shown that reduced CREB–CBP activity might contribute to the pathogenesis of sporadic Alzheimer's disease whereas elements which enhance CRE-dependent gene expression might be beneficial for the treatment of Alzheimer´s disease (Beglopoulos & Shen 2006). The relation of CBP to MDD is not well-established, but the comorbidity of Alzheimer´s disease and MDD is high (Spalletta et al. 2012), suggesting an involvement of CBP in the pathophysiology of anhedonia in the CMS-V group.

Another putative target of miR-366 is SRCAP. SRCAP has the ability to bind CBP and thereby potentiate the ability of CBP to activate transcription (Monroy et al. 2003). SRCAP acts as a co-activator of CREB-mediated transcription and a disruption of the interaction between SRCAP and CBP results in inhibition of CREB-mediated transcription (Monroy et al. 2001) which has been implicated in the etiology and pharmacotherapy of MDD (Serretti et al. 2011). Although the literature does not provide direct linkage between CBP, SRCAP, and MDD decreased synaptic plasticity and neurogenesis are common findings in depressed individuals suggesting that stress-induced decreases of CBP and SRCAP levels occur in the LHb during MDD. Therefore, an upregulation of miR-336 in the LHb may contribute to the induction of anhedonia in the CMS model of depression.

5.2.3 | Markers of Recovery

Two different expression patterns were investigated according to recovery (figure 16A-B), and this revealed the identification of four miRNAs; miR-130b, miR-331-5p, miR-327, and miR-331-5p (table 5). Only miR-331-5p and miR-327 will be discussed due to their molecular importance. These miRNAs have several predicted targets, where synapsin 1 (SYN1), CREB, both regulated by miR-327, and methyl-CpG binding domain protein 2 (MBD2), regulated by miR-331-5p, appeared relevant for recovery considering their functional roles.

The CMS-induced upregulation of miR-331-5p was reversed following antidepressant treatment with escitalopram. In addition, it was downregulated in the CMS-Res group, and thus a marker of recovery. Interestingly, this miRNA was also related to stress-resilience (table 5), as seen by similar expression levels in both the U-V and CMS-V group when compared to the CMS-Res group. Hence, it seems likely that a low expression level of miR-331-5p is essential for the healthy state of rats exposed to the CMS regime.

SYN1 may be downregulated in CMS-V rats whereas chronic escitalopram treatment reverses this regulation. Synapsines are a family of neuron-specific phosphoproteins localized in the presynapse where they are believed to anchor synaptic vesicles to the cytoskeletal framework, and thereby regulate synaptic vesicle homeostasis. SYN1 is shown to control the availability of synaptic vesicles to exocytosis and neurotransmitter release (Perlini et al. 2011). In addition, SYN1 is involved in neural plasticity (Farisello et al. 2012). Phosphorylation of SYN1 is shown to control the interaction between synaptic vesicles and the cytoskeleton, and thus, has an important role in the regulation of neurotransmitter release. SYN1 is found upregulated in the hippocampus of healthy rats subjected to electroconvulsive seizures (ECS). ECS is reported to lead to decreased phosphorylation during brief seizure activity in the hippocampus, and after termination of the seizure activity phosphorylation increases dramatically. It has been emphasized that the increase of SYN1 phosphorylation following ESC may lead to a dysregulated interaction between presynaptic vesicles and the cytoskeleton, and thus, enhanced neurotransmitter release (Elfving et al. 2008). Defects in the vesicular mediated transport system are also shown affected in hippocampus in the CMS model of depression (Henningsen et al. 2012, Holm et al. 2011). Thus, I speculate that a downregulation of SYN1 in the LHb, may contribute to the development of anhedonia in the CMS model of depression, whereas chronic treatment with escitalopram reverses the CMS effect on SYN1. However, the consequence of a downregulated SYN1 level in the LHb is still unclear.

CREB may be downregulated during the CMS regime in CMS-V rats and upregulated following successful antidepressant treatment. As described in section 1.1.3 and 5.2.2, CREB is important for synaptic plasticity and neurogenesis. In hippocampus, CREB appears to be a crucial mediator of antidepressant effects since an increased activity of CREB is observed following antidepressant treatment (Vaidya & Duman 2001). The

antidepressive effect could be due to an upregulation of the CREB-regulated target gene, BDNF, thereby inducing neurogenesis. Another feature demonstrating the role of CREB in MDD is that cAMP signalling mediators have antidepressant effects (O'Donnell & Zhang 2004). The increased level of miR-327 observed in the CMS-V group is reversed by chronic escitalopram treatment, further indicating a specific role of miR-327 in the LHb to induce an anhedonic-like status of rats exposed to the CMS model of depression. I speculate that an increased level of miR-327 influence neural plasticity and neurogenesis in the LHb and this regulation could cause the anhedonic-like status observed in CMS-V rats.

MBD2 may be upregulated in the CMS-Res and CMS-Esc-R groups and downregulated in the CMS-V group. MBD2 is a putative demethylase which binds to methylated CpGs on DNA and remove the methyl group in the presence of water. The removal of the methyl group leads to the formation of non-methylated CpG bearing DNA (Bhattacharya et al. 1999). Recently, interest in epigenetic modifications has been considered in the pathophysiology of MDD and antidepressant action. It is believed that epigenetic changes can explain several aspects of MDD, including high discordance rates between monozygotic twins, individual differences among inbred rodents, the chronic relapsing nature of the illness, and the strikingly greater prevalence of depression in women (Krishnan & Nestler 2008). DNA methylation and maternal behaviour seem to be important in the regulation of the GR in hippocampal tissue. Offspring receiving low maternal care show increased methylation of the GR compared to offspring receiving high maternal care. It is suggested that the increased methylation of GR affect the negative feedback mechanism on the HPA axis and thereby promoting hypercortisolism during stress (Weaver et al. 2004). The reduced level of miR-331-5p observed in CMS-Res and CMS-Esc-R, and thereby an increased level of MBD2 could influence the methylation rate of GR in the CMS model of depression. Thus, miR-331-5p could function as an antidepressant miRNA, determining the individual response to chronic stress and chronic escitalopram treatment.

The large number of regulated miRNAs after antidepressant treatment with escitalopram when compared to CMS-V was unexpected since escitalopram display high selectivity to the serotonin transporter. Another study investigating antidepressive effects of escitalopram have confirmed this (Christensen et al. 2011). However, the effect of chronic escitalopram treatment has not been investigated neither on the LHb nor at a miRNA level. Thus, it is not possible to evaluate the effect of escitalopram in the LHb by comparing to similar studies neither in humans nor in animal models.

5.3 | miRNA Expression Profiling Associated with Treatment Resistance

The segregation of escitalopram treated CMS rats into responders and non-responders allows the investigation of miRNAs associated with treatment resistance. Refraction of antidepressant treatment is a common problem in the clinic, affecting 30-40% of the patients (Lanni et al. 2009). Therefore, the acquaintance of biomarkers or knowledge about the mechanisms responsible for drug resistance is important for the discovery of more efficient antidepressants. According to the expression pattern illustrated in figure 16D, one miRNA was identified, miR-546. This hypothesis compares miRNA expression levels of the CMS-V group with CMS-Esc-R and CMS-Esc-NR groups.

The miRNA expression level of miR-546 increased between the CMS-V and the CMS-Esc-R group, while the expression level between CMS-Esc-R and CMS-Esc-NR increased further, see figure 17. Treatment resistance in the CMS model of depression may be caused by the high upregulation of miR-546, whereas a minor increment would induce recovery from anhedonia. A large increment of a miRNA may result in a high

interaction with target mRNAs and thereby a high degree of degradation or translational inhibition, whereas a minor increment will result in minor interaction with target mRNAs. This may explain the increased miR-546 level observed between CMS-Esc-R and CMS-Esc-NR. A minor increase of miR-546 may induce treatment response, whereas a high increase may results in treatment resistance due to inhibition of additional target mRNA.

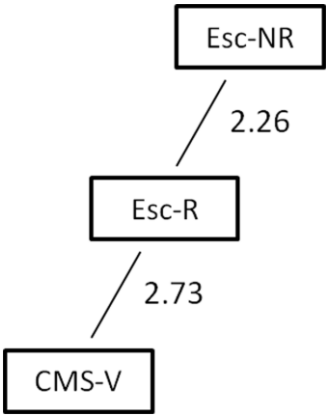

Figure 17 | miRNA expression pattern of miR-546, involved in treatment resistance. The lines indicate a significant difference between the groups. The individual fold changes are listed next to the line. CMS-V: CMS vehicle, Esc-R: CMS escitalopram responders, Esc-NR: CMS escitalopram non-responders.

As depicted in figure 15 only one miRNA was significantly regulated in the CMS-Esc-NR group. This suggests that the CMS-Esc-NR at the miRNA level is highly similar to the CMS-V group. However, in the hierarchical clustering analysis, depicted in figure 13, a clear segregation between CMS and treatment groups is observed. Small non-significant miRNA regulations caused by treatment with escitalopram may be an explanation of this segregation, since these regulations are integrated in the hierarchical clustering analysis. Although the CMS-Esc-R and CMS-Esc-NR groups have a different behavioral output from the sucrose consumption test, the overall drug effect segregates the escitalopram treated rats from CMS rats.

5.4 | miRNA Expression Profiling Associated with Stress Resilience

To investigate potential factors rendering individuals less vulnerable to CMS exposure, the CMS-Res group was compared to the U-V and CMS-V group (Figure 16E). Comparative analyses of miRNAs from these groups, according to the applied constraints revealed the identification of two individual miRNAs, miR-331-5p and miR-409-5p (Table 5). These miRNAs could either exert a compensatory effect caused by the CMS regime - an effect that is absent in CMS-V rats – or they could be associated with a status-specific miRNA in CMS-Res rats. miR-331-5p and miR-409-5p have several putative target. Since putative targets for miR-331-5p already are described in section 5.2.3, only predicted targets for miR-409-5p will be described, including apolipoprotein e receptor 2 (ApoER2) and dihydropyrimidinase-like 2 (DRP-2). The miR-409-5p targets described in the following are all upregulated in the CMS-Res group.

DRP-2 is a path-finding and guidance protein for axonal outgrowth during the formation of neuronal connections and for the maintenance of neuronal communication (Owen et al. 2009). The protein itself has no enzymatic activity, but interacts with binding partners, and thereby affects neurite outgrowth and retraction, neural differentiation, and neurotransmitter release (Hensley et al. 2011). Additionally, DRP-2 is involved in repairing and maintenance of the plasticity of neuronal connections in aged brains. A depletion of DRP-2 cause dysfunctions of the repairing activity in the brain and leads to neuronal abnormalities (Owen et al. 2009). A study by Johnston-Wilson et al. (2000) showed a decreased level of DRP-2 in the prefrontal cortex in post mortem brains from patients suffering from schizophrenia, bipolar disorder or MDD

(Johnston-Wilson et al. 2000). Another study showed that the expression level of DRP-2 is decreased in hippocampus in patients suffering from Alzherimer´s disease leading to loss of synaptic plasticity (Sultana et al. 2006). These findings indicate that DRP-2 has a significant role in the development of psychiatric and neurodegenerative disorders. As described in section 1.1.3 the synaptic plasticity is affected in MDD, and thus, the increased level of DRP-2 and the decreased level of miR-409-5p in the LHb may contribute to the healthy state observed in CMS-Res, whereas a downregulation of DRP-2 in the LHb may contribute to the anhedonic-like phenotype observed in CMS-V rats.

ApoER2 is a member of the low-density lipoprotein receptor family and is expressed postsynaptically. ApoER2 is the dominant receptor for reelin, an extracellular matrix protein, and binding of reelin to apoER2 is essential for neuronal survival, synaptic plasticity, and neurogenesis (Sentürk et al. 2011, Marzolo 2012). A downregulation of ApoER2 may thus influence the activity of reelin. In a recent study, ApoER2 in peripheral blood lymphocytes was significantly lower in patients suffering from MDD when compared to controls (Suzuki et al. 2010). Additionally, a study by Lussier et al. 2011 investigated whether a developmental genetic deficit in reelin signalling could influence the vulnerability to a depressive state using injections of corticosterone. They used a strain of haploinsufficient heterozygous reeler mice, which have approximately 50 % of normal brain levels of reelin. They observed no differences between the vehicle-injected reelin deficient mice and wild-type mice. On the contrary, corticosterone injected reelin deficient mice displayed increased immobility and decreased swimming in the forced swim test when compared to corticosterone injected wild-type mice (Lussier et al. 2011). These findings suggest that impaired reelin signalling may show vulnerability to the depressive effects of glucocorticoids. In the present study, I found a downregulation of miR-409-5p group in the LHb in CMS-Res rats, which may regulate the level of ApoER2. This suggests an implication of miR-409-5p in mechanisms relevant for stress-coping. A downregulation of ApoER2, as demonstrated in CMS-V rats, may cause an inhibited reelin function and explain the anhedonic-like phenotype. Since wild-type mice, in the study by Lussier et al. 2011, did not show depressive behaviour after corticosterone injection the stress-resilient mechanism of reelin may not function as a coping strategy caused by the CMS regime, but instead caused by a protein deficiency in stress-susceptible rats.

Several studies have demonstrated that the stress-resilient phenotype is associated with a heightened degree of molecular plasticity, demonstrated by a larger number of gene regulation in the stress-resilient phenotype (Krishnan et al. 2007, Christensen et al. 2011). This suggests that several active neurobiological processes contribute to the stress-resilient phenotype. In the present study, a larger number of miRNAs were regulated in the CMS-Res group when compared to the CMS-V group. The hierarchical clustering analysis in figure 13 confirms this observation, showing that U-V and CMS-V are closer related than U-V and CMS-Res. Hence, the upregulation of reelin may not be the only factor contributing to the stress-resilient phenotype, but further studies are needed to clarify the exact mechanism responsible for the stress-resilient phenotype.

5.5 | Methodological Considerations

In the present study a combination of TaqMan® Low Density Arrays and LCM was used to investigate miRNA correlates of CMS and antidepressant treatment, treatment resistance, and stress-resilience in the LHb. Many of the unique characteristics of miRNA pose challenges to an accurate detection and quantification. For instance, the length of mature miRNAs is insufficient for annealing to traditional PCR primers. In addition, mature miRNA lack the poly(A) that can be used as a universal primer-binding site for reverse

transcription. Furthermore, pri-miRNAs and pre-miRNAs contain the RNA sequence of the mature miRNA, thus the detection of miRNAs must be selective (Pritchard et al. 2012). Taqman® Low Density Arrays are an established method and have been widely used in order to investigate large scale miRNA profiling of different diseases. The method is sensitive and specific, and can be used for absolute quantification. In addition, the Taqman® Array-based approach uses the TaqMan® probe to measure miRNA expression, which reduces the likelihood of false fluorescence signalling during qPCR that arises from mis-priming (Pritchard et al. 2012, Armstrong et al. 2012). Contamination is a common and inevitable problem when performing region-specific analyses on macrodissected tissues. Because of the size of LHb this problem would have been unavoidable. LCM was thus used as the method of tissue isolation in the present study. The LCM technique provide a rapid and simple method for isolating homogenous populations of cells from heterogeneous tissue sections and is compatible with miRNA and gene expression profiling and proteomic analysis (Pritchard et al. 2012, Espina et al. 2006).

To identify putative miRNA targets TargetScan Version 6.1 was used. I only used TargetScan to identify putative targets, because a direct comparison of the putative miRNA targets revealed from different target prediction tools do not overlap well. TargetScan considers only stringent seeds, which increase specificity and the amount of false-positive is diminished (Saito & Saetrom 2010). However, TargetScan only focuses on the 3´UTR region, and increased evidence suggests that miRNA also has the ability to induce degradation or translation inhibition through targeting within the 5´UTR and protein coding region. This may results in predicted target genes being biased toward the 3´UTR region, and therefore ignoring many potential targets (Lytle et al. 2007). The targets generated from TargetScan are only predicted targets; hence, further validation of the targets is needed to clarify the exact role of individual miRNAs involvement in MDD.

6 | Conclusion

The present study was initiated to clarify miRNA aberrations in the LHb during depressive-like states and after antidepressant treatment in the search for novel disease targets of MDD. Furthermore, the study includes a search for habenular biomarkers involved in treatment resistance and stress-resilience in order to investigate mechanisms underlying antidepressant drug refraction and stress-coping strategies. To my knowledge this is the first study to investigate the impact of a stress paradigm on large scale miRNA profiling in the LHb. Based on the present findings it is difficult to determine the exact role of the LHb in depression-related behaviour. However, several of the predicted mRNA targets are involved in mechanisms already associated to depression, including neuronal plasticity, neurogenesis, synaptic release, and demethylation. To clarify the exact role of the LHb in the pathophysiology of depression, further investigations are required to confirm the present results and to explore the individual miRNAs involvement in MDD.

7 | Future Prospects

Performance of the present study and analysis of the corresponding results give rise to several considerations which provide issues of interest for further investigation. Future experiments that might prove useful for the confirmation and knowledge extension of the present study are listed below:

- Confirmation of the individual miRNA found to be regulated. The method of validation could be Northern Blot.
- Validation of putative targets relevant for MDD by microarray. This part has already been executed, but data analysis has not yet been terminated.
- Validation of putative targets relevant for MDD using a Luciferase reporter system.
- Investigation of miRNA sponges in the CMS model of depression. This allows investigation of miRNA regulation.
- Test the differences in miRNA profiling between the medial and the lateral habenula, since the medial part also seems affected by the CMS regime.

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Appendix I

In the following the chemicals and instruments used throughout the present study are listed.

Materials and Chemicals:

- CapSure Macro caps (Molecular Devices, USA)
- Cresyl violet acetate (Sigma Aldrich)
- Megaplex™ RT Primers (Applied Biosystems)
- miRNeasy Mini Kit (Qiagen)
- Polyethylene napthalate (PEN) glass slides (Molecular Devices, USA)
- RNA, MS2; from bacteriophage MS2 (Roche Applied Sciences)
- TaqMan® Array Rodent MicroRNA Card A (Applied Biosystems)
- TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems)
- TaqMan® PreAmp Master Mix Kit (Applied Biosystems)
- TaqMas® Universal PCR Master Mix, No AmpErase® UNG, 2X(Applied Biosystems)

Apperatus:

- 2100 BioAnalyzer (Agilent Technologies)
- Applied Biosystems 7900HT Fast Real-Time System
- Centrifuge Minispin plus (eppendorf)
- Cryostat (CM3050S, Leica Microsystems, GmbH, Germany)
- Nanodrop ND-1000 (Thermo Scientific)
- Veritas Microdissection Instrument model 704 (Molecular Devices, USA)

Appendix II

Tabel 6 | Sucrose Consumption Test for Control Rats

Animal number	Baseline	Stress Week 1	Week 2	Week 3	Week 4	Treatment Week 5	Week 6	Week 7	Week 8
25	11,2	8,70	19,30	12,80	18,10	13,30	15,70	8,60	14,50
26	18,8	17,10	22,50	19,00	26,10	15,10	22,90	9,50	15,60
27	13,4	15,90	21,10	15,10	18,80	15,40	19,90	15,80	10,30
29	11,7	8,80	17,70	14,00	21,50	12,60	19,20	6,10	16,80
32	13,9	14,10	15,70	15,80	18,30	10,80	16,10	11,80	19,60
33	15,8	17,70	17,90	15,50	14,90	14,80	22,30	12,50	18,40
34	14,0	7,60	13,80	7,80	17,00	16,00	13,60	15,30	15,20
36	9,3	7,30	17,20	6,10	13,60	9,50	18,30	14,30	11,60
37	19,0	19,70	19,40	21,40	21,60	15,10	19,70	17,90	20,60

Tabel 7 | Sucrose Consumption Test for Anhdeonic-like Rats

Animal number	Baseline	Stress Week1	Week2	Week 3	Week 4	Treatment Week 5	Week 6	Week 7	Week 8
280	17,4	10,00	10,30	10,30	11,80	8,30	12,90	10,60	10,00
297	14,2	7,40	6,60	3,90	4,80	4,90	5,90	3,60	4,75
299	14,8	10,20	9,80	7,50	6,50	7,00	5,20	9,00	7,10
306	17,1	9,50	11,90	4,30	9,00	5,10	6,10	8,10	7,10
307	11,5	10,30	9,90	3,40	7,30	7,50	5,80	3,90	4,85
313	16,8	8,00	9,40	6,40	6,80	5,40	6,90	4,00	5,45
362	18,5	11,80	17,40	7,00	11,00	8,50	12,90	8,80	12,70
108	18,3	15,20	12,90	8,40	9,60	10,40	10,90	9,60	9,60
109	11,8	9,70	5,30	4,20	6,20	3,40	5,50	4,00	5,40

Tabel 8 | Sucrose Consumption Tests for Resilience Rats

Animal number	Baseline	Stress Week 1	Week 2	Week 3	Week 4	Treatment Week 5	Week 6	Week 7	Week 8
361	15,2	20,90	26,20	18,10	24,20	19,30	21,70	19,70	23,60
375	14,2	11,80	15,20	16,90	15,70	19,50	21,30	18,90	23,10
377	12,6	14,30	11,90	10,80	18,20	11,30	11,90	13,70	11,40
380	18,0	18,20	23,40	19,00	25,60	20,60	20,80	25,60	24,60
408	13,6	14,30	18,70	12,70	19,30	19,00	15,80	22,50	21,20
424	17,0	19,70	22,90	17,40	19,60	17,00	20,20	18,20	28,00
434	14,4	12,40	16,20	14,30	18,40	18,20	20,80	20,30	20,50
456	12,1	11,10	12,20	12,10	11,30	11,10	17,00	14,70	17,50
473	15,1	18,60	19,10	14,80	22,30	17,70	17,20	14,30	17,00

Tabel 9| Sucrose Consumption Test for Non-responders

Animal number	Baseline	Stress Week 1	Week 2	Week 3	Week 4	Treatment Week 5	Week 6	Week 7	Week 8
206	18,4	10,80	5,40	7,30	5,60	6,00	7,20	6,20	13,80
222	16,5	6,30	10,20	6,90	7,70	8,80	11,20	9,10	10,10
230	20,2	10,00	9,10	8,00	11,40	10,80	9,70	9,30	9,10
246	17,2	15,50	5,10	10,50	11,80	7,40	9,50	7,10	6,70
242	19,1	13,40	11,20	6,70	9,00	9,70	9,80	8,90	11,60
418	12,4	8,80	12,50	3,70	5,70	4,50	6,40	4,80	8,90
419	11,5	10,10	11,00	3,60	5,90	5,70	4,30	5,60	6,20
461	16,2	13,90	14,80	8,00	8,30	8,40	9,90	7,20	14,20
477	14,1	9,70	9,90	6,70	6,30	10,10	7,80	7,70	9,20

Tabel 10 | Sucrose Consumption Test for Responders

Animal number	Baseline	Stress Week 1	Week 2	Week 3	Week 4	Treatment Week 5	Week 6	Week 7	Week8
157	15.2	7.80	12.60	2.70	6.60	7.40	5.80	13.10	15.00
213	12.8	9.20	9.30	8.50	8.80	11.80	10.30	10.70	9.20
421	13.5	12.90	13.40	12.10	8.70	14.10	11.70	12.50	15.40
437	14.0	11.00	13.40	8.60	9.30	11.70	11.80	9.40	17.40
455	12.2	7.70	10.30	6.80	8.00	7.70	6.30	10.60	10.30
466	12.7	13.00	11.20	5.70	5.90	7.10	15.40	12.20	10.50
468	14.1	12.90	10.90	10.40	7.20	16.00	11.10	14.00	20.00
474	13.3	10.70	10.80	7.20	7.60	8.90	18.10	12.60	11.40
475	14.2	12.90	12.70	6.90	6.60	9.90	7.80	9.80	16.10

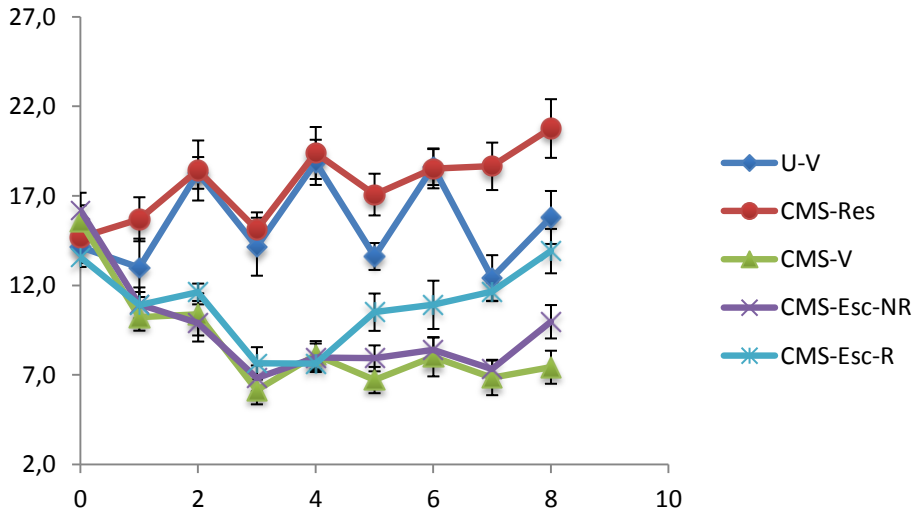

Figure 18 | Sucrose intake during 4 weeks of chronic mild stress (CMS) followed by 4 weeks of escitalopram or vehicle treatment with continuous exposure to CMS.