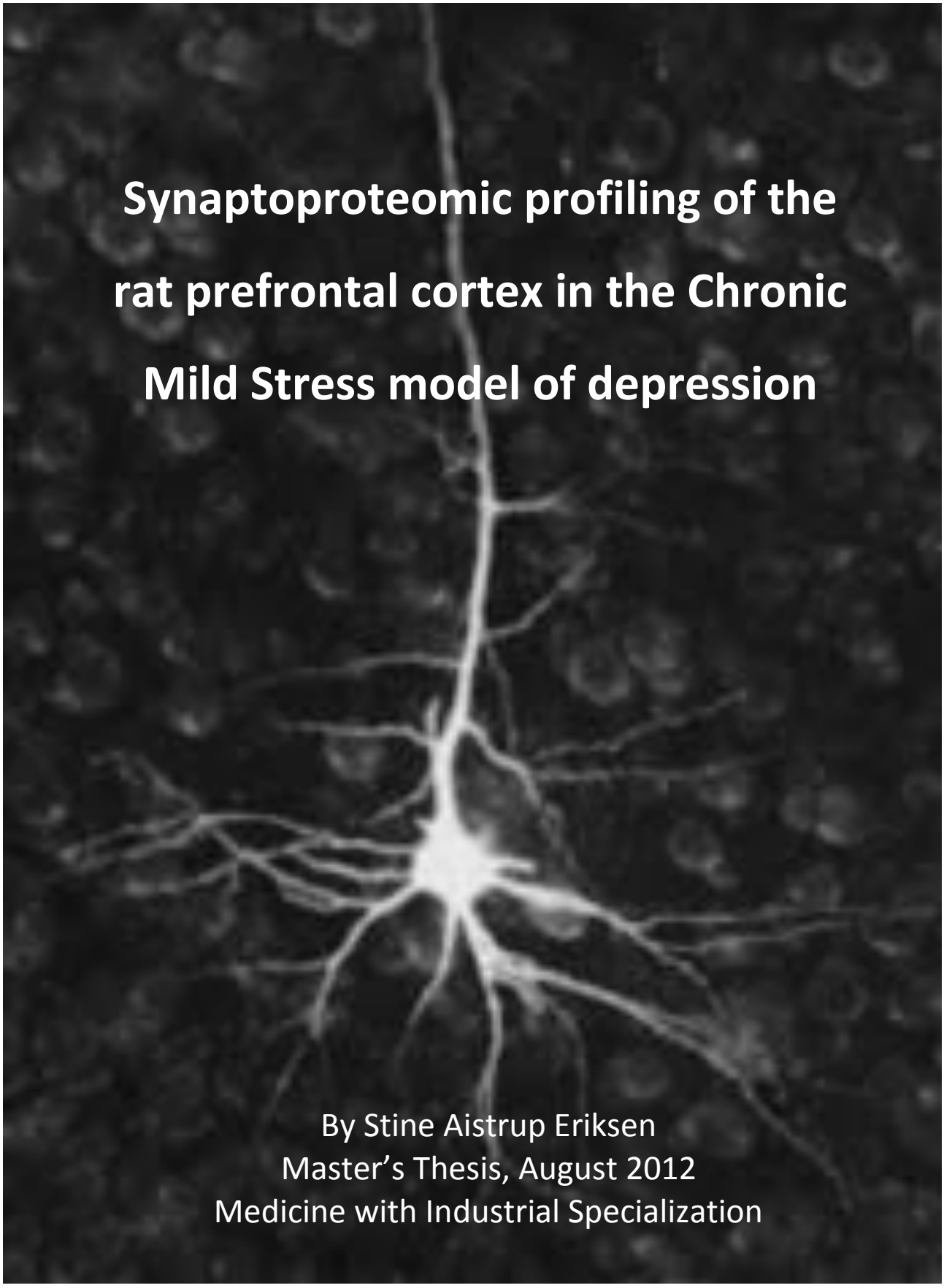

Synaptoproteomic profiling of the rat prefrontal cortex in the Chronic Mild Stress model of depression

By Stine Aistrup Eriksen

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Ove Wiborg

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Abstract

Major depressive disorder (MDD) is a common mood disorder and a major cause of disability worldwide. It is a debilitating illness which, if left untreated, carries high levels of morbidity and mortality. Due to the heterogenic nature of this disease, the underlying pathophysiology is still largely unresolved. It is well established that life stressors pose major environmental risk factors for developing MDD. A complex interaction between environmental factors and the genetic makeup is likely to underlie differences in stress-sensitivity, and may explain why some people are more prone to develop MDD than others after severe adversity. MDD has a neurobiological basis with complex molecular alterations, associated with functional and structural brain abnormalities, with the prefrontal cortex (PFC) as one of the brain areas showing profound alterations.

Extensive preclinical research has focused at unravelling the underlying pathophysiology of MDD through genetic and more recently proteomic approaches. Mass spectrometry-based proteomics provides a robust and sensitive identification of the global protein expression within a given tissue or cell. This approach is highly advantageous for investigating a complex disease as MDD, since proteins are likely the most ubiquitous molecules affected in disease. Many molecular alterations associated with the pathophysiology of MDD reside within the synapse. Thus, to reduce the complexity of the proteome analysed and to enrich for less abundant synaptic proteins, nerve terminals (synaptosomes) can be purified by differential centrifugation on a Percoll gradient and analysed by iTRAQ coupled to tandem mass spectrometry.

The aim of the present study was to investigate quantitative changes in protein abundance in PFC synapses using a highly validated animal model, the Chronic Mild Stress (CMS) model of depression. This model generates two stress-response phenotypes, reflecting depressive-like behaviour (anhedonia) and stress-resilience. The large-scale, non-hypothesis driven proteomic analysis of the two phenotypes was applied in order to investigate MDD-associated markers of stress-susceptibility and stress-resilience. Furthermore, the behavioural task, the odour span task, was set up in a pilot study with the aim of investigating the effect of CMS on working memory.

Proteins involved in the biological pathway, synaptic transmission, were primarily regulated between stress-resilient compared to anhedonic-like rats and control rats, whereas proteins involved in synaptic transduction primarily were regulated between anhedonic-like rats compared to control rats. Proteins involved in metabolism were significantly regulated in resilient rats compared to anhedonic-like rats. Finally, proteins involved in cytoskeletal organisation and oxidative stress were pathways shown to be affected in both phenotypes; however, the proteins involved in these biological pathways, in each phenotype, were of different types.

In conclusion, stress-resilient and anhedonic-like rats showed a clear segregation in synaptic proteome profiles reflecting the two hedonic responses to CMS. Stress-susceptibility and stress-resiliency were associated with several proteomic aberrations, particularly those related to metabolism, cytoskeletal organisation, synaptic transmission and signal transduction. These proteins should be further investigated to confirm their relevance to depression.

Preface

This masters' thesis was written by Stine Aistrup Eriksen 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 Research Unit for Molecular Medicine (MMF), Aarhus University Hospital. Supervisors of the project were Jacek Lichota and Ove Wiborg.

The reference list of publications cited can be found at the end of the thesis. References are cited in parentheses, with author´s last name and publication year. A list of the 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. Furthermore, I would like to thank Johan Palmfeldt for his great support during laboratory work and for always taking the time to help me. Additionally, I would like to thank all my great colleagues in Skejby for their support throughout the year. Especially, I would like to thank Christina Fuhr Bisgaard, Kim Henningsen and Line Jensen for sharing their expertise, for our many discussions, and for their extensive support whenever needed! At least, I would like to thank all at Centre for Psychiatric Research, Skejby, for having a great time both at work and after working hours!

Table of Content

Introduction

Major Depressive Disorder

Depression, also known as major depressive disorder (MDD) is a highly prevalent mood disorder affecting approximately 121 million people worldwide and WHO has projected this disorder to become the leading cause of burden of disease by the year 2030 (WHO (1); WHO 2004). MDD is a debilitating illness manifested by biological, cognitive and psychological symptoms with far reaching personal and public consequences, and if left untreated depression is a life threatening disease associated with high levels of morbidity and mortality. Depressed individuals are at higher risk of serious physical health problems including coronary artery disease and diabetes as well as worsening of the prognosis of other medical conditions (Palazidou, 2012). In spite of many years of research, the heterogenic character of MDD hampers the efforts to explain the full picture of the underlying pathology, contributing to the various clinical challenges associated with MDD. The major challenges in the clinic are posed by a high resistance to treatment reported in about 40% of people receiving pharmacological treatment, and patients responding to treatment experience a delay in the therapeutic effect of up to six to eight weeks (Lanni, Govoni, Lucchelli, & Boselli, 2009; Meyer, 2012). Furthermore, there exist a high frequency of recurrent depressive episodes, with approximately 20-50% of people experiencing such within two years of remission (Meyer, 2012).

Due to the heterogenic nature of MDD no objective diagnostic tests are available, and the diagnosis of the disease is therefore most often based on a highly variable set of symptoms described by the Diagnostic and Statistical Manual of Mental disorders (DSM-IV, 2000) or the International Classification of Disorders, ICD-10 (WHO (2)). The core symptoms of MDD are 1) Anhedonia; described as the inability to experience pleasure from activities previously experienced as rewarding, and 2) depressed mood; unmotivated sadness and lack of interest in most things. Subsidiary symptoms are feelings of guilt, worthlessness and hopelessness, irritability, restlessness, appetite disturbance, weight loss or weight gain, suicidal thoughts, insomnia or hypersomnia, fatigue and decreased energy (Chopra, Kumar, & Kuhad, 2011). Additionally, cognitive impairments are associated with MDD including diminished ability to think and concentrate, or indecisiveness, with devastating effects on short- and long-term learning and memory, and execute functions (Eriksson et al., 2012). To diagnose MDD one core symptom and at least four subsidiary symptoms, or both core symptoms and three subsidiary symptoms, must be present every day for at least two weeks in row and the symptoms are disrupting normal social and occupational functioning (Chopra et al., 2011).

Gene-Environment Interaction

Increasing evidence indicates that the interaction between environmental factors and genetic predisposition influences the individuals' risk for developing MDD (Caspi & Moffitt, 2006; aan het Rot, Mathew, & Charney, 2009). Stressful life events, e.g. being divorced, or losing a loved one, have long been recognised to constitute major environmental factors highly implicated in the development of MDD (R. T. Liu & Alloy, 2010). This is illustrated by the fact that the onset of the first episode of MDD is preceded by a severe stressful life event in approxi-

mately 70-80% of cases. An association between stress and depression has been found for both acute and chronic stress, and may as well be implicated in the induction of depressive relapses (G Hasler & Northoff, 2011; Wager-Smith & Markou, 2011). Epidemiological studies show that there exists a highly genetic vulnerability for MDD accounting for approximately 40-50% of individuals diagnosed with this condition (Nestler et al., 2002). However, elucidating MDD-specific genes have been challenging. A factor that might contribute to this is the restrictive nature of the diagnostic criteria determined by ICD-10 and DSM-IV, which likely encompass a group of disorders that are heterogeneous with respect to aetiology and pathophysiology (Gregor Hasler, Drevets, Manji, & Charney, 2004).

Pre-existing abnormalities related to genetic predisposition including susceptibility and/or lack of protective genotypes together with early life adversity or other causes may act as vulnerability factors. It has been speculated that the higher the genetic load and other vulnerability factors, the lower the amount of life stress required to induce a depressive episode and vice versa (Palazidou, 2012). Due to the complexity of MDD the specific patterns of gene-environment interaction have not yet been established. An explanation can be that MDD may result from the combined effect of multiple small genetic changes with a variety of environmental factors. In addition to this, genetic and environmental factors may interact with each other in complex ways to influence the phenotype of depression (Tsuang, Bar, Stone, & Faraone, 2004), further hampering the efforts to couple specific gene-environment interactions to MDD.

Stress Susceptibility and Stress-Resilience

Humans display great heterogeneity in their response to stress and adversity. As previously mentioned, a great amount of depressive episodes can be causally attributed to stressful life events, however, these events in themselves raise only moderately the risk of developing depression. Hence, some individuals are stress-sensitive and prone to develop MDD in response to modest stressors, while others are stress-resilient, remaining symptom free after severe adversity (Hjemdal, Vogel, Solem, Hagen, & Stiles, 2011). Compared to the large body of research describing stress-induced maladaptive neurobiological changes, relatively little attention has been devoted to understand how most individuals adapt well in face of adversity, also described as resiliency to stress. However, in recent years stress resiliency has gained a lot of attention because information of more defined depressive phenotypes might lead to a better understanding of diverse depressive behaviours (V. Krishnan & Nestler, 2008).

The resilient phenotype has been indicated to represent a distinct active neurobiological process and not simply the absence of vulnerability (Henningsen et al., 2012a). Investigating stress responses at multiple levels, both behavioural as well as neurobiological measurements, could help in the identification of new potentially predictive and protective targets. Stress-resilience may be mediated by adaptive changes in several neural circuits that regulate reward, fear, emotion reactivity and social behaviour, which together are thought to mediate successful coping with stress (V. Krishnan & Nestler, 2008).

Hypothesis of Depression Pathology

The involvement of stress-induced depression has primarily emerged from various studies showing a dysfunctional stress response by the hypothalamic pituitary adrenal (HPA) axis in patients suffering from MDD. These observations have led to one of the most established hypotheses that attempt to unravel the pathophysiology of MDD; the neuroendocrine hypothesis (Carmine M Pariante & Lightman, 2008). Other established hypotheses describe the involvement of altered levels of different substrates in the brain such as monoamines, neurotropic and neurogenic factors (S. Lee, Jeong, Kwak, & Park, 2010), and inflammatory mediators (Miller, Maletic, & Raison, 2009). All of these hypotheses are based on alterations observed in both depressed individuals and in animal models of depression, as well as changes of systems and structures produced by antidepressant treatment within different brain areas. Each hypothesis describes important aspects of the pathophysiology of MDD, however, they cannot individually explain the full extent of this condition. Instead the pathological changes may be interconnected in complex meshwork and including yet unknown molecular mechanisms that have to be established to fully understand MDD pathology (Wager-Smith & Markou, 2011). The following describes different depression hypotheses presented as a short overview.

The Neuroendocrine Hypothesis

Responses to stress are controlled primarily by the HPA axis. the main function of this axis is to regulate glucocorticoid stress hormones via negative feedback loops. Chronic exposure to stress may however impair this feedback system and resulting, at least partly, in hyperactivity of the axis and ultimately producing a persistent and abnormal high secretion of the glucocorticoid cortisol. Several years of research have demonstrated that HPA hyperactivity is one of the most consistent findings in MDD patients, but the precise mechanisms underlying HPA abnormality are yet unknown (Carmine M Pariante, 2006; Carmine M Pariante & Lightman, 2008; Skynner et al., 2006).

The HPA axis is governed by the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus in response to stress. CRH stimulates the secretion of the adrenocorticotropic hormone (ACTH) from the pituitary and this hormone promotes the release of glucocorticoids from the adrenal glands (Fig. 1). Glucocorticoids mediate their effect through two receptors; a high affinity mineralocorticoid receptor (MR) and a low affinity glucocorticoid receptor (GR). An increase in the level of glucocorticoids saturates the MR and activates GR, which triggers the negative feedback reaction on the HPA axis. Both of these receptors are present in high concentrations in the prefrontal cortex (PFC) and hippocampus, which have been suggested to be highly implicated in the pathology of depression (Skynner et al., 2006). A prolonged elevation of cortisol can produce neuronal dysfunction with decreased glucose uptake, reduced dendritic arborisation, neuronal death and cell loss within these brain areas (Morales-Medina, Sanchez, Flores, Dumont, & Quirion, 2009; Radley et al., 2006; Skynner et al., 2006). The neuronal damage caused by persistent hypercortisolaemia in chronic stress is thought to be connected to voltage-gated ion channels. These allow increasing amounts of calcium entry into the activated neuron, which is beneficial for a shorter period, however, during chronicity it will cause damage to the nerves (Palazidou, 2012).

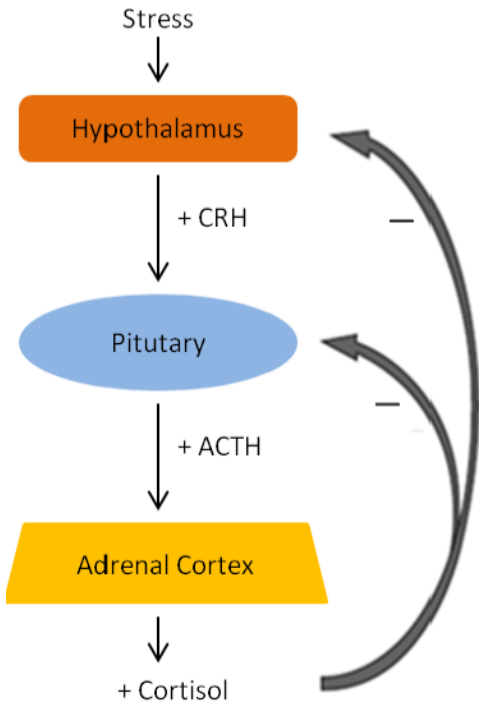

Fig 1: Shows the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress triggers the hypothalamus to increase the secretion of corticotropin releasing hormone (CRH). CRH acts on the pituitary which releases adrenocorticotrophic hormone (ACTH). ACTH stimulates the adrenal cortex to increase the release of glucocorticoids including cortisol. 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. (modified from (Chopra et al., 2011))

In MDD patients the impaired glucocortico-mediated negative feedback mechanism is demonstrated in studies where systemic administration of the synthetic glucocorticoid dexamethasone does not produce a subsequent decrease in cortisol secretion, as is observed in healthy individuals (with no pathology in endogenous cortisol production) (C M Pariante & Miller, 2001). Furthermore, successful antidepressant treatment is associated with resolution of the impaired HPA axis, whereas persistence of an impaired HPA axis is associated with high risk of relapse (Carmine M Pariante, 2006). In spite of this, a dysfunctional HPA axis is not universal for MDD pathology as it is only observed in approximately 50% of patients. Therefore, additional factors must be considered as described by others hypothesis (Palazidou, 2012).

The Monoamine Hypothesis

The monoamine hypothesis has played a central role in depression research for the last 50 years (S. Lee et al., 2010). It suggests a deficiency or imbalance in the monoamine neurotransmitters including serotonin, dopamine and norepinephrine as the cause of depression. This is based on the observation that most antidepressant drugs act by increasing the levels of monoamines in the synaptic cleft (Andrade & Rao, 2010). Most pharmacological treatments available today are designed to increase monoamine transmission acutely; however, symptoms of recovery are delayed for up to six to eight weeks. Further, the drug efficacy is suboptimal as earlier stated with only 60-70% of MDD patients gaining an effect of the treatment (Lanni et al., 2009; S. Lee et al., 2010). Finally, studies of monoamine depletion in healthy people do not induce depressive symptoms, whereas such depletion in depressed people exacerbates depression-associated symptoms. Thus, discrepancies like these indicate that there is probably not a simple relationship between biogenic amines and MDD (Delgado, 2006; Shansis et al., 2000). The time lag for symptoms to improve, suggest that long-term adaptions in neuro-

transmitter systems and/or their downstream targets might be necessary for therapeutic effects. It is believed that an increased concentration of synaptic monoamines cause secondary and slower neuroplastic changes involving transcriptional and translational alterations (V. Krishnan & Nestler, 2008). How significant the role of these monoamine systems is in the complex pathophysiology of MDD remains to be determined as well as the role of other neurotransmitters. In addition to serotonin, GABAergic and glutamatergic neurotransmitter systems have likewise been shown to be involved in MDD (Palazidou, 2012).

Glutamate – A Novel Hypothesis of Depression?

In addition to the monoaminergic systems, there is growing evidence that the glutamatergic system plays an important role in the neurobiology and treatment of MDD (Hashimoto, 2009; Sanacora, Treccani, & Popoli, 2012). Several observations in the clinic have suggested that MDD pathophysiology is associated with dysfunctions of the glutamatergic system (Auer et al., 2000; Hashimoto, Sawa, & Iyo, 2007; Mitani et al., 2006; Sanacora et al., 2004) as well as malfunction in the mechanisms regulating clearance and metabolism of glutamate (Bernard et al., 2011; Choudary et al., 2005). Concurrently, several studies have shown an association between different types of environmental stressors and the release and transmission of glutamate in limbic and cortical areas (Moghaddam, 2002; Moghaddam & Jackson, 2004; Tordera et al., 2011). Moreover, it has been suggested that glutamatergic transmission plays a central role in mediating the complex emotional and cognitive changes associated with depression. Drugs that target glutamine release, clearance or receptors have been shown to produce antidepressant effects, some of which has been reported to induce rapid and sustained antidepressant efficacy in both depressed and treatment-resistant patients (Sanacora et al. 2012; Pittenger & Duman 2008,) as opposed to the delay in efficacy of conventional antidepressant drugs (Lanni et al., 2009). Based on these observations the authors of a recent review suggest that the glutamatergic system should be recognised as a primary mediator of psychiatric pathology, and that a paradigm shift from a monoamine hypothesis of depression to a hypothesis focused on glutamate will provide advancement in the research for new drugs and therapies (Sanacora et al., 2012).

The Neurotrophic Hypothesis

Neurotrophic factors are important regulators of plasticity and survival of neurons and glial cells. Evidence for volumetric decreases in numerous brain regions including the hippocampus and PFC are found in depressed individuals (V. Krishnan & Nestler, 2010) and have been associated with a lack of neurotrophic support (Nestler et al., 2002). Volume loss in these areas, shown by structural magnetic resonance imaging (MRI), are in accordance with subtle atrophic changes such as reduced neuronal size, local reductions in dendrite densities, decreased glial cell number, and trophic factors in postmortem biopsies (V. Krishnan & Nestler, 2010). The neurotrophic hypothesis states that MDD is caused by deficiencies of neurotrophic factors and that antidepressants neutralise this deficit (Nestler et al., 2002). The theory is mainly based on changes reported for one of the major neutrophins of the brain, the brain derived neurotrophic factor (BDNF)(V. Krishnan & Nestler, 2008), which is found in high concentrations in the hippocampus and frontal cortex under normal circumstances (Szapacs et al., 2004). Three main observations have led to the hypothesis. First, hippocampal postmortem samples from MDD

patients show reduced levels of BDNF. Second, an impaired signalling of BDNF within the hippocampus produces certain depression-related behaviours such as anhedonia (Monteggia et al., 2007), and impairs the actions of antidepressants. Lastly, experimental increment of the hippocampal BDNF level produces antidepressant-like effects (V. Krishnan & Nestler, 2010; S. Lee et al., 2010). Furthermore, several studies report an antidepressant-induced increase in BDNF expression in various brain regions including PFC and hippocampus (Palazidou, 2012) after chronic administration of antidepressants. Chronic treatment with antidepressants produce changes in BDNF expression which parallels the aforementioned time delay of up to six weeks to gain an effect from the treatment (Lanni et al., 2009), indicating that BDNF may oppose the effects of chronic stress and might be critical for therapeutic recovery (Balu et al., 2008; Duman & Monteggia, 2006).

Besides acting as a neurotrophic factor in the brain, BDNF is suggested to modulate other neuronal signalling molecules including the monoamine, amino acid, and peptide neurotransmitters (Szapacs et al., 2004). More specifically, BDNF has been suggested to augment serotonergic neurotransmission. Infusion of BDNF directly into the brain is known to influence the survival and function of serotonergic neurons and potentiate activity-dependent release of serotonin, however, the molecular mechanisms of which BDNF modulate the serotonin system are yet unknown (Szapacs et al., 2004). Even though several studies show a close relation between changes in neurotrophic factors and depression, there exist likewise several discrepancies among results from preclinical studies which have failed to show these patterns of changes induced by stress and by antidepressants, or have shown the opposite effects. This emphasises that the neurotrophic hypothesis is too simplistic to fully explain the pathology of MDD (V. Krishnan & Nestler, 2008).

The Neuroinflammatory Hypothesis

Immunological mechanisms have also been implicated in the pathophysiology of depression. This association is based on the observations that proinflammatory cytokines elicit sickness behaviour, which resembles symptoms of MDD including anhedonia, alterations in mood, fatigue, lethargy and cognitive deficits (Chopra et al., 2011). In addition, symptoms of depressive illness are recognised adverse events in patients receiving treatment with interferon. In severe cases of MDD an activation of the immune system can occur with a concomitant increase in cytokine concentrations. This activation may affect the function of other systems believed to be implicated in the pathogenesis of MDD. It is suggested that an increased proinflammatory cytokine concentration causes peripheral tryptophan (the precursor of serotonin) depletion, influences noradrenergic activity, and stimulates the HPA axis (Palazidou, 2012). One possible explanation for this HPA axis regulation is that chronic exposure to cytokines, due to chronic physical illness or chronic stress, may impair the glucocorticoid receptor function and thereby affect the negative feedback regulation of the HPA axis. This increases the chance of developing glucocorticoid resistance, which subsequently may cause further increase in inflammation. Finally, cytokines have been suggested to diminish the neurotrophic support and decrease the level of hippocampal neurogenesis (Miller et al., 2009; Pace, Hu, & Miller, 2007).

The complexity of MDD becomes evident from the diverse hypotheses described above. As previously mentioned, none of the established hypotheses are able to explain the exact inter-

play between the various pathological changes observed in MDD patients or in animal models of depression. A combination of the different hypotheses demonstrates a dynamic interaction between different molecules, pathways and systems, where dysfunctions can lead to depression, and antidepressant treatment may normalise function leading to recovery. This emphasises the need for the discovery of yet unknown factors, which could improve our understanding of this complex disease. However, the involvement of different brain regions further complicates this matter. Brain imagining technology has shown that various parts of the brain elicit different levels of activity and structural changes in the depressed state. These brain structures are mostly involved in emotional stability and regulation of neurotransmission, autonomic and neuroendocrine function. Communication within this neurocircuit and its regulatory effects on other parts of the brain is yet far too complex for a comprehensive mapping. Nevertheless, a large body of evidence suggests that the cortex of the anterior frontal lobe termed the PFC play a central role in the pathophysiology of MDD (Palazidou, 2012; Shansky & Morrison, 2009).

Prefrontal Cortex and Depression

The PFC has long been a central area of depression research presumably due to its role in cognition. Dysfunction of the PFC and the circuitry associated with it, is assumed to partly underlie the impairment of cognitive function and executive processes, including working memory and decision-making which constitute common symptoms of MDD (Koolschijn, van Haren, Lensvelt-Mulders, Hulshoff Pol, & Kahn, 2009; Palazidou, 2012). Symptoms such as rumination, poor concentration, and negative affect also suggest a PFC dysfunction in MDD patients (Shansky & Morrison, 2009). PFC is necessary for the generation of emotions and decreased activity within this area has been associated with anhedonia (Der-Avakian & Markou, 2012) and psychomotor retardation. In addition, PFC is involved in the regulation of autonomic and neuroendocrine responses, pain modulation, aggression, sexual, and eating behaviours; all observed to be dysregulated in MDD (Palazidou, 2012). Furthermore, the PFC contains high levels of glucocorticoid receptors and regulates HPA activity under behaviourally stressful conditions, which further emphasises a possible association between a dysfunctional PFC and MDD pathology (Radley et al., 2006).

Structural Alterations

Both postmortem and functional imaging studies of patients with MDD implicate areas within the PFC as sites of abnormal structure and function, suggesting a potential vulnerability to stress in this region. Specifically, *in vivo* human brain images of this area have demonstrated altered function in depression with significant reduced activity and decreased PFC volume (Fitzgerald, Laird, Maller, & Daskalakis, 2008; Juckel, Mendlin, & Jacobs, 1999; Sibille et al., 2004). Consistent with this, postmortem studies show abnormalities in PFC cell morphology of MDD patients with reductions in the density and size of both neurons and glia (Banasr & Duman, 2008; Cotter et al., 2002; Cotter, Hudson, & Landau, 2005; Stockmeier & Rajkowska, 2004). Similar findings are observed in animal models of depression, showing evidence of stress-induced atrophy of PFC neurons. This includes a decrease in the density of spines, as well as a decrease in the number and length of dendrite branches, which might explain the reduction in PFC volume (Brown, Henning, & Wellman, 2005; Radley et al., 2006; Shansky & Morrison, 2009).

A study by Radley et al. 2006 observed a stress-induced reduction of one-third of all axospinous synapses on apical dendrites of the medial PFC pyramidal neurons (layer II/III). Moreover, a significant decrease of 16% in apical spine density, and a reduction of 20% in total apical dendritic length in medial PFC pyramidal neurons were found in this study. It is suggested that loss of PFC dendritic spines may not only produce a decrease in the total population of axospinous synapses, but may also impair the capacity for biochemical compartmentalisation and plasticity, in which dendritic spines play a major role. Dendritic atrophy and spine loss may account for important cellular features of stress-related MDD (Radley et al., 2006).

Glial Cells

An involvement of glial cells in MDD pathology have been suggested based on post-mortem studies from MDD patients which showed both reduced number and altered morphology of glial cells most prominently within the PFC compared to other brain regions. Recent studies provide evidence that stress exposure may induce some of the reported glial cell pathologies. This has been demonstrated by decreased glial density in the hippocampus and reduced density of astrocytes in the PFC in animals exposed to chronic stress. These results suggest that deficient or compromised glial function in the PFC may contribute to the symptoms of depression, and this may result from impaired gap junctional intercellular communication in astrocytes resulting in altered neuronal function within the PFC. This could possible lead to malfunction in cortical and subcortical circuits closely connected to the PFC, contributing to MDD pathology (Sun, Liu, Yuan, Li, & Chen, 2012).

The Limbic System

PFC is closely connected to the limbic system and this interconnection creates a neuronal network known to play an important part in emotional behaviour (Fig. 2). This network includes the cingulate cortex, hippocampus, hypothalamus, amygdala, and the nucleus accumbens (Berton & Nestler, 2006; Morgane, Galler, & Mokler, 2005). Within this network, PFC and hippocampus may mediate anhedonia (Der-Avakian & Markou, 2012), memory impairments, feelings of worthlessness, hopelessness, guilt, and suicidality. Nucleus accumbens and amygdala might be important for emotional memory, anxiety, and reduced motivation. Finally, the hypothalamus may play a role in neurovegetative symptoms of depression such as regulation of sleep, appetite, energy, and sexual behaviour (Berton & Nestler, 2006). Both structural and functional abnormalities associated with MDD have been identified in most of these areas (Koolschijn et al., 2009; Marchand & Dilda, 2005).

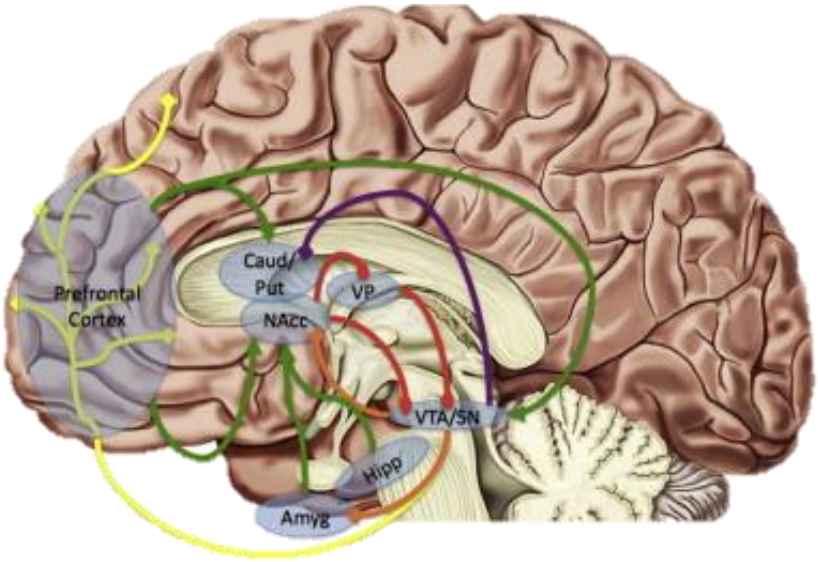

Fig. 2. Shows a sagittal section of the human brain with a schematic illustration of neuronal projections between the prefrontal cortex and limbic structures (Amyg, amygdala; Caud, caudate; Hipp, hippocampus; NAcc, nucleus accumbens; Put, putamen; SN, substantia nigra; VP, ventral pallidum; VTA, ventral tegmental area) (modified from (Treadway & Zald, 2011)).

Innervation between the brain areas of the PFC-limbic neurocircuit is largely monoaminergic, GABAergic or glutamatergic. An association between dysfunctions of these neurotransmitter systems and abnormal cognitive processing of emotional stimuli has been suggested (Eriksson et al., 2012; Sarter, Bruno, & Parikh, 2007). This is illustrated in different studies of MDD affected individuals showing that functional impairments of PFC-dependent cognition may be induced by stress-related alterations in noradrenergic and dopaminergic inputs. This was suggested to reflect local post- or presynaptic changes specific to the cells that are the source of these projections (Liston et al., 2006). Furthermore several post-mortem studies have documented a serotonergic dysregulation in PFC in depressive patients (Klempan et al., 2009; Puig & Gulledge, 2011; Savitz, Lucki, & Drevets, 2009). The prominent innervation by serotonin neurons and the dense expression of serotonergic receptors in the PFC suggest that serotonin is a major modulator of its function (Puig & Gulledge, 2011). Moreover, tryptophan depletion induces relapse of depressive episodes correlating with decreased prefrontal cortical activity (Bremner et al., 1997). However, despite decades of research the role of serotonin in PFC function is still largely unresolved (Palazidou, 2012; Puig & Gulledge, 2011).

It is hypothesised that PFC has a significant inhibitory regulatory effect on the limbic structures, which becomes compromised in the depressed state and disrupting the balance between the structures within the neurocircuit, probably as a result of decreased activity in the PFC. This dysregulation may be responsible for clinical symptoms such as altered behaviour and cognition and the associated neuroendocrine, neurotransmitter, autonomic and immunologic disturbances characterised for MDD. Antidepressants increase monoaminergic neurotransmission and BDNF concentrations and reverse some of the structural changes, and have a beneficial modulatory effect on the disrupted PFC-limbic neurocircuit. Collectively, these functions underscore the role of PFC in shaping responses to salient environmental events during stressful situations. However, further research must be conducted to establish specific factors linking stress-induced alterations of the PFC with MDD development (Palazidou, 2012).

Modelling Major Depressive Disorder

Investigating molecular changes in different brain areas associated with MDD require availability of brain tissue, which obviously cannot readily be obtained from patients diagnosed with the disease, emphasising the great advantages of applying animal models of depression in this setting. Animal models pose the possibility to monitor both *in vivo* and *in vitro* neurobiological changes in brain tissue with accessibility from a large population of animals within a controllable environment. Observations from such models constitute a useful complementation to human brain imaging and post-mortem studies investigating MDD aetiology and treatment. Second, animal models of depression provide the ability to investigate the action of antidepressants and to test novel ones, which is not possible in healthy animals, as antidepressants do not have any effect on mood in this group (Kellner et al., 2008).

The Chronic Mild Stress Model of Depression

It is difficult to develop an animal model of depression that perfectly reproduces all symptoms of the disease. Self-consciousness and self-reflection are believed to be unique to human cognition, hence, symptoms such as decreased self-esteem, depressed mood, negative thinking and suicidality are impossible to monitor in an animal model. However, animal models of depression offer the possibility to model certain features of the disease. Anhedonia, which is one of the core symptoms of MDD (Chopra et al., 2011), is mimicked in the Chronic Mild Stress (CMS) Model of depression. A stress-induced development of anhedonic-like behaviour is monitored by measuring the animal's sucrose consumption during periods of chronic mild stress and chronic antidepressant treatment. A reduction in sucrose intake is indicative of a stress-induced decrease in sensitivity to reward, since palatable sweet solutions are considered to have rewarding properties and CMS is known to suppress sucrose consumption. This diminished sensitivity to reward is suggested to model anhedonia observed in the clinic (Paul Willner, 2005). A valid animal model of depression must exhibit behavioural features of depression, should model a core symptom, employ realistic inducing conditions and finally respond to antidepressant medication. Three major criteria have been translated from these conditions:

1) **Face validity**: how well the model resembles symptoms of the condition or disease
2) **Construct validity**: (also known as the aetiological validity) how well the model simulates symptoms caused by the same aetiology that trigger the condition or disease in humans.
3) **Predictive validity**: how well the model responds favourably to clinically established drugs

These parameters are used for the validation of animal models to ensure a more general conclusion to how well the model can function as simulations for investigating the psychobiology of depression and as an antidepressant screening test. The CMS model of depression fulfils all of these three criteria (P Willner, 1997).

Face Validity

The primary behavioural readout from the CMS model of depression is anhedonia, which is one of the core symptoms in clinical MDD. Furthermore, several subsidiary symptoms and parameters associated with depression have been induced by CMS including increased activity of the HPA axis (Konkle et al., 2003), loss of body weight (P Willner, Moreau, Nielsen, Papp, & Sluzewska, 1996), decreased sexual and aggressive behaviours (D'Aquila, Brain, & Willner, 1994), decreased locomotor activity (Gorka, Moryl, & Papp, 1996), disturbances of diurnal (D'Aquila, Newton, & Willner, 1997), and circardian rhythms (J.-L. Moreau, Scherschlicht, Jenck, & Martin, 1995), and immunological dysfunction (Grippo, Francis, Beltz, Felder, & Johnson, 2005).

Construct Validity

The rationale is that exposure to stress induces anhedonia and rests on two assumptions: First, the CMS causes a generalised decrease in reward sensitivity. Second, the sucrose intake is a valid measure of reward sensitivity (P Willner, 1997). This has been validated by the observations that CMS does not affect the animal's thirst of normal drinking water (Muscat, Papp, & Willner, 1992). Additionally, a two-bottled preference test (sucrose and water) confirmed that the level of sucrose consumption is a preference phenomenon rather than an issue of thirst (P Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). The decline in sucrose consumption is unrelated to the caloric content of sucrose, shown by a similar reduction in the intake of a calorie-free saccharin solution. Moreover, reductions in sucrose consumption were observed in both food-deprived and non-deprived animals (P Willner, Muscat, & Papp, 1992). Both water and food intake was not decreased by CMS (Muscat et al., 1992). Finally, animals exposed to CMS show increased thresholds in intracranial self-stimulation (ICSS) (J. L. Moreau, Jenck, Martin, Mortas, & Haefely, 1992; J.-L. Moreau et al., 1995). ICSS is another measure for reward sensitivity, and is a powerful technique because it reflects the direct activation of the brain reward system (Carlezon & Chartoff, 2007).

Predictive Validity

A wide range of antidepressants have been tested in the CMS model. Different types of antidepressants shown to be effective in reversing CMS-induced anhedonia include tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRI´s), and noradrenaline reuptake inhibitors (P Willner, 1997). Moreover, electroconvulsive treatment (ECT) has been shown to restore normal responsiveness to reward (J.-L. Moreau et al., 1995). Litium and carbamazepine, which is lesser conventional antidepressants but clinically effective drugs, are shown to reverse CMS-induced anhedonia (Papp, Moryl, & Willner, 1996). A time delay of 3-5 weeks of treatment to gain full recovery of CMS-induced anhedonia is observed, which is compatible with the clinical time course observed for MDD patients. In addition, unchallenged control rats do not gain any effect by antidepressant treatment (P Willner, 1997). Non-antidepressants such as neuroleptics, anxiolytics, amphetamine and morphine have no effect on CMS-induced decrease in sucrose consumption (Muscat et al., 1992; Papp et al., 1996; P Willner, 1997).

All together, these observations indicate that the CMS model of depression is a valid model for investigating the pathophysiology of MDD as well as to investigate potential novel antidepressants (Hill, Hellemans, Verma, Gorzalka, & Weinberg, 2012).

Biomarkers of Depression

As mentioned previously, no specific biologic pathways or marker(s) for MDD have yet been identified and approved for clinical use despite extensive research efforts aimed at understanding the neurobiological underpinnings of the disease. Diagnosis and the evaluation of treatment are therefore based solely on relatively subjective assessments of symptoms (Domenici et al., 2010). A biomarker is a measurable feature of an individual that represents indicators of a disease, or outcome of treatment. Biomarkers can provide a basis for the selection of lead candidates for clinical trials, for contribution to the understanding of the pharmacology of candidates, and for characterisation of the subtypes of disease for which a therapeutic intervention is most appropriate (Atkinson et al., 2001). Biomarkers often have a biological feature, e.g., genome variation and protein variation (Schmidt, Shelton, & Duman, 2011). Identifying biological markers of MDD and treatment response would make a significant difference in the establishment of more precise disease diagnoses, eliminating subjectivity and reliance of patient's self-report of symptoms, and in the development of improved antidepressant treatment. Biomarkers often have a biological feature, e.g., genome variation, protein variation etc. (Schmidt et al., 2011).

In molecular terms, proteins are likely the most ubiquitous molecules affected in disease, they are target for treatment response and recovery, and can be isolated directly from the affected organs or cells. As brain biopsies are not readily accessible for clinical diagnosis of MDD, an alternative approach is the use of discovery-based analysis of protein abundance using animal models or human post mortem brain tissue. Subsequently, results from this may be translated to biological fluids such as blood, urine and cerebrospinal fluid (CSF). This is of special interest in the search for potential biomarkers because of the ease in accessibility and obtainability by minimal invasion as well as low cost compared to tissue samples obtained from biopsies or surgical removal. Additionally, these fluids exchange important protein components with several organs and tissues in the body (Thongboonkerd, 2008).

Several studies have searched for specific MDD biomarkers based on the hypothesis of monoamine dysfunction, the neuroendocrine, and the immune-inflammatory hypothesis. These investigations mostly relied on the selection of single markers, which have generated a number of putative biomarkers, however, require replication in larger studies. Due to the aetiological heterogeneity and the overlap of dimensions across mood disorders, it has been speculated that individual biomarkers cannot pose a specific or applicable MDD marker. Therefore, application of large-scale profiling approaches can depict more accurately behavioural phenotype changes and thus may provide more promising results (Domenici et al., 2010). Quantitative, high-throughput, non-hypothesis driven experimental approaches constitute a valuable approach to identify panels of candidate biomarkers. Simultaneous analysis of the global protein expression of brain tissue from post-mortem MDD patients or animal models of depression can be successfully applied to identify altered protein signatures applicable for MDD diagnosis and disease/treatment monitoring. Proteomic-based methods are well suited for this

purpose as it enables simultaneous detection of hundreds of proteins with high sensitivity and accuracy (Filiou, Turck, & Martins-de-Souza, 2011).

Proteomics and Depression

Proteomics refers to a systematic analysis of all expressed proteins within a cell or tissue. The proteome defines the pool of proteins encoded by the genome of an organism at a specific point in time, including the set of isoforms and post-translational modifications. This gives proteomic research a great advantage of revealing the full variety of gene expression, therefore facilitating more closely reflections of pathophysiological processes underlying the clinical phenomenology of MDD as compared to genomic approaches. Proteomics provide the chance to determine the whole proteome in a given tissue without a priori assumption about candidate molecules. This research method may contribute to the discoveries of biomarkers of MDD by comparing protein expression in patients and control subjects as well as patients receiving different therapeutic interventions. Proteomic methods based on mass spectrometry are relatively new research approaches within psychiatric research and neuropharmacology, and are expected to gain crucial significance within this field over the coming decade (Taurines et al., 2011).

Mass spectrometry (MS) is one of few methods that are capable of detecting proteins in a high throughput manner and is therefore a widely utilised tool for profiling proteomes. One standard approach is separation of proteins or peptides by Liquid Chromatography (LC) columns, and then detection, identification and quantification by MS. Peptides can be chemically labelled by isobaric tags for relative and absolute quantification (iTRAQ). This labelling method allows simultaneous determination of both identity and relative abundance of peptides in tandem mass spectra and has gained popularity due to its high sensitivity and robustness, and because it allows simultaneous measurement of up to eight samples (Bantscheff et al., 2008). The great number of proteins expressed by cells, however, challenges this methods' ability to separate and identify all proteins in the sample. Additionally, the detection of low abundant proteins that mediate key pathways affected in MDD (i.e. neurotransmitters, neurotransmitter receptors and transcription factors) can be masked by the more abundant proteins such as cytoskeletal proteins (Filiou et al., 2011; Taurines et al., 2011). The digestion of proteins to peptides which is required for iTRAQ labelling further increases the complexity of the sample (Voshol, Glucksman, & van Oostrum, 2003). A strategy to overcome this problem is to reduce the complexity of the proteome, prior to protein digestion and labelling, by subcellular fractionation of the sample examined (Mallei et al., 2011).

Proteomic Research Using Synaptosomes

One subcellular fraction, termed synaptosomes, is produced by differential centrifugation of homogenised brain tissue and consists of nerve terminals isolated within a membranous sac containing most of the synaptic material of the *in vivo* nerve terminals. These reconstructed nerve terminals enclose the machinery to produce ATP, enzymes and proteins and synaptic vesicles that can take up and release neurotransmitters. Furthermore, their plasma membranes contain functional ion channels, carriers and receptors, which allow them to maintain membrane potentials and ion homeostasis (Mallei et al., 2011). Therefore, synaptosomes are

enriched with proteins involved in synaptic function and thus an ideal tool to investigate the synaptic proteome which is critical to understand complex synaptic dysfunctions related to depressive phenotypes. Synaptic proteins represent many human disease genes and drugs targets, including targets for antidepressant treatments. Aberrations in synaptic transmission associated with MDD (Crisafulli et al., 2011; Duric et al., 2012), potentially involve extensive regulation of the synaptic proteome, and thus provide important information regarding depression pathology.

Aim of Project

Through several years of research, a large body of evidence indicates that the PFC plays a significant role in the pathophysiology of MDD. However, due to the complexity of the disease, the molecular mechanisms and their multifaceted interactions in the PFC, and other important brain regions leading to depression, are yet far from elucidated. Thus, it is of great importance to identify biomarkers that have the potential to classify different pathological findings into more homogeneous groups, enabling MDD diagnosis and treatment based on both molecular and symptomatic findings.

The aim of the present study was to identify potential protein biomarkers for stress-induced depression by analysing the global protein expression of the synaptic proteome within the PFC, in the search for novel disease targets. Furthermore, the study included a search for biomarkers involved in stress-resilience in order to investigate the molecular background of this important phenotype. The highly validated CMS model of depression was used in the present study to investigate the involvement of different protein pathways involved in anhedonia, as opposed to general stress-effects, as well as stress-coping strategies by including the stress-resilience phenotype. Proteomic methods based on mass spectrometry hold special promise for the discovery of novel biomarkers of MDD, and subproteomics has emerged to reduce sample complexity and potentially increase the information yield, characterising enriched fractions of synaptic proteins such as synaptosomes (Mallei et al., 2011). In the present study iTRAQ labeling coupled to tandem mass spectrometry provided basis for the non-hypothesis driven, large-scale analysis of PFC synaptosomes (synaptoproteomics), facilitating an unbiased approach for novel biomarker research among stress-susceptible and stress-resilient animals. To my knowledge, this is the first study investigating the PFC synapse proteome in the CMS model of depression.

The PFC show profound alterations in response to stress and is closely involved in cognitive function such as working memory (Holmes & Wellman, 2009), thus providing important information regarding changes in both global protein expression and working memory task performances in response to CMS. A pilot study was conducted to apply a PFC-dependent behavioural test, the odour span task which assesses working memory. The aim was to grade performances among the three experimental groups in order to investigate the effect of CMS on PFC function, and subsequently couple these results with findings of the synaptoproteome study. To my knowledge, no other studies have previously applied the odour span task to test working memory in the CMS model of depression.

Experimental Procedure

Animals

6-7 weeks old male Wistar rats were purchased from Taconic M&B, Denmark. The animals were singly housed, food and water was available *ad libitium*, and the animals were kept on a standard 12-h light/dark cycle except when one of these parameters was changed according to the stress regime. All procedures involving animals were accepted by the Danish National Committee for Ethics in Animal Experimentation (2008/561-447).

Sucrose Consumption Test

Animals were adapted for five weeks to consume a palatable sucrose solution (1.5%). In this period, the sucrose test was performed twice a week during the first three weeks and once a week during the last two weeks. The animals were food and water deprived 14 hours prior to the sucrose test. The test consisted of a one hour period of free access to a bottle of the sucrose solution. The sucrose consumption was measured by weighing the bottles pre- and post-testing. Baseline sucrose consumption was calculated for each animal and is defined as the mean sucrose consumption from the last three sucrose tests prior to stress start. During the stress period, the sucrose consumption test was performed once a week.

Chronic Mild Stress

Based on the baseline sucrose consumption animals were divided into two matched groups, control and CMS, and placed in separate rooms. The CMS group was exposed to eight weeks of chronic mild stressors. Briefly, this procedure consisted of seven different stressors and lasted from 10 to 14 hours. The mild stressors consisted of interchanging periods of intermittent illumination, stroboscopic light, grouping, food and/or water deprivation, soiled cage, 45° cage tilting and no stressors according to the week schedule illustrated below (table 1). The control group was left unchallenged except for 14 hours food and water deprivation before sucrose consumption test.

Time of day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning	Intermittent illumination	Water deprivation	Stroboscopic light	No stress	Sucrose test	Food deprivation	Cage tilting
Afternoon	No stress	Cage tilting	Soiled cage	Water /food deprivation	Grouping	Cage tilting	Soiled cage

Table 1. The chronic mild stress regime. The stressors are applied according to this weekly schedule during the entire regime.

Grouping consisted of animals being housed with different partners, with the individual rat alternatively being a resident or an intruder. Following exposure to stress for four weeks, rats were categorised as anhedonic-like (defined as a > 30% within-subject decrease in sucrose intake) or stress-resilient (defined as a < 10% within-subject decrease in sucrose intake). Rats with a sucrose consumption not corresponding to either category were excluded from the experiment.

Tissue Preparation

Three groups of animals; control (n=9), anhedonic-like (n=9) and stress-resilient (n=9), were decapitated and brains were dissected with immediate isolation of the PFC. This was performed by removing the olfactory bulb and making a coronal section of the brain to isolate the PFC according to Paxinos and Watson, 1998 (Paxinos & Watson, 1998). Immediately hereafter the tissue was snap-frozen on dry ice and stored at -80 ^{o}C until further processing. Within each group three PFCs were pooled resulting in three subgroups; control (n=3), anhedonic-like (n=3) and stress-resilient (n=3), to ensure sufficient amounts of tissue sample for synaptosomal fractionation.

Synaptosome Preparation

Isolation of synaptosomal fractions was performed by centrifugation through four Percoll gradients according to the method developed by Peter Dunkley, Neil Sims and Mary McKenna (Peter R Dunkley, Jarvie, & Robinson, 2008a; Sims & Anderson, 2008), adapted by Katarzyna Kulej et al. for whole brain preparation applied in the present study with minor modifications for fractionation of PFCs.

The three pooled PFCs were homogenised in 5 mL ice cold 0.32 M sucrose buffer [1.0 mM EDTA, 5.0 mM Tris-HCL pH 7.4, complete EDTA-free protease inhibitor cocktail tablet, 0.28 mM DTT], using a prechilled 15-ml Potter-Elvehjem type Teflon-glass tissue grinder (19x84mm, chamber clearance 0.1-0.15 mm) and electrical laboratory stirrer at 700 rpm/min. The homogenate was centrifuged at 3200 rpm (800 x g) for 10 minutes at 4^{o}C to remove nuclei and cell debris as a pellet, leaving the bulk of the synaptosomes in the supernatant. The supernatant was collected and 500 µL, termed *total protein*, was stored at -80^{o}C for Western blot analysis. The reminder, approximately 4 mL, was equally divided and loaded on top of two discontinuous Percoll gradients (fig. 3A). From the top down, the Percol gradient comprised 2 mL each of 3%, 10%, 15% and 23% Percoll (vol/vol) in 1.28 M sucrose buffer [4 mM EDTA, 20 mM Tris-HCL, pH 7.4]. The gradients were centrifuged at 20,000 rpm (31,400 x g), 4 ^{o}C for 5 minutes (excluding time for acceleration and deceleration time) using a Sorvall Evolution RC Superspeed Centrifuge (Thermo Scientific) with a SA300 rotor head, producing four visible fractions in the interfaces between the Percoll layers termed (from top down): F2, F3, F4 and F5 (fig. 3B). According to the literature an additional fraction F1 will appear between the interfaces of 0% and 3% Percoll if the tissue/buffer-ratio is higher and contains mostly membranes. Synaptosomes can be obtained from each fraction, however most viable and homogeneous synaptosomes are located in F3 and F4. F2 is enriched by damaged synaptosomes and glial material and contain mostly myelin, membranes, and membranous vesicles. F3 contains pre-

dominantly sealed synaptosomes with synaptic vesicles and few mitochondria and contains numerous membranes and membranous vesicles. F4 mostly contains large diameter synaptosomes with numerous synaptic vesicles and higher mitochondrial content and contains as well few extrasynaptic mitochondria. F5 contains predominantly extrasynaptic mitochondria (P R Dunkley et al., 1988; Peter R Dunkley et al., 2008a; Harrison, Jarvie, & Dunkley, 1988). Each faction was carefully collected using a disposable glass Pasteur pipette and washed twice to remove Percoll by centrifugation at 16.000 rpm (20,000 x g) for 15 minutes in 8 mL ice cold 0.32 M sucrose buffer [1.0 mM EDTA, 5.0 mM Tris-HCL, pH 7.4], leaving the fraction as a pellet in the bottom of the tube. The two corresponding fractions produced from each homogenate were pooled and stored at -80 ^{o}C until further processed.

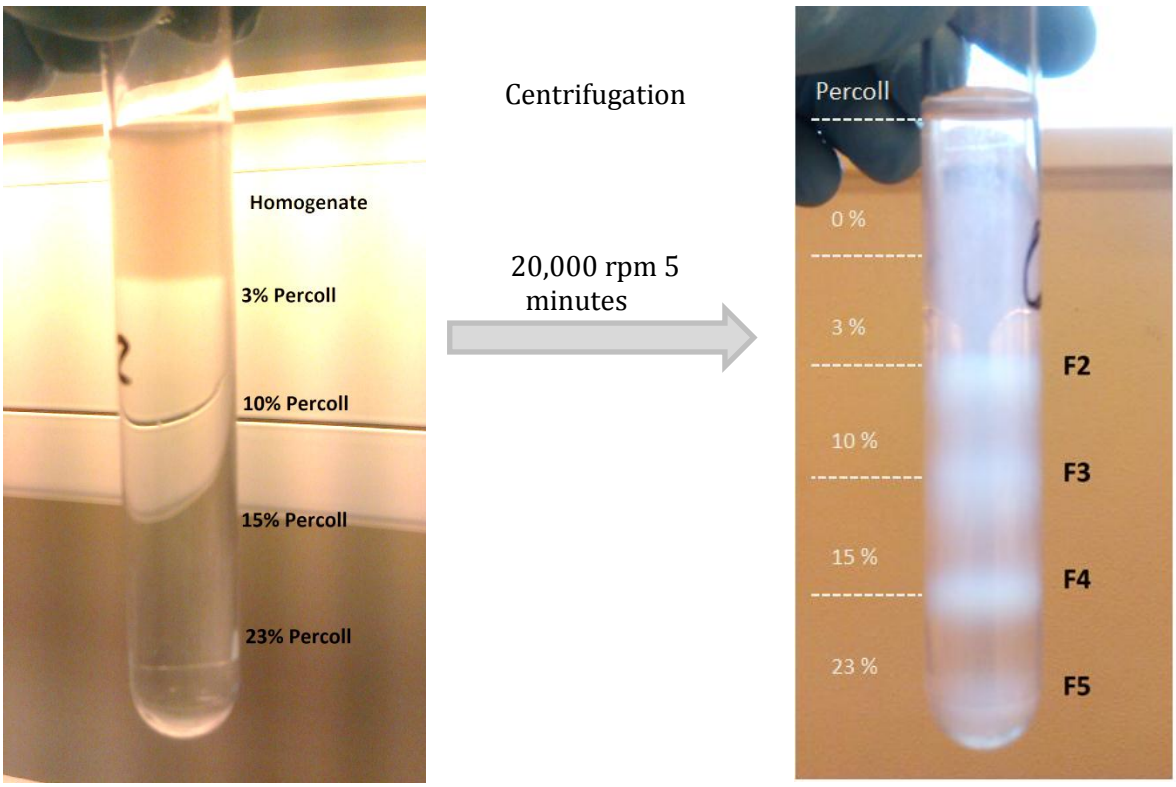

Fig. 3. A) Discontinuous Percoll gradient consisting of four different concentrations of Percoll (3, 10, 15 and 23 %) layered on top of each other. The homogenised prefrontal cortices were layered on top of the gradient and centrifuged to produce four fractions. B) The four fractions are located in the interfaces of the different Percoll gradients.

Comparison of Percoll Fractions – A Pilot Study

Two pilot studies were conducted to make a quantitative comparison of the synaptic protein abundance among the collected Percoll fractions. Three PFC's isolated from three rats excluded from the CMS regime were homogenised and fractionated according to the method described in the previous section (*Synaptosome preparation*). Protein concentration for each of the four collected fractions was determined by the Bradford assay (Bio-Rad Laboratories). Western blot analysis was performed on total protein, F2, F3, F4 and F5 samples using antibodies for detection of the pre- and postsynaptic protein markers, syntaxin2 and PSD-95, respectively. This was conducted to confirm that synaptic proteins were up-concentrated in the

sub-fractions by use of the Percoll gradient as compared to the homogenate sample (total protein). Subsequently, a large-scale protein abundance comparison between F2, F3 and F4 was performed using proteomics. According to the literature these three fractions contain the highest content of synaptosomal material (P R Dunkley et al., 1988; Peter R Dunkley et al., 2008a; Harrison et al., 1988). iTRAQ labeling and quantitative nanoLC-MS/MS separation and identification was conducted and analysed for a representative selection of synaptic proteins and proteins involved in different pathways associated with the synapse (determined according to biological categorisation of identified proteins using Panther Pathway software (Pantherdb.org). The method applied in this pilot study (Pilot Study 2) is described in detail later on (*Synaptoproteomic Analysis)*.

Pilot Study 1 - Western Blot Analysis

5.2 µg protein for each of the fractions F2, F3, F4 and F5 was lysed in 6x sample buffer [0.09% bromophenol blue, 0.09% xylenecyanol FF, 60% Glycerol] for 15 minutes at 65° C and loaded on a Criterion TGX AnykD Gel (Bio-Rad Laboratories) for separation. For western blotting, the proteins were transferred from gel to PVDF using Bio-Rad Trans-Blot Turbo equipment and Trans-Blot Turbo Transfer Pack (Midi Format, 0.2 µm PVDF). The transfer was performed using preprogrammed protocols at 2.5A and 25V in 7 min. The PVDF membrane was blocked for 1h in a 5% skimmed milk solution [5% dry powered non-fat milk, PBS with 0.1 % Tween 20 (TBS)] at room temperature and incubated overnight with primary antibody diluted 1:2500 in TBS: rabbit Syntaxin-2 (Abcam), rabbit PSD-95 (Abcam) and mouse β-actin (Santa Cruz). The membranes were washed in TBS and incubated for 1h with the secondary antibody diluted 1:10,000 in TBS. Secondary antibodies were HRP-conjugated polyclonal goat anti-rabbit or anti-mouse immunoglobulins (Dako). The membrane was washed in TBS and the blot signal was detected using ECL kit (Amersham Bioscience) and the chemiluminiscence was visualised on ImageQuant LAS 4000, and analysed in ImageQuant TL software (GE Healthcare).

Synaptoproteomic Analysis

The method applied for proteomic analysis of Percoll subfractions are described in the following and is equal for both pilot study 2 (quantitative comparison between F2, F3 and F4) and the synaptoproteomic analysis (quantitative comparison between the three experimental groups; anhedonic-like, stress-resilient and control). Only one factor differed between these two studies; the pilot study was conducted for one comparison between fractions producing one iTRAQ labeling series, whereas the comparison between experimental groups was conducted in triplicates producing three iTRAQ labeling series.

Both pilot studies indicated a higher yield of proteins associated with the synapse in F2 and was therefore initially chosen among the four Percoll gradients for the quantitative comparison between the experimental groups. Further details are described in results (Pilot Studies of Percoll Fractions).

iTRAQ labeling, IEF separation and Purification of Peptides

Protein concentrations were measured by the Bradford assay (Bio-Rad Laboratories) as recommended by the manufacturer. For each of the three fractions: F2, F3 and F4 in the pilot study, and for each of the three experimental groups, of each triplicate; anhedonic, resilient and control in the synaptoproteomic study of F2, 110 µg protein was processed and labeled with a specific iTRAQ reagent in accordance with the manufacturer's instructions (Applied Biosystems). In brief, proteins were precipitated in acetone x10 overnight and hereafter the proteins were denaturated, reduced and blocked at cysteine residues. Each protein sample was digested by trypsin 2 µg (Trypsin Gold, Promega) overnight at 37°C in iTRAQ sample buffer. Each peptide sample was labeled with one of the different iTRAQ labels termed 114, 115, 116 or 117. Tagging the different samples with these specific labels and subsequently mixing the samples enables relative quantification of the peptides in each digest. In the pilot study labeled peptides of the three fractions were combined in one series, e.g. F2 sample peptides labeled with 114, F3 peptides to 115 and F4 peptides to 116 combined and mixed in one tube. For the synaptoproteomics study labeled peptides were combined in three series, in such way that each series consisted of one of each experimental group; control, resilient and anhedonic (fig. 4). These series were purified on a Strong Cation Exchange (SCX) chromatography column, (Strata from Phenomenex). Before loading, 10 mM phosphoric acid with 25% acetonitrile (AcN), pH 3, was added by at least a factor ten to each sample, ensuring a low pH for optimal purification. This buffer was also used in the washing step. Peptides bound within the chromatography column were eluted with a buffer containing 5% ammonia and 30% methanol and subsequently vacuum-dried. The dried peptides were resuspended in rehydration buffer constituting 8 M Urea, 0.5% IPG buffer pH 3-10 (GE Healthcare), 2.5 mg/mL DTT, 0.002% bromophenol blue and rehydrated overnight.

The peptides were separated on an Immobiline Drystrip gel, pH 3-10 (GE Healthcare) using isoelectric focusing (IEF) on a Multiphor II unit (Pharmacia Biotech AB). IEF was run with the applied current set to a maximum of 5W and 2mA and the following program: 1 min gradient from 0-500V, 1.5h gradient 500-3500V, 16h at 3500V and 10 min gradient at 3500-800V. After separation, the gel strip was cut into ten equal sized pieces and the peptides were extracted from the gel in two steps, with a buffer containing 0.5% trifluoracetic (TFA) and 5% acetonitrile (ACN). Finally the peptides were purified on PepClean C-18 Spin Columns (Pierce, Rockford) according to manufacturer's protocol prior to nanoLC-MS/MS analysis.

Nano-liquid Chromatography and Mass Spectrometry Analysis

The peptides were separated by liquid chromatography (Easy nano LC from Proxeon) coupled to mass spectrometry (MS)(LTQ-Orbitrap, Thermo Fisher Scientific, Waltham, USA) through a nano-electrospray source with stainless steel emitter (Proxeon). The peptide-separation was performed on a reverse phase column, 75 µm in diameter and 100 mm long, packed with 3.5 µm Kromasil C18 particles (Eka Chemicals) at a flow of 300 nL/minute using a 100 minutes gradient of AcN in 0.4% acetic acid; starting with 5% and ending with 35% AcN. The mass spectrometry detection consisted of full scan (m/z 400–2000) with Orbitrap detection at resolution R = 60,000 (at m/z 400), followed by up to four data-dependent MS/MS scans with linear ion trap (LTQ) detection of the most intense ions. Dynamic exclusion of 25

sec was employed as well as rejection of charge state +1. Pulsed Q dissociation (PQD) fragmentation was performed with activation time of 0.1 sec, ion accumulation time of 140 ms, a target value of automatic gain control of 40,000, one microscan and activation Q of 0.7. For efficient fragmentation and detection of iTRAQ reporter ions, normalised collision energy of 33 was used.

Database Searches

The raw data files were processed using extract_msn.exe (Thermo Fisher Scientific, released 2/15/2005) to generate peak lists of the tandem spectra. The processed data were searched with Mascot from www.matrixscience.com (Matrix Science version 2.2.04), which was used for protein identification and iTRAQ reporter quantification. For the pilot study the ten different peptide fractions from IEF were MS-analysed and all generated peak lists were merged. For the synaptoproteomic study, the ten different peptide fractions from IEF, for each of the triplicate series, were MS-analysed. All generated peak lists from the same series were merged and searched against SwissProt_2011x database with taxonomy filter Rattus containing 7687 sequences, using the MudPIT scoring algorithm of Mascot. Full scan tolerance was 5 ppm and MS/MS tolerance was 0.75 Da. Setting of trypsin digestion was cleavage at C-terminal of lysine and arginine except before proline, and up to two missed cleavages were accepted. Fixed modifications were those originating from iTRAQ protocol: iTRAQ-4plex of lysine and N-terminal and methylthio modification of cysteines, whereas oxidation of methionine and iTRAQ-4plex of tyrosine were set as variable modifications. The significance level of protein identifications was set to 0.001, which resulted in a false discovery rate of less than 0.003 when searched in Mascot against the decoy database of random sequences. iTRAQ quantitation was performed in Mascot where normalisation to summed intensities was applied to compensate for possible variation in starting material. When identification of a protein yielded several possible protein isoforms, all of them were considered for quantification. For the pilot study iTRAQ-ratios between the three fractions were calculated using F4 as the standard (=100%). The relative values were used to compare the abundance of certain proteins among the three fractions. No statistical analysis was performed in the pilot study.

For the synaptoproteomic study stringent criteria' were set for the selection of data that accounted for the most reliably quantitative output: 1) iTRAQ values were reported for proteins with five or more measured iTRAQ scan values from peptides with expectation values of 0.02 or below 2) In the quantitative calculations, only protein isoforms with iTRAQ values in all three replicates were included. 3) Only proteins detected from at least two peptides were included in the statistical calculations. The iTRAQ-ratios between the three experimental groups were calculated for each protein using the average value obtained from the three biological replicates from each independent group. Ratios for each protein were reported as significantly different from 1.0 if they passed two tests: 1) a two-tailed student's t-test for equal variance, $p<0.05$ data and 2) a fold change ≥20%. False discovery rate (FDR) for differential expression was calculated to be approximately 11.3% using Benjamini and Hochberg's statistics with extra stringency from the fold change criterion (Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001).

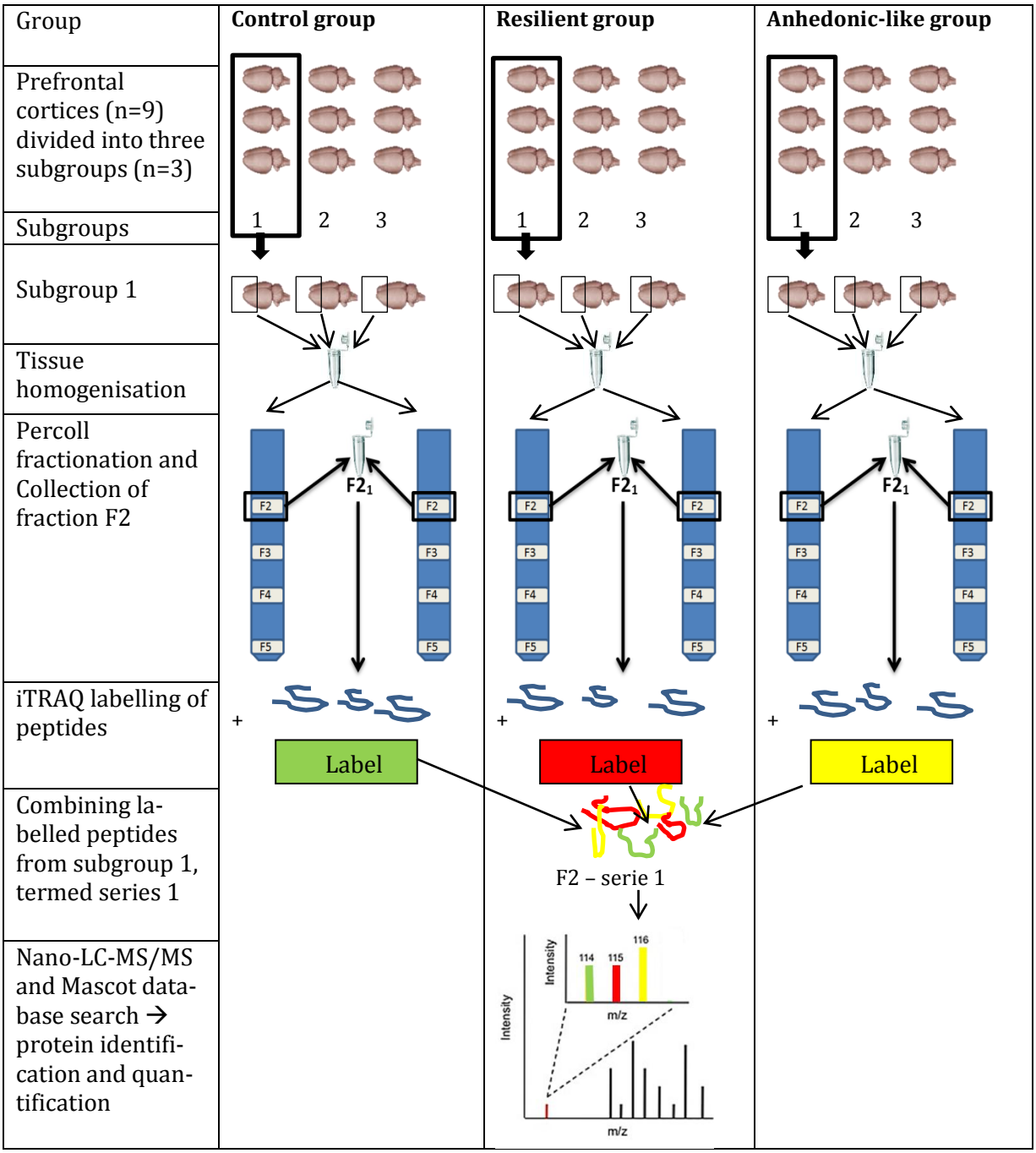

Fig. 4. Schematic illustration of the experimental setup for F2 synaptoproteomic analysis. The figure outlines the procedure for the first series consisting of the comparison between experimental groups of the first subgroup of three. This setup was repeated two times more using the last two subgroups of prefrontal cortices and was termed F2 - series 2 and F2 - series 3, respectively. iTRAQ quantitative data were based on the average value from the triplicate produced by the three series.

Cluster Analysis

A cluster analysis, performed for synaptoproteomic results, was used to separate the three experimental groups based on between-group similarities in global protein expression profiles. The clustering approach orders objects in a treelike structure and provides information about relations among groups. The cluster analysis was performed with Cluster software version 2.11. Hierarchical clustering was performed by clustering genes and arrays. Subsequently, the cluster analysis was visualised by Tree View software version 1.60 (http://rana.lbl.gov/EisenSoftware.htm).

Data Analysis and Statistics

Sucrose Consumption Test

A two-tailed student's t-test was used to analyse the overall stress-effect at the initial four weeks of the stress regime and was estimated by comparing all CMS exposed animals in the trial to all control animals. Weekly sucrose measurements for the graded stress response were analysed by multivariate analysis of variance (MANOVA). Group-wise comparisons for eight weeks of CMS were performed by Bonferroni post hoc tests to adjust for multiple comparisons. The statistical level of significance was set at $P<0.05$. SPSS Statistics Version 19 was used for the statistical analyses.

Synaptoproteomics Data Analysis

A two-tailed student's t-test for equal variance was applied to measure differences in the relative protein abundance between anhedonic-like, resilient and control animals. Data was based on iTRAQ quantitative data of triplicate values (from the three series) for each identified protein. Proteins that passed significance and fold change criteria were listed according to function and biological process determined through UniprotKB database (uniprot.org), pathway analysis (string.embl-heidelberg.de, pantherdb.org) and PubMed literature searches.

Behavioural Testing - Pilot Study

Apparatus and Materials

The odour span task applied in this study was modified from the method described by Young, J. W. et al. 2007 (Young et al., 2007). In brief, 19 different odours was used in the experiment (cinnamon, fennel, tarragon, clove, cumin, nutmeg, coriander, curry, thyme, dill, oregano, sage, lime leaves (crushed), rosemary, paprika, thyme, coffee powder, chamomile tea, cocoa (Santa Maria, Kilic, X-tra)), most of which was used in previous studies (Young et al., 2007), for which rodents showed no preference. Scented mixtures were prepared by mixing 0.5 mg of the individual odour with 100 mg fine-grained sand. 19 glass cups (seven cm in diameter and five cm high) were each filled with a different odour. Rats were given one session (and in special cases two) per day and each training session consisted of several trials (the amount depended of how quickly the animal completed each task). All behavioural testing was performed on a 90 x 90 cm square black platform elevated one meter above the ground. During each trial the

scented cups were placed randomly on the platform, ensuring that rats choose cups according to odour and not location. The behavioural experiment consisted of habituation, training, odour non-matching to sample training, no reward probe and the odour span task.

Habituation

Male Wistar rats (n=20), 10-15 weeks old, were used in this pilot study. Rats were randomly selected among control and CMS rats that had been excluded for further experiments and exposure to CMS based on sucrose data. A subgroup (n=10) was used for testing of different setups of the behavioural test and the second subgroup (n=10) was applied for optimisation and protocol design. The animals were singly housed and kept on a standard 12-h light/dark cycle and tested during the light phase of the cycle. During testing sunlight was blocked completely and an electrical dim light source was used instead. Water was available *ad libitum*. The animals were given 85% of their free feeding weight three days before and during behavioural testing. The rats were placed in the experimental room three days before the training sessions were initiated and during the remainder of the period to ensure habituation of the experimental settings. Habituation of the testing platform was applied by placing each rat on the testing plate for approximately 30 min per day. One cup filled with sand (no odour) and a food reward (either one Guldkorn (Valora Trade Denmark A/S) or half a Cherious weed loop (Nestle) laid on top was placed on the testing platform during the habituation sessions. If the rat consumed the food reward, it was replaced with a food reward covered half in sand. If the rat likewise consumed this, the next food reward was covered almost completely by sand in such way that only a small area of it was visual.

Training

Only rats that had consumed the visible food reward at least in two consecutive sessions during habituation were used in the training session. In the first day of this session rats learned to dig in sand (no odour) to retrieve food rewards by covering one food reward with sand in such way that the top was visible, and five food rewards hidden approximately one cm deep in the sand. The rat was left for 20 minutes to examine and consume the content in the baited cup and those rats that did not find any of the hidden food rewards were given an additional session later that day. The second day, the rats were introduced to scented cups containing half visible food reward and half hidden. When the animal retrieved and consumed the food rewards the cup was removed and a new cup was introduced to the rat containing a different odour and one hidden food reward. Training sessions lasted 20 minutes and was repeated until the rat learned to reliably dig in the cup.

The Odour Non-Matching to Sample Test

In this session rats was trained on a non-matching to sample (NMS) test. In each trial the animal was presented with a scented cup containing ½ food reward hidden in the sand. After the animal had retrieved the food reward the same cup and one new cup with a different odour was presented at the plate simultaneously. This time the food reward was hidden only in the new cup. This test is required to generate a working memory span capacity, whereas matching to sample only requires rats to remember an individual odour, and therefore only assess olfac-

tory discrimination, not memory. This session was performed as many times as possible for 20 minutes. The session was performed until the rat reliably selected the new odour (75% correct responses for two consecutive days). During the training sessions rats should be introduced to all 19 different odours.

The No-Reward Probe Test

To test for the possibility that animals may have used the scent of the reward or marked cups or sand, the no reward probe was tested. This was performed in continuation of the NMS applied only for those rats that successfully chose the new odour in three or more consecutive trials. In this test odours that had not been used in the previous NMS was used. The test was equal to the NMS except for the newly introduced cup did not contain a hidden food reward. Instead, if the rat successfully dug in the new cup, the food pellet was dumped on top of the sand by the experimenter and the rat could consume its reward. This was performed five times and the performance noted. Changing in behaviour e.g. the rat did not show interest in the cups presented in this test would indicate that the food reward was not masked sufficiently by the sand in the former test, and alternatives should be considered. This could be testing another type of food reward or mixing the scented sand with the food reward that had been crushed into fine grains.

The Odour Span Task

After acquisition of the non-matching to sample, animals were introduced to the odour span task. This test was similar to the NMS, however after the animal successfully retrieved the food reward from the newly presented odour a third odour was presented to the rat. Each time the rat correctly selected the new odour one new scented cup was placed on the test platform such that increasingly amounts of cups with different odours were presented to the rat for every correct choice (fig. 5). The trial ended when the animal dug in a cup it had previously retrieved a food reward from in the same session. This was characterised as an incorrect response. The number of cups that the animal was successfully able to remember before making its first incorrect response was noted as the span for that trial. Because there is no memory requirement when the first cup is presented, the span is equal to the number of cups presented and chosen sequentially without error minus one. After making an incorrect choice a new trial was started; beginning again with a single cup and increasing the number of cups for each correct selected cup (only using odours that had not been used in the previous trial). This was repeated for 30 minutes or until all 19 odours had been used in the same session. If the animal selected the new odour without making mistakes the session was ended after odour number 12. This session was performed for three consecutive days, and each performance was monitored such that numbers of trials performed and spans for each session was noted. The performing score for each rat was calculated based on the average spans for each of the three sessions. The maximum performance was a span score of 11 (12-1), that is e.g. in all three sessions the rat conducted flawless span tasks (choose the new odour in every span until 12 different odours had been remembered in all of the three sessions).

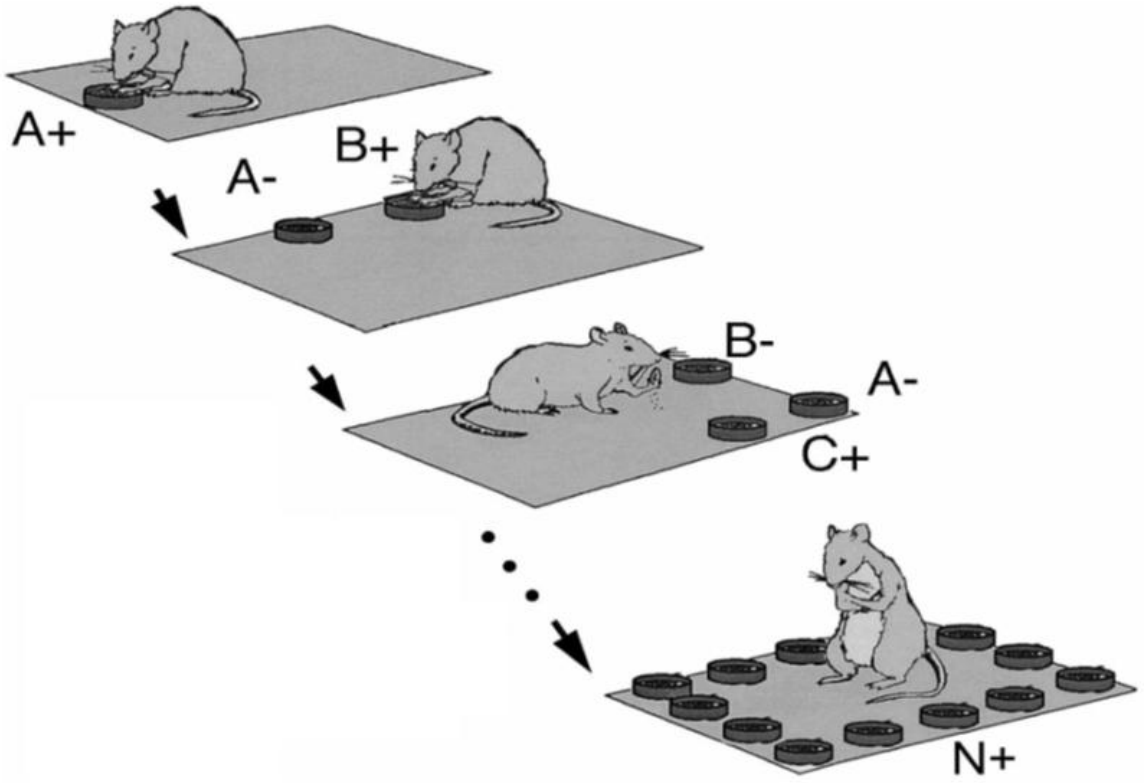

Fig: 5. Showing the design of the odour span task with an initial sand cup with odour (A) baited (+). When the rat has retrieved the hidden food reward, the same cup, now with no food reward (A-) and a novel odour baited (B+) is introduced to the rat. Hereafter, a third odour baited (c+) and the two former odours (non-baited) (A-) and (B-) are presented to the rat, and so forth. (modified from (Turchi & Sarter, 2000))

Results

Sucrose Consumption

At day 0 of the CMS regime all animals were randomly segregated into two groups; CMS (n=257) and controls (n=80). The effect of CMS on the hedonic response was evaluated through weekly sucrose consumption tests. Sucrose data (log10 transformed) from the unchallenged control rats and the CMS rats followed a normal distribution as tested by Q-Q plots and the two groups showed no statistical differences at baseline outputs confirming that these groups were well matched (data not shown). A significant overall decrease in sucrose consumption was observed for CMS animals as compared to control animals *(P<0.0001).*

The sucrose output from CMS animals was used to monitor the graduation of the stress-induced segregation into anhedonic-like and stress-resilient phenotypes. After four weeks of exposure to the CMS regime 35% of the stressed rats showed a more than 30% decrease in sucrose intake, indicating a stress-induced decrease in sensitivity to reward, and were defined as anhedonic-like. On the contrary, 29% of the stressed rats did not decrease their sucrose intake and were designated stress-resilient. Rats not corresponding to either criterion were denoted as intermediate group and were excluded from the experiment. Control rats did not decrease their sucrose intake. A subset of the anhedonic-like (n=9), stress-resilient (n=9) and control (n=9) rats was used in the present study. In these subgroups anhedonic-like rats significantly differentiated from control rats (P<0.0001) and resilient rats (P<0.0001) at week eight of CMS (fig. 6). Furthermore, no significant difference between control and stress-resilient animals were observed except the last week of the CMS regime (P=0.011).

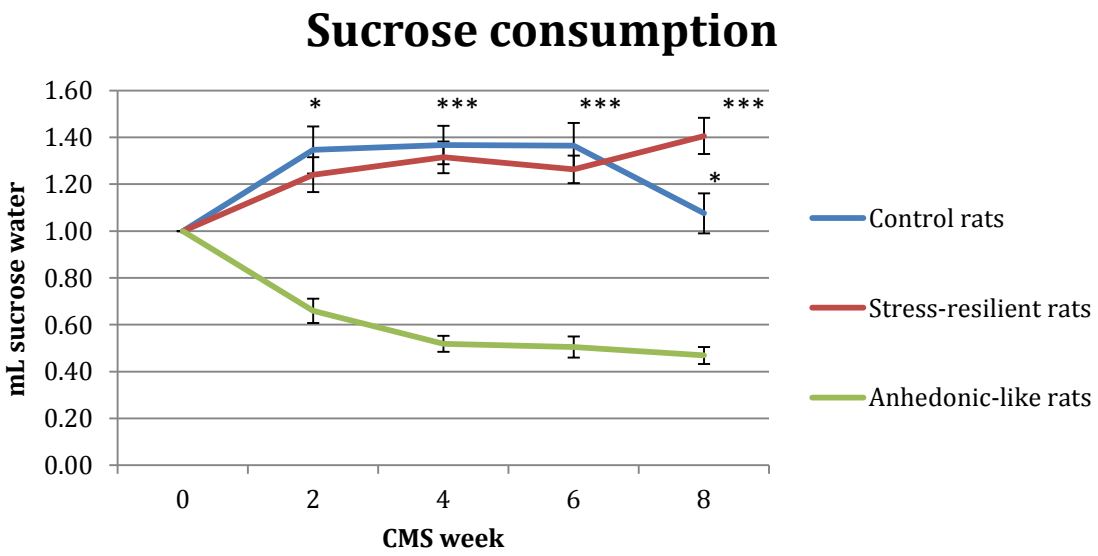

Fig. 6. Mean group sucrose intake indexed to baseline for the three subgroups measured for eight-week CMS regime. Significant differences are determined by one-way MANOVA, (***) P<0.001, (*) P<0.05 for control versus anhedonic-like, control versus stress-resilient and, for week eight only, stress-resilient versus control.

Pilot Studies of Percoll Fractions

Purified synaptosomes extracted from a pool of PFCs of (n=3) control animals were collected from four different fractions of the Percoll gradient: F2, F3, F4, and F5. These fractions were subjected to different forms of analysis to evaluate sample quality and quantity of synaptic proteins, described below.

Pilot Study 1 – Western Blot Results

Western blot analysis was performed on total protein, F2, F3, F4 and F5 samples using antibodies for detection of the pre- and postsynaptic protein markers, syntaxin2 and PSD-95, respectively. This was conducted to confirm that synaptic proteins were up-concentrated in the sub-fractions by use of the Percoll gradient as compared to whole extract fraction (total protein).

Western blot results showed that PSD-95 was enriched in all fractions except for F5 as compared to total protein (fig. 7A). F2 contained the highest concentration of PSD-95 which corresponded to a 40 % enrichment of this protein as compared to total protein. F3 and F4 were 30% and 6% enriched for PSD-95, respectively. On the contrary, syntaxin 2 showed a decreased concentration in all fractions compared to total protein with a decrease ranging from 40% for F2 to 90% for F5 (fig. 8A). F4 was diluted in five different concentrations to produce standard curves for both synaptic proteins (fig. 7B, 8B), and a subsequent estimation of the total amount of PSD-95 and Syntaxin 2, isolated in the four different fractions, was performed based on the volume collected for each fraction, and listed as the percentage-wise distribution among fractions (table 2).

PSD-95

Fig. 7. Western blot results for PSD-95. A: 5.2 µg proteins loaded of crude protein sample (total protein) and the four Percoll fractions F2, F3, F4, and F5. The highest concentration of this protein was found in F2. By use of a Percoll gradient method PSD-95 was up-concentrated in the three fractions F2, F3 and F4 as compared to total protein. B: Dilution series of Percoll fraction F4.

Syntaxin 2

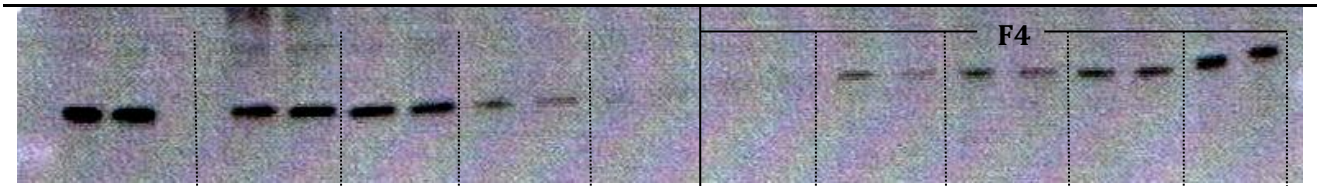

Fig. 8. Western blot results for Syntaxin 2. A: 5.2 µg proteins loaded of crude protein sample (total protein) and the four Percoll fractions F2, F3, F4, and F5. According to these results syntaxin 2 was not purified by the Percoll gradient as total protein showed the highest intensity. Of the four Percoll fractions F2 showed the highest content of Syntaxin 2. B: Dilution series of Percoll fraction F4.

Fraction	PSD-95	Syntaxin 2
F2	63%	51%
F3	20%	18%
F4	12%	21%
F5	4%	10%

Table 2. The distribution of the synaptic proteins PSD-95 and Syntaxin 2 presented as percentages of the total protein loaded across the four Percoll fractions.

Pilot Study 2 – LC MS/MS Results

iTRAQ results for F2, F3, and F4 were merged in the Mascot database search and analysed to assess the quantitative output between these fractions. As iTRAQ is based on relative values, one fraction must be set as standard; here F4 was set as standard in order to obtain relative values among the three fractions. The Panther Pathway software program was used for a systematical categorisation of the list of LC-MS/MS-identified proteins and 28 proteins were categorised as involved in transport, which is a biological process closely related to the synapse. The relative iTRAQ values for those proteins were compared in between the three fractions as illustrated in figure 9. The relative abundance of proteins associated with the synapse was highest for F2 as compared to F3 and F4. Moreover, the relative abundance of synapse-associated proteins exhibited a trend to a negative correlation as fractions are collected down the Percoll gradients, that is; highest abundance in F2 followed by F3 and lowest abundances in F4, consistent with the western blot results.

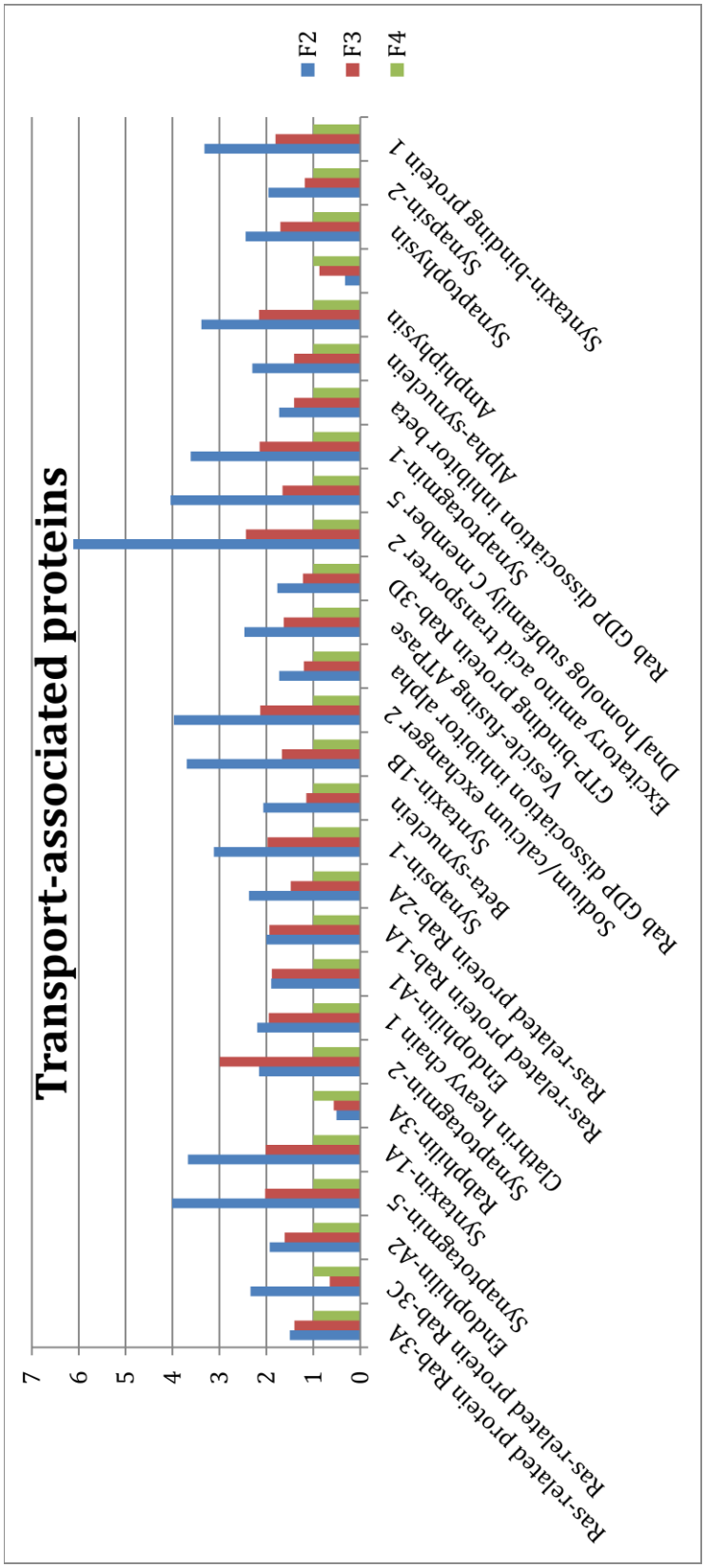

Fig. 9. Relative abundance between F2, F3, and F4 for identified proteins categorised as transport-associated (by Panther Pathway analysis). F4 was set as standard.

In summery findings for both western blot and LC-MS/MS pilot study analysis suggested that the yield of synaptic proteins was highest for fraction F2 of the four Percoll gradients. Based on these results, F2 was applied as the key fraction for analysis of differences in protein abundance between the three experimental groups of the synaptoproteomic study, in order to search for putative biomarkers of depression.

Synaptoproteomic Analysis

Purified synaptic proteins of the PFC from anhedonic-like, stress-resilient and control animals was collected from the F2 interphase of the Percoll gradient and subsequently subjected to large-scale proteomics to identify the protein abundance signatures. Quantitative data was obtained from 267 different proteins using iTRAQ, LC-MS/MS analysis. Using Panther Pathway software, the molecular functions of these proteins were mainly characterised as being involved in catalytic-, binding-, structural molecule- or transport activity.

Hierarchical Clustering Analysis

The treeView cluster analysis illustrated in figure 10 was based on quantitative iTRAQ data from 267 different proteins. This illustrated a clear segregation of the anhedonic-like group from stress-resilient and control groups, indicating an overall change in protein abundance pattern in anhedonic-like rats compared to controls.

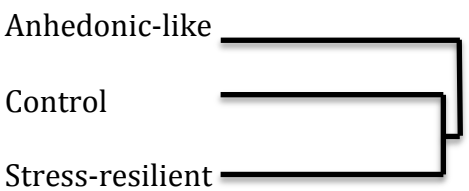

Fig. 10. Hierarchical cluster analysis based on quantitative iTRAQ data of all identified proteins (n = 267). The cluster analysis indicates a clear segregation between animals responding to stress (anhedonic-like) and those that show resilience to stress (resilient). This is illustrated by a closer relation between resilient rats and control rats as compared to anhedonic rats.

Subsequent comparison of relative protein abundance between the experimental groups that fulfilled the criteria of significant regulation ($p \leq 0.05$) and a fold change ≥ 20% revealed 26 proteins (table 3) with a false discovery rate of 5%. These proteins were mainly classified as synaptic communication proteins involved in synaptic transmission and signal transduction. Additionally, proteins involved in biological processes such as metabolism, antioxidant activity and cytoskeleton organisation were identified in this study (fig. 11).

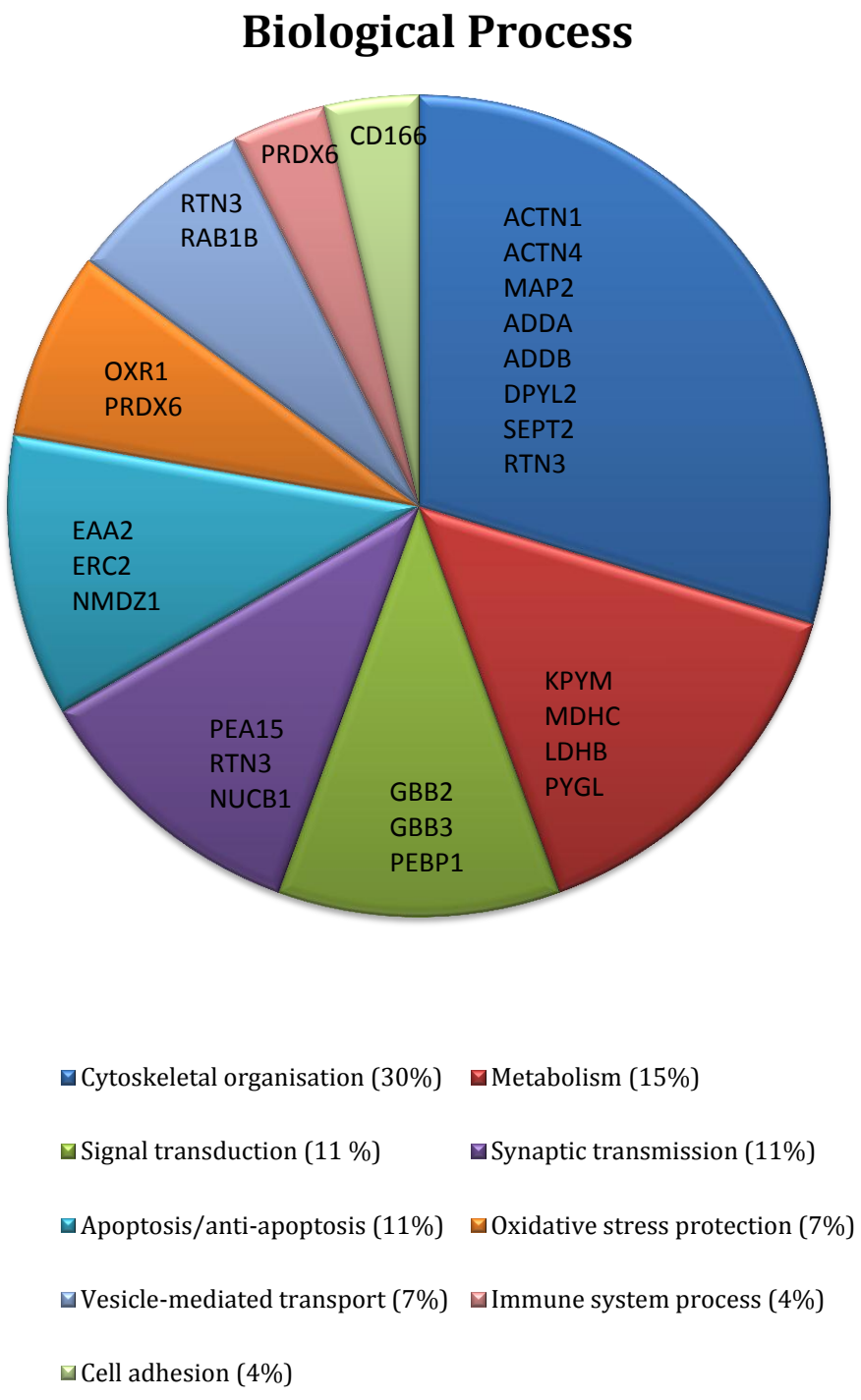

Fig. 11. Proteins identified by iTRAQ that fulfilled the selection criteria for further analysis (n=26), was classified according to biological processes and plotted into a pie chart to illustrate the percentage-wise distribution of these proteins.

ID	Protein name	Biological process	Fold change in %			p-value
			A vs C	R vs C	R vs A	
ACTN1	Alpha-actinin-1	Cytoskeletal organisation	-40			0.000
ACTN4	Alpha-actinin-4	Cytoskeletal organisation	-40			0.011
PEA15	Astrocytic phosphoprotein PEA-15	Anti-apoptosis	-30			0.001
GBB2	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	Signal tranduction	-20			0.016
GBB3	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	Signal tranduction	-20			0.030
MAP2	Microtubule-associated protein 2	Cytoskeleton organisation	-20			0.016
RTN3	Reticulon-3	Cytoskeleton organisation, vesicle-mediated transport, anti-apoptosis	-20			0.032
RAB1B	Ras-related protein Rab-1B	Vesicle-mediated transport	-20			0.004
CD166	CD166 antigen	Cell adhesion	20			0.018
OXR1	Oxidation resistance protein 1	Protection against oxidative stress	30			0.034
PRDX6	Peroxiredoxin-6	Antioxidant, immune system process		-20		0.042
ADDB	Beta-adducin	Cytoskeleton organisation		-20		0.038
ADDA	Alpha-adducin	Cytoskeleton organisation		-20		0.026
RTN3	Reticulon-3	Cytoskeleton organisation, vesicle-mediated transport, anti-apoptosis		-20		0.001
EAA2	Excitatory amino acid transporter 2	Synaptic transmission		20		0.007
PEBP1	Phosphatidylethanolamine-binding protein 1	Signal transduction			-20	0.048
KPYM	Pyruvate kinase isozymes M1/M2	Metabolism			-20	0.020
MDHC	Malate dehydrogenase, cytoplasmic	Metabolism			-20	0.002
LDHB	L-lactate dehydrogenase B chain	Metabolism			-20	0.021
DPYL1	Dihydropyrimidinase-related protein 1	Cytoskeletal organisation			-20	0.015
NUCB1	Nucleobindin-1	Calcium binding, apoptosis			-20	0.047
ACTN4	Alpha-actinin-4	Cytoskeletal organisation			20	0.031
ERC2	ERC protein 2	Synaptic transmission			50	0.022
NMDZ1	Glutamate [NMDA] receptor subunit zeta-1	Synaptic transmission			90	0.006
SEPT2	Septin-2	Cytoskeletal organisation			30	0.018
PYGL	Glycogen phosphorylase, liver form	Metabolism			30	0.010

Table 3. List of proteins significantly altered between groups as assessed by a two tailed student's T-test and a fold change ≥20%. A vs. C (anhedonic-like (n=3) compared to control (n=3)), R vs. C (resilient (n=3) compared to control (n=3), R vs. A (resilient (n=3) compared to anhedonic-like (n=3)). All data are based on quantitative iTRAQ data.

Behavioural Testing – The Odour Span Task

An attempt to implement the behavioural test, the odour span task, to our laboratory, was performed by applying a pilot study to test different setups of the study design, inspired by the studies by Young et al., 2007 and Dudchenko et al., 2000 (Dudchenko, Wood, & Eichenbaum, 2000; Young et al., 2007). A heterogeneous group of rats (both CMS and control animals (n=20)) was included in the pilot study of the odour span task, to produce a protocol for the subsequent assessment of working memory of the three experimental groups; control, resilient and anhedonic. The aim was to grade the performances of this test after CMS exposure and couple these results with biological findings from the proteomic analysis.

Habituation and Training

In the pilot study two subgroups of each ten rats were tested in different experimental setups to achieve the most appropriate procedure for both habituation and training sessions. It became evident in habituation and training sessions in both subgroups that male Wistar rats are extremely sensitive to the environmental settings. As a result in the initial subgroup (n=10) only half of the animals had not consumed the visible food reward after five days of food restriction (85% of free feeding weight) and habituation which corresponded to a total of approximately 100 minutes on the testing platform (table 4). Food reward consumption was independent of CMS exposure prior to the experiment. Neither was the type of food reward influencing the interest to consume it, as all rats received the different types of food rewards in their cage, which they immediately hereafter consumed. Further food restriction for three days (and no habituation sessions) did not change the motivation among the rats that had not previously consumed the food reward. Increasing the habituation sessions with 10 minutes each day for the second subgroup (n=10), however, increased the number to eight of rats that had consumed the visible food reward after five days of habituation (table 5). Nevertheless, results for both subgroups combined (n=20) showed that only three rats (15%) learned to dig in the sand to retrieve the hidden food reward in two consecutive training sessions, corresponding to approximately 145 minutes of habituation and training. Two of these rats achieved only a maximum of four food reward retrievals per session (each consisting of 25 minutes). This was due to a quick loss of interest in the cup and the rat spent instead most of the time exploring the platform. The third rat was the only one that achieved the task of digging in the sand cup to retrieve the hidden reward. This task was learned relatively fast, however, and after 100 minutes of habituation and training it had reliably dug and retrieved the hidden reward in two consecutive days. Thereafter, this rat was introduced to the non-matching to sample task. The remainder of animals was given either habituation or digging-sessions for an additional week, depending on previous performance. None of these rats however exhibited a stable digging task performance and were excluded for further experiments, as they could not fulfil the criteria for further testing.

Day	1	2	3	4	5	6	7	8	9	10
Min. of habituation	10	10	30	30	20	-	-	20	20	20
Food reward retrieval in %	0	10	40	50	50	-	-	50	70	80

Table 4. Initial subgroup (n=10) study design, showing the amount of rats in percentages that at least once consumed the visible food reward. 85% of free feeding weight was given every day.

Day	1	2	3	4	5
Min. of habituation	30	30	30	30	30
Food reward retrieval in %	30	50	70	70	80

Table 5. Second subgroup study design, showing the amount of rats in percentages that at least once consumed the visible food reward. 85% of free feeding weight was given every day.

Non-Matching to Sample and the No-Reward Probe Session

The single rat that fulfilled the criteria for further testing performed the non-matching to sample task with a correct response (chose the newly presented odour) in approximately every trial. After five successive trials the no-reward probe session was performed, which revealed that the odour of the hidden food reward, Guldkorn, was not successfully masked by the sand, and could therefore be scented by the rat. The non-matching to sample was repeated with another type of cereal, Cherious weed loop, which could not be scented by the animal when hidden in the sand. Hence this food reward was used in further experiments.

In summary, results from this pilot study suggested that only a small fraction (15%) of the rats included in the study design of the odour span task would learn the digging task after habituation and training in five consecutive days given approximately 30 minutes sessions each day. Of those rats that learned the task of digging for hidden food rewards, only a single one was able to retain a stable interest in this task and thus only one reaching a stable performance level required to precede to the next task: the non-matching to sample, during two weeks of habituation and training. Based on these results, the odour span task was not further optimised or applied for the graded stress-response groups of the CMS regime.

Discussion

The Chronic Mild Stress Model of Depression

As mentioned in the Introduction stress play a prominent role in MDD aetiology and the establishment of this causality is important for developing a theoretical model of depression, because it indicates that stress can initiate a cascade of biological events that lead to depression (Wager-Smith & Markou, 2011).

In the present study the highly validated CMS model of depression was used to mirror symptoms of stress-induced depression and to analyse molecular mechanisms underlying the pathology of MDD. CMS induces diminished sensitivity to rewarding stimuli, which mimics anhedonia, the core symptom of MDD. This stress-induced anhedonic-like behaviour is measured as a decrease in consumption or preference for a sweet solution, such as sucrose (P Willner et al., 1987; Paul Willner, 2005). A unique feature of this model is that rats exposed to CMS can be segregated into two groups, based on hedonic readouts from the sucrose measurements; anhedonic-like (stress-susceptible) and stress-resilient. This is further confirmed in the present study by the hierarchical cluster analysis of the global synaptoproteome, showing a differentiated protein expression pattern for stress-resilient and anhedonic-like groups (fig. 10), thereby strengthening the hedonic segregation.

Segregation into these two CMS-induced phenotypes has been confirmed at different levels elsewhere. Behavioural tests confirm a segregation of these phenotypes in both conditioned place preference (Bergstrom et al., 2008) and in step-down avoidance learning test (Strekalova & Steinbusch, 2010). In addition, anhedonic-like rats display hypercorticosolism during the time course of the CMS-regime (Christiansen, Bouzinova, Palme, & Wiborg, 2012) as well as increased glutamate levels (Delgado y Palacios et al., 2011) compared to stress-resilient rats. Furthermore, differences in gene (Christensen, Bisgaard, & Wiborg, 2011) and protein expression profiles (Henningsen et al., 2012b) are observed between the two groups. However, some similarities have as well been observed between the two phenotypes. Both anhedonic-like and stress-resilient rats show impairment in working memory as assessed by the spontaneous alternation behaviour (SAB) task, a hippocampal- and PFC-dependent behavioural task (Henningsen et al., 2009; Moustgaard, Hau, & Lind, 2008). Furthermore, a CMS-induced decrease in cell proliferation in the hippocampal dentate gyrus has been observed in both phenotypes (Jayatissa, Henningsen, Nikolajsen, West, & Wiborg, 2010). These results suggest that stress-resilient rats are affected by stress exposure; however, this is not reflected by the hedonic state which remains unchanged. The mechanisms responsible for the hedonic segregation are unknown, emphasising the importance for integrating the stress-resilient phenotype in depression research. The segregation into two stress-responses in hedonic state is similar to the variability in human stress-coping responses and MDD vulnerability (Kendler, Karkowski, & Prescott, 1999). Thus, the occurrence of the resilient phenotype improves the translational value of the CMS model of depression, and provides the opportunity to study the different phenotypes of stress reactivity, thus providing valuable information about vulnerability to chronic stress and stress coping strategies. In addition, CMS simulates realistic conditions for human depression and generates multiple behavioural changes similar to those observed clini-

cally (Paul Willner, 2005), thus supporting the hypothesis that molecular alterations found using this model also occur in patients with stress-induced affective disorders.

Synaptoproteomics

To obtain information about the two stress-sensitivity phenotypes, the synaptic protein profiles of the PFC from the three CMS groups, anhedonic-like, stress-resilient and control, were established and compared. iTRAQ labeled peptides were separated and identified by LC-MS/MS which provided a sensitive and robust quantitative proteomic platform to identify synaptic markers of stress-reactivity. Analysis of significantly regulated proteins produced refined information about stimulated or impaired biological pathways specific for development of anhedonic-like behaviour or resilience. Based on pathway analysis and PubMed literature searches some of these proteins were selected for a more detailed description with respect to their potential implication in the pathogenesis of MDD. It is important to note, that the application of an appropriate validation method such as selected reaction monitoring (SRM) is work in progress and the results discussed here, and the conclusions drawn, are based on non-validated data. The following will provide suggestions of putative protein profiles specific for the two stress-response phenotypes and their potential involvement in MDD, based on various animal depression models using different study designs.

Synaptic Protein-Markers of Stress-Reactivity

Among the three experimental groups most of the significantly regulated proteins were found between the anhedonic-like rats compared to control rats, and between stress-resilient rats compared to anhedonic-like rats. This, combined with the clear segregation of anhedonic-like rats from both stress-resilient and control rats in the hierarchical cluster analysis, indicate that the progression of an anhedonic-like response to stress is more complex than a resilient response, and that the two stress phenotypes show differences in the abundance for several different types of proteins. Some of the biological processes indicated to be heavily affected by CMS are metabolism and cytoskeletal organisation which previously have been shown to be implicated in stress-responses and in MDD (Beasley et al., 2006; Bisgaard et al., 2007; Bisgaard Fuhr, Bak, et al., 2012; Bisgaard Fuhr, Palmfeldt, Henningsen, Gregersen, & Wiborg, 2012; Henningsen et al., 2012b; Mallei et al., 2011; Piubelli, Carboni, Becchi, Mathé, & Domenici, 2011).

Metabolism

An association between deficiencies in energy metabolism has been speculated to be involved in MDD pathology (Harvey, 2008; Tagliari et al., 2010) based on clinical findings of alterations in cerebral blood flow in brain areas coupled to the disease, including the PFC (Palazidou, 2012; Videbech, 2000), as well as decrements in molecular markers of neuronal metabolism (Moretti et al. 2003) and altered levels of mitochondrial enzymes (Beasley et al., 2006; Mallei et al., 2011). Glucose metabolism constitutes the main energy source of the brain and is necessary for normal brain functioning and synaptic transmission, including transport of glutamate, GABA, dopamine, and serotonin (Tagliari et al., 2010). Moreover, a correlation has been found between glucose utilisation and dynamic neural activity as well as cognitive

function (Ishida, Noda, & Ueda, 2009). This is illustrated through preclinical findings where cognitive deficits can be induced by chronic stress and these deficits correlate with disruptions in brain metabolism (Tagliari et al., 2010). As MDD symptomatology includes impairment of cognitive function tightly coupled to PFC (Palazidou, 2012) it may be speculated that metabolic changes of this brain area, caused by chronic stress, could implicate important pathological processes of the disease.

Stress-Resilient and Anhedonic-Like Animals Differ in Markers of Metabolism

In the present study, all the proteins involved in glucose metabolism (KPYM, MDHC, LDHB), except for one (PYGL), were down regulated in stress-resilient rats compared to anhedonic-like rats, indicating a possible difference in the glycolytic pathway and citric acid cycle between the two stress-responses. Similar observations have been found by Mallei et al. 2011, showing altered levels of proteins involved in metabolism in PFC, and that such alterations could be restored with antidepressant treatment (Mallei et al., 2011). In the present study, pyruvate kinase isozymes M1/M2 (KPYM) was identified as being less abundant in stress-resilient compared to anhedonic-like rats. Pyruvate kinase is a critical enzyme for glucose metabolism (Tagliari et al., 2010), and there is evidence for an association between pyruvate kinase and the facilitation of neurotransmitter uptake into synaptic vesicles. It has been suggested that glycolytic ATP-generating enzymes, including pyruvate kinase, may be important for this transport of neurotransmitters, and thus critical for normal synaptic transmission (Ishida et al., 2009).

A study by Piubelli et al. 2011 reported a similar significant down regulation of KPYM in a proteomic analysis of both the frontal lobe and the hippocampus of Flinders resistant rats experiencing early-life stress (maternal separation). The decrement was highest within the PFC compared to the hippocampus, and the decrement of KPYM was paralleled by a decrease in several other proteins involved in metabolism (Piubelli et al., 2011). A possible involvement of KPYM in different stress-responses has likewise been observed in two proteomics studies of the rat hippocampus one using repeated social defeat to induce a depressive-like state (L Carboni et al., 2006) and another using a pharmacological approach to induce a depressive-like phenotype (Rubino et al., 2009). Both of these studies showed altered KPYM abundances. Furthermore, KPYM was significantly regulated in the hippocampal proteome in a mouse line selectively bred for high stress reactivity (Knapman et al., 2012). Collectively, this indicates that KPYM may play a role in stress-reactivity, as shown in the present study.

Oxidative Stress

Together with the glycolytic pathway and the citric acid cycle, the process of oxidative phosphorylation is essential for the production of cellular energy in the brain, and deficiencies in the respiratory complex activities are suggested to be associated with oxidative stress, which may underlie alterations in energy metabolism and possibly induce cellular degeneration (Tagliari et al., 2010). A substantial amount of data supports an implication for oxidative stress mechanisms in the pathogenesis of MDD (Behr, Moreira, & Benicio N. Frey, 2012; Chopra et al., 2011; Kodydková et al., 2009; Lucca et al., 2009). This association has theoretical

appeal, as the brain is particularly vulnerable to oxidative damage due to its comparatively high oxygen utilisation that generates free radical by-products such as reactive oxygen species (ROS) (Michel et al., 2007). ROS have beneficial effects within the cell in low concentrations; however, can at high concentrations cause cell damage through several mechanisms such as enzyme inactivation, lipid peroxidation and DNA modification. Under normal conditions ROS are metabolised into less toxic molecules by the action of non-enzymatic and enzymatic antioxidants. Furthermore, structural repair and replacement mechanisms exist to protect the organism against excessive ROS-induced damage. Oxidative stress results from increased production of ROS, decreased antioxidant defense mechanism or failure to repair oxidative damage (Michel et al., 2007; Tagliari et al., 2010). In the present study proteins involved in antioxidant mechanisms or cellular protection against oxidative damage were identified as being differently expressed in response to CMS.

Both anhedonic-like and stress-resilience phenotypes showed altered levels of such proteins compared to controls, indicating an implication of oxidative stress as a general stress response; however affecting different proteins in the respective phenotypes. A coupling between exposure to stressors and altered levels of oxidative stress is confirmed from other preclinical studies (Lucca et al., 2009; Tagliari et al., 2010).

Among anhedonic-like animals, an up regulation was observed for the oxidation resistance protein 1 (OXR1) compared to controls after exposure to the CMS regime. OXR1 is a vital neuroprotective protein that is induced under oxidative stress (Elliott & Volkert, 2004) and function through control of neuronal cell sensitivity to these reactive species. Conversely, loss or mutations of the gene encoding this protein (Oxr1) causes cell death (Oliver et al., 2011; Volkert, Elliott, & Housman, 2000). Mutations of other individual genes that prevent or repair oxidative damage do not show similar results of oxidation sensitive phenotypes, which suggest that the Oxr1 gene product may play an important and unique role in neuronal protection (Elliott & Volkert, 2004). No direct relation of this protein and MDD has previously been shown; however, based on the above-mentioned findings, one might speculate that the exposure to CMS in the present study might have produced an increase in oxidative stress within PFC neurons of anhedonic-like rats, possibly resulting in a concurrent induction of OXR1 as a compensatory mechanism. Further studies must be conducted to confirm this relationship though.

Further suggestion for an involvement of oxidative stress in response to CMS is depicted by the down regulation of the antioxidant peroxiredoxin-6 (PRDX6) in stress-resilient animals as compared to control animals. PRDX6 is an antioxidant enzyme that reduces various cellular peroxide substrates and thereby protects cells from ROS-induced cell damage and apoptosis together with the reduction of redox-sensitive cell signaling involved in processes such as cell proliferation, differentiation, and transformation (Fenwick Gardiner, Pryce Gaynor, & Shelley A. Phelan, 2010). Peroxiredoxins have recently been considered part of the most important enzymatic peroxide-removal systems in living cells among the well-characterised antioxidant enzymes including superoxide dismutase (SOD) and catalase. Supporting this view, studies of peroxiredoxin knockout/knockdown mice demonstrated that deficiency in any of the six isoforms (PRDX1-6) produced an increased level of free radical species. Moreover, altered expression levels of peroxiredoxins, and posttranslational modifications that render the proteins

inactive, have been involved in different neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Goemaere & Knoops, 2012), which both show a high comorbidity with depression (K. R. R. Krishnan et al., 2002). A study by Lucca et al. used the CMS paradigm on rats to investigate oxidative stress parameters in response to stress and reported an increased protein peroxidation as a marker of oxidative damage together with decreased SOD activity in different brain areas implicated in MDD, including PFC. The observed decrease in SOD was suggested to be a result of CMS-induced activation of the HPA axis that may have negatively affected the number of SOD molecules and/or the degree of activation of this enzyme (Lucca et al., 2009). This coupling may be supported by the suggestion that cortisol-induced alteration of mitochondrial membranes, potentially results in a release of mitochondrial content and subsequently inducing neuronal apoptosis. Neuronal cell death as a consequence of mitochondrial changes appears to be related with PFC and hippocampal atrophy associated with MDD (Tagliari et al., 2010). Similar findings have been described in a study by Skynner et al. 2006, which found decreased levels of PRDX6 in the mouse cortex after chronic corticosterone treatment with a concurrent observation of altered cell morphology and neuronal cell death (Skynner et al., 2006). It is well established that the CMS regime, as applied in the present study, causes increased levels of circulating cortisol (Christiansen et al., 2012) that may be speculated to contribute, as one factor, to oxidative stress and possibly neuronal damage in the PFC along with changes in the level of antioxidants as observed for PRDX6.

Cytoskeletal Proteins

Panther pathway analysis and literature searches for significantly regulated proteins among the three experimental groups revealed that 30% of these proteins were categorised as cytoskeletal proteins: MAP-2, ACTN1, ACTN4, ADDA, ADDB and SEPT2, DPYL2 and RTN3. In the comparison between animals exposed to CMS and control animals, all proteins belonging to this category were down regulated. Furthermore, the abundances of the cytoskeletal-associated proteins ACTN4 and SEPT2 are lower in anhedonic-like animals when compared to stress-resilient animals, suggesting a possible involvement of structural proteins in the processes underlying stress and stress vulnerability. This association is confirmed in both preclinical and clinical studies (L Carboni et al., 2006; Nakatani et al., 2007; Piubelli et al., 2011), showing a relation between cytoskeletal remodeling and depressive-like phenotypes as well as changes in cytoskeletal-associated protein expression levels upon treatment with antidepressants (Massimiliano Bianchi et al., 2009; Lucia Carboni et al., 2006; Yang, Wang, Wang, Liu, & Wang, 2009). The cytoskeleton plays a key role in preserving neuronal cell morphogenesis and in synaptic plasticity (Luo, 2002), and altered plasticity has been speculated to be a result, at least partially, of altered function and/or levels of cytoskeletal-associated proteins (M Bianchi, Hagan, & Heidbreder, 2005; Cereseto et al., 2006). MDD pathology, and the impaired functioning of the hippocampus and PFC among other brain areas, have been associated to alterations in neuronal plasticity (Andrade & Rao, 2010; Dwivedi, 2011). Various proteins contribute to the organisation and regulation of the neuronal cytoskeleton (Luo, 2002).

Cytoskeletal Alterations in Anhedonic-Like Rats

Four isoforms of α-actinins exist, where α-actinin-1 (ACTN1) and α-actinin-4 (ACTN4) are expressed by most non-muscle cells including the neuronal cells (Dixson, Forstner, & Garcia,

2003). α-actinins are bundling proteins that cross-link actin filaments which comprise the principal cytoskeleton of dendritic spines. Although the literature does not provide a linkage between α-actinins and depression, it has been shown that these proteins are involved in both AMPA and NMDA receptor signaling (Krupp, Vissel, Thomas, Heinemann, & Westbrook, 1999; Merrill et al., 2007; Schulz et al., 2004), which have been found altered in both preclinical and clinical studies of MDD (Boyce-Rustay & Holmes, 2006; Feyissa, Chandran, Stockmeier, & Karolewicz, 2009; G Hasler & Northoff, 2011; Y. Liu, Yang, & Zuo, 2010).

In the present study the dendritic marker (Reinés, Cereseto, Ferrero, Bonavita, & Wikinski, 2004) microtubule-associated protein (MAP2) was less abundant in anhedonic-like animals compared to control animals. MAPs constitute an abundant group of cytoskeletal components, and the dynamic instability of the cytoskeletal microtubular system is essential for neuronal organisation, and is dependent on the phosphorylated state of neuronal MAPs(Soetanto et al., 2010; Yang et al., 2009). Moreover, decreased microtubular dynamics as well as altered functionality of proteins involved in the regulation of microtubule dynamics have been observed in response to stress in preclinical studies. Moreover, antidepressants have shown to affect the expression of microtubular proteins (M Bianchi et al., 2005). Another study by Yang et al. 2009, found no changes in MAP2 levels in the hippocampus after chronic unpredictable mild stress, however, they observed a decreased level of phosphor-MAP2 which were suggested by the authors to illustrate a stress-induced impairment of microtubule dynamics (Yang et al., 2009). As data obtained in the present study is based on quantitative iTRAQ outputs from both modified (e.g. phosphorylated) and non-modified proteins, it is not possible to conclude whether the observed down regulation of MAP2 in the present study is a result of less phosphorylated MAP2 proteins; however, these observations combined suggest that MAP2 might be affected by CMS-induced alterations in the present study.

Cytoskeletal Alterations in Stress-Resilient Rats

Exposure to the CMS regime in the present study caused a down regulation of α-adducin (ADDA) and β-adducin (ADDB) in stress-resilient animals compared to controls. Adducins are a family of membrane skeleton proteins highly expressed in the brain, possibly concentrated at dendritic spines and growth cones (Porro et al., 2010). In dendritic spines adducins are involved in the dynamic assembly/disassembly of actin cytoskeleton primarily through recruitment of spectrin to the growing ends of actin filaments, as well as promoting actin capping and bundling (Porro et al., 2010; Rabenstein et al., 2005). Through this action, adducins play an important role in synaptic plasticity and ADDB have been found to be critical for long-term learning and memory (Bednarek & Caroni, 2011). This provides support for an important role of cytoskeletal-associated proteins in neuronal functioning and synaptic transmission, and that stress-induced alteration of these proteins, may have severe consequences for neuronal survival. In the present study, different types of cytoskeletal proteins were affected in each of the stress-response phenotypes and may play different parts in the development of a stress-susceptible behaviour or a stress-coping behaviour that yet have to be elucidated.

Synaptic Transmission in Stress-Resilient Animals

An interesting finding of the present study is that all three proteins associated with synaptic transmission (EAA2, NMDZ, and ERC2) were up regulated specifically for the stress-resilient phenotype as compared to control and anhedonic-like rats. All these proteins are directly (EAA2, NMDZ1) and indirectly (ERC2) involved in glutamate transport (Ko et al., 2006; Zink, Vollmayr, Gebicke-Haerter, & Henn, 2010), which has been confirmed by several studies to be altered in MDD (Boyce-Rustay & Holmes, 2006; G Hasler & Northoff, 2011). In this relation, both alterations of the NMDA receptor, as well as glial glutamate transport have shown to play prominent roles in pathological processes associated with depression (Choudary et al., 2005; Feyissa et al., 2009; Hashimoto, 2009). More specifically, changes in expression of different NMDA receptor subunits have been reported in MDD patients (Feyissa et al., 2009; Karolewicz, Stockmeier, & Ordway, 2005). Both decreased levels of the glutamate [NMDA] receptor subunit zeta-1 (NMDZ1/NR1) (Beneyto & Meador-Woodruff, 2008; Hashimoto, 2009), as observed in the present study, as well as decreased levels of other NMDA receptor subunits, NR2A and NR2B, have been reported within the PFC of MDD patients, and have been suggested to produce hypofunctional NMDA receptors and possibly underlie some of the pathological processes of MDD (Feyissa et al., 2009). Correspondingly, decreased levels of the NMDZ1 in the mouse frontal cortex has been reported in a stress model of depression and a genetic model of deficient glutamate function linked to depressive-like behaviour (Tordera et al., 2011).

The excitatory amino acid transporter 2 (EAA2), also termed GLT-1, SLC1A2, or EAAT2, is a glial high affinity glutamate transporter and is essential for terminating the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft (Choudary et al., 2005). This transport of glutamate is important to protect neurons from glutamate excitotoxicity (W. Chen et al. 2002). Compelling evidence have coupled down regulation of EAA2 to MDD both through clinical observations and preclinical observations (Bernard et al., 2011; Choudary et al., 2005; John et al., 2012). One of these studies reported a down regulation of EAA2 in human PFCs using both microarray and *in situ* hybridisation histochemistry analysis (Choudary et al., 2005). Moreover, antidepressant treatment has been shown to induce EAA2 expression in both cortical regions and in hippocampus, and this induction was suggested to be caused by a counterbalance in the tonus of glutamatergic neurotransmission (Zink, Rapp, Donev, Gebicke-Haerter, & Thome, 2011). Interestingly, a study by Bisgaard et al., 2012 found the related glutamate transporter protein EAA1 to be up regulated in the hippocampus of stress-resilient rats as compared to control and anhedonic-like rats using the CMS model of depression (Bisgaard Fuhr, Palmfeldt, et al., 2012). These findings, coupled to the up regulation of EAA2 in resilient animals compared to anhedonic-like animals observed in the present study, one might speculate that an up regulation of proteins involved in glutamate uptake from the synapse might indicate a neuronal protection mechanism as part of a stress-coping process specific for the stress-resilient phenotype.

In addition to the pathological consequences of oxidative stress, excessive activation of glutamate receptors are converging and represent sequential as well as interacting processes that together produce cell vulnerability in the cell. One theory is that CNS stress can lead to high levels of exogenous glutamate both as a result of direct release from the cell and enzymatic conversion of high extracellular glutamine to glutamate. Moreover a shutdown of nerve and glial glutamate uptake systems by pro-oxidant conditions may occur concurrently (Schubert & Piasecki, 2001). Calcium overload in the neuron may be a result of an increased stimulation of

NMDA receptors which can produce ROS accumulation in the cell and possibly neuronal death (Y. Liu et al., 2010).

In the present study, the increased abundance of proteins closely coupled to glutamate neurotransmission as a specific profile for stress-resilient animals as compared to both control and anhedonic-like animals, could be speculated to pose one of several features of this stress-response phenotype that accounts for differences reported between stress-susceptible and stress-resilient rats of the CMS model of depression. Based on the observations that antidepressants increase EAA2 levels (Zink et al., 2011), and drugs that targeting the NMDA receptor show antidepressant-like effects (Sanacora et al., 2012) as well as different classes of antidepressants have shown to bind to NR1 (Raabe & Gentile, 2008), one might speculate that the increased abundance of those proteins in stress-resilient rats may be involved in a stress-coping mechanism in these rats. However, further investigation is needed to establish such a relation.

Synaptic Transduction in Anhedonic-Like Rats

In the present study, the anhedonic-like rats showed decreased abundances of the G protein subunits β 2 (GBB2) and 3 (GBB3) as compared to control rats. Guanine nucleotide-binding proteins (G proteins) are involved as modulators or transducers in various transmembrane signaling systems, and hundreds of cell-surface receptors for neurotransmitters and other ligands use G proteins to transduce intracellular signaling pathways. G proteins comprise a heterotrimeric complex of Gα-, Gβ-, and Gγ-subunits (H.-J. Lee et al., 2004). Growing evidence suggests an association between G proteins and mood disorders based on alterations in the concentration or function of G proteins in peripheral blood elements in patients with bipolar disorder and MDD (Avissar & Schreiber, 2006).The Gβ-subunits are targets for some antidepressant drugs and the gene (GNB3) encoding GBB3 has been established in a meta-analysis by Lo´pez-Leo´n *et al*., 2008 as a MDD susceptibility gene. Here, a single-nucleotide polymorphism (SNP) of C825T in exon 10 of GNB3 has been associated with depression with a significantly higher frequency of the T allele in MDD patients than in healthy controls (López-León et al., 2008). Moreover, the TT genotype is associated with altered responsiveness to antidepressants (H.-J. Lee et al., 2004). As none of these studies have reported of altered levels of the gene product, a coupling between such gene alteration and the lower abundance level of GBB3, as found in the present study, is not possible. On the other hand, these results point towards G-proteins as important components in depressive behaviour.

Another protein involved in synaptic transduction, phosphatidylethanolamine-binding protein 1 (PEBP1 or HCNp-pp), was found in higher abundance in the anhedonic-like group compared to the stress-resilient group in the present study. Interestingly, PEBP1 functions as a precursor protein of the hippocampal cholinergic neurostimulating peptide (HCNP), and a decreased expression of PEBP1 has been suggested to affect acetylcholine production in the hippocampus. PEBP1 has been described as differently expressed in preclinical studies of depression (Kim & Kim, 2007; Piubelli et al., 2011), and after chronic corticosterone treatment (Feldmann et al., 2008). PEPB1 was found down regulated in studies that analysed abundance of this protein in the hippocampus (Feldmann et al., 2008; Kim & Kim, 2007), whereas the study involving the frontal lobe showed a down regulation of the same protein (Piubelli et al.,

2011). This indicates that this protein might pose interesting features relevant for stress-induced pathological processes associated with MDD, and with a possibly involvement of brain area specific changes, however, more specific and extensive research must be conducted to confirm this.

In summary, the protein profiles of anhedonic-like and stress-resilient rats differed from each other as a great amount of proteins were found differently expressed among these two groups. Pathways that overlap for both stress-phenotypes were cytoskeleton organisation and oxidative stress associated protein, however, these two common pathways ware based on different protein profiles from each phenotype. Pathways that did not overlap between the two phenotypes were metabolism, synaptic transmission and synaptic transduction. The various differences observed between anhedonic-like and stress-resilient rats in the present study may represent differences in important active biological processes specific for stress-vulnerability and stress coping-strategies, respectively. This segregation of the two phenotypes at the protein level is well reflected by the two hedonic responses of the CMS model and confirms the importance of including these two stress-phenotypes in the search for MDD-associated biomarkers.

Methodological Considerations

Proteomics in Major Depressive Disorder

Proteomic technologies provide valuable tools for investigating altered protein signatures rather than single proteins which is a necessary approach to identify pathological processes associated with a complex psychiatric disorder such as depression (Filiou et al., 2011). Moreover, proteomics has the great advantage of facilitating analysis at the protein level including post-translational modifications and thereby reflecting more closely the pathological processes underlying the disease as compared to genetic approaches. In general, in a given tissue, a proteome of several thousand proteins could be expected, but so far only a fraction has been detected with current proteomic methods (Taurines et al., 2011). The reason is that the resolution of mass spectrometers does not reach the dynamic range of proteins in cells, and thus lacking the potential to profile the complete proteome. This inevitably hampers the detection of low abundant proteins (Wiśniewski, 2008), which may often be the most biologically relevant in a complex disease such as MDD, where many and small changes have been suggested to underlie the disease (Filiou et al., 2011; Taurines et al., 2011; Tsuang et al., 2004). Moreover, the existence of several different proteomic platforms, brain areas and subfractions tested in the search for MDD biomarkers as well as various rodent strains and stress-models of depression, makes it difficult to compare these studies. This may constitute the main reason for the great viability of results published from proteomic studies conducted both in depressed patients or in animal models of depression, as well as no single protein has been directly linked to the cause of the disorder. However, when looking at the involved pathways and biological processes in these studies combined, a more uniform direction emerges in the coupling of proteins to depression. Here, biological processes related to altered protein abundance are synaptic vesicle trafficking (endo- and exocytosis), energy metabolism (mitochondrial), cytoskeletal organisation, signal transduction, transcription, neurogenesis, and inflammation (Beasley et al., 2006; Bisgaard et al., 2007; L Carboni et al., 2006; Henningsen et al., 2012b; Kedracka-Krok

et al., 2010; Mallei et al., 2011; Mu et al., 2007; Piubelli et al., 2011). This feature of diverging results on the protein level as opposed to similar results on the pathway level demonstrates proteomics as a useful guidance tool, especially suitable in the investigation of a multifactorial disorder like MDD. Biological patterns and trends identified, using discovery-based large-scale proteomics to create a proteome signature, can subsequently be investigated more deeply in hypothesis-based studies. Despite some limitations posed by proteomic methods, the advantages render this approach extremely informative and well suited for analysis of global protein abundance.

Isolation of Synaptosomes

A discontinuous Percoll gradient was used in the present study to isolate synaptosomes. Synaptosomes are isolated nerve terminals which are greatly enriched in proteins involved in synaptic functions, thus providing an ideal tool to analyse disease-related changes in protein abundance at synaptic sites where many biological processes associated to MDD and targets of antidepressants are located (Mallei et al., 2011). Furthermore, subproteomics, that is characterising proteomes of a subfraction such as synaptosomes, reduces sample complexity and potentially increases the information yield (Zhang, 2010).

According to the protocol by Dunkley et al. 2008 five visible fractions between the interfaces of the four Percoll concentrations are produced when whole rat brain are used for the homogenate (Fig.12)(Peter R Dunkley, Jarvie, & Robinson, 2008b). In the present study the lower amount of tissue of the three PFCs resulted in only four visible fractions using the Percoll gradient, lacking the most upper fraction (F1)(Fig. 3). However, a pilot study (data not shown) was conducted using double the amount of brain tissue, and this produced five visible fractions. According to the literature F1 contain mostly membranous material which may produce a visible band only reaching a certain amount of material.

Fraction three (F3) and four (F4) of the Percoll gradient have been described as being enriched in metabolically and functionally viable synaptosomes that should contain most of the synaptic material including synapse-associated proteins. However, synaptosomes are located in all five Percoll fractions and most of the damaged ones have been shown to localise in the upper fractions. According to the literature, fraction F2 consists mostly of myelin and membranous material (P R Dunkley et al., 1988; Peter R Dunkley et al., 2008a; Harrison et al., 1988). As it is assumed that abundant proteins masks less abundant proteins, in the case of F2 in the present study, proteins associated with the synapse should be masked by myelin- and membranous-associated proteins, which was not the case. Instead results from the two pilot studies indicated that F2 quantitatively contained more synaptic proteins as compared to F3, F4, and F5. Moreover, it was confirmed that the Percoll fractionation method successfully produced fractions enriched with synaptic proteins as compared to the crude sample (total protein sample). Based on these results, the discontinuous Percoll gradient was used as the method to isolate synapse-associated proteins using F2 as the subfraction used for the LC-MS/MS analysis of PFCs for the three experimental groups in the search for MDD-associated biomarkers.

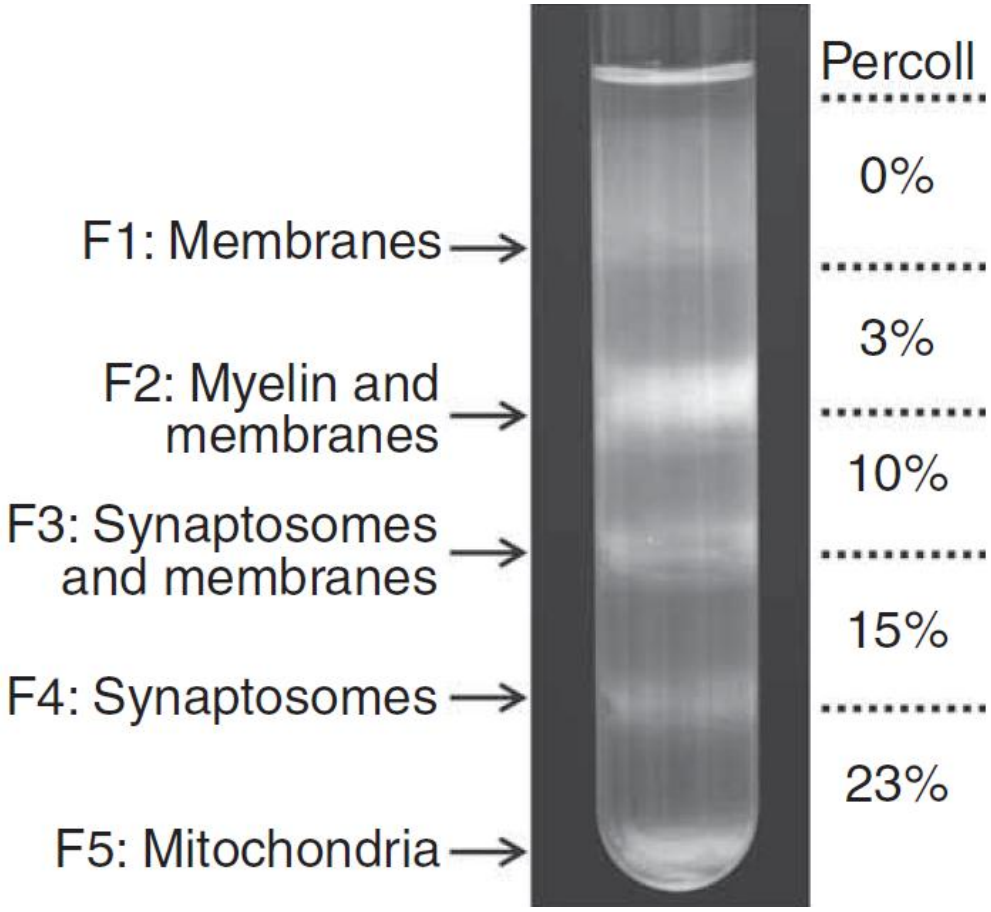

Fig. 12. Showing five fractions (F1-F5) produced through centrifugation using a discontinuous Percoll gradient as described by (Peter R Dunkley et al., 2008a).

Behavioural Testing – A Pilot Study

Odour Span Task

A pilot study of the odour span task was performed to produce a protocol for assessment of working memory in the three experimental groups; control, stress-resilient and anhedonic-like rats. The aim was to grade the performances of this test after CMS exposure and couple these results with biological findings from the proteomic analysis. The odour span task has been shown to be a novel paradigm for assessing working memory in rodents (Young et al., 2007) and it was hypothesised that rats exposed to the CMS paradigm have an impaired working memory performance, as assessed by this task. This is based on results from previous studies testing PFC-related behaviour in animals exposed to chronic stress, including spatial delayed alternation task in a T maze (Hains et al., 2010), water maze-task (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007; Quan et al., 2011) for stress-susceptible rats, and spontaneous alternation behaviour (SAB) task for both stress-susceptible and stress-resilient rats (based on hedonic state) (Henningsen et al., 2009). Previous studies have confirmed an involvement of PFC in

olfactory working memory (Cui et al., 2011; Rushforth, Steckler, & Shoaib, 2011; Turchi & Sarter, 2000). A great advantage of assessing working memory using olfaction is 1) rodents preferentially attend to olfactory cues (Young et al., 2007) and 2) olfactory working memory have likewise been coupled to the PFC in humans (Dade, Zatorre, Evans, & Jones-Gotman, 2001), increasing the translational value of the task. Furthermore, working memory consist of both spatial and non-spatial span capacity mediated by the hippocampus and the PFC, respectively (Yoon, Okada, Jung, & Kim, 2008). Performance in the odour span task is independent of spatial working memory, hence independent of hippocampal function (Cui et al., 2011), and this task can therefore be considered to assess PFC-related working memory specifically. To my knowledge, no other study has used the odour span task to assess the effect of chronic mild stress on working memory in rats.

The results in the present study indicate that male Wistar rats are extremely sensitive to novel environments and require extensive habituation before showing interest and/or motivation to consume the food reward, which is an essential part of the task. The lack of interest in food reward retrieval has not been reported by other studies applying a similar protocol (Dudchenko et al., 2000; Turchi & Sarter, 2000; Young et al., 2007). Most of these use mice or different rat strains such as Fischer-Brown Norway rats and Long-Evans rats. Different rat stains are likely to have different behavioural patterns (Stryjek, Modlińska, & Pisula, 2012) possibly accounting for the lack of motivation observed in the present study as compared to other studies. Results from the present study clearly showed that a high and stable motivation level was required for the rat to achieve the task of dig reliably in a sand bowl for reward retrieval and subsequently learn the role of the non-matching to sample task. The latter task is described as the most time consuming task to learn by previous studies which account approximately six days of training to achieve this task. Taking all of this into consideration it is likely that the male Wistar rats require several weeks of habituation and training before the odour span task can be applied as a measure of working memory after exposure to the CMS regime. This is problematic as it is not possible to initiate the behavioural testing before the CMS regime has been terminated, and there is a risk that the stress-effect will decrease within those weeks. This phenomenon has been observed previously using the CMS model of depression and is termed spontaneous recovery.

In conclusion, the different setups applied in this pilot study did not provide results as expected and must be repeated to establish an appropriate protocol for male Wistar rats. The odour span task requires long and sustained habituation and training, which might influence the behavioural outcome of this task in CMS animals. Alternatively, a behavioural task that is less dependent on habituation and on a high motivation level should be considered for assessment of PFC-dependent changes in working memory.

Conclusion

The present study was initiated to investigate global protein aberrations in the PFC during depressive-like states in the search for MDD biomarkers. This large-scale, non-hypothesis driven analysis of differential protein profiles provided a powerful strategy for investigating the complex pathophysiology underlying the two stress-response phenotypes of the CMS model of depression. Within this analysis, the yield of synaptic proteins were successfully increased through fractionation, and thus increasing the chance of identifying low abundant proteins, believed to play an important role in MDD pathology. Both the global protein pattern of the three experimental groups, as well as proteins fulfilling the criteria of significance, showed a clear segregation of stress-resilient and anhedonic-like rats that reflect the segregation of the hedonic response to CMS. In the present study, stress-susceptibility and stress-resiliency were associated with several proteomic aberrations, particularly those related to metabolism, cytoskeletal organisation, synaptic transmission and signal transduction. These proteins should be further investigated to confirm their relevance to depression.

Future Prospects

Results from the present study provided basis for further analysis of interesting protein profiles for each of the phenotypes that must be validated before any correlation between these profiles and MDD pathology can be confirmed. In this setting, selected reaction monitoring will provide an established method for proteomic verification of selected proteins (work in progress). Immunohistochemistry can be applied in subsequent studies to investigate PFC subregional localisations of proteins of interest. Moreover, the application of different functional studies such as knock-down or protein overexpression systems may confirm a potential role in depression for individual validated proteins.

Another important aspect, that is enabled by the CMS model of depression, is the inclusion of antidepressant treatment prior to proteomic analysis in the search of MDD associated biological processes. This provides the opportunity to investigate several additional aspects of the disease including identification of proteins involved in treatment-induced recovery as well as those involved in treatment resistance, and thus strengthening the translational value of the study.

From a proteomic view, each fraction of the Percoll gradient may contain enrichments of various low abundant proteins with special features e.g. synapse-associated mitochondria, hence the proteome of other fractions besides F2 may provide valuable information in the search of MDD biomarkers (work in progress).

Finally, a behavioural task should be applied to assess PFC function enabling the coupling of altered protein profiles with behavioural changes. The water maze-task could be applied as a behavioural task for assessing PFC-dependent working memory.

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Appendix I

Chemicals:

- Sucrose ($C_{12}H_{22}O_{11}$, Sigma, cat. no. #S9378),
- EDTA disodium salt ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$, Sigma cat. no #E-9884),
- DL-1,4-DTT (Cleand's Reagent, $C_4H_{10}O_2S_2$, Sigma, cat. no. #D5545),
- Trishydroxymethylaminomethane, (Trizma®), (Tris, $(HOCH_2)_3CNH_2$, Sigma cat. no. #T6066),
- Hydrocgloric acid, HCl (Chem-Supply, cat. no. AC0744),
- Percoll® (GE Healthcare, cat. no. #17-0891-01),
- Complete EDTA-free protease inhibitor cocktail tablet (Roche, cat. no. 11873580001)
- ECL Plus Western Blotting Detecting System (GE Healthcare, cat. no. RPN2132)
- Rb pAb Syntaxin 2, 50 µg (1µg/µL), ab 12369, lot. Gr 5596-2 (Abcam)
- Rb pAb PSD-95, 100 µg (1µg/µL), ab 18258, lot. Gr 1360-1 (Abcam)
- Beta-actin, 200 µg/µL, (C4) SC-47778, lot. B2410, mouse monoclonal IgG (Santa Cruz)
- Precision Plus Protein All Blue Standards, 500 µL, cat. no. 161-0373
- Criterion™ TGX Any kD™ Precast Gel cat. no. #567-1125 (Bio-Rad)
- Bio-Rad Trans-Blot Turbo transfer pack, midi format 0.2 µm, cat. no. #170-4157
- iTRAQ® Reagents Multiplex Kit P/N 4352135 lot. 0903101
- Pep Clean C-18 Spin Colum, prod. #89870, lot. #LC 12929910
- Protein Assay Bradford cat. no. #500-0006 (Bio-Rad)

Appendix II

Table 6. Sucrose consumption test for Control rats

Animal ID	Baseline	Week 1	Week 2	Week 3	Week 4	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

Table 7. Sucrose consumption test for stress-resilient rats

Animal ID	Baseline	Week 1	Week 2	Week 3	Week 4	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

Table 8. Sucrose consumption test for anhedonic-like rats

Animal ID	Baseline	Week 1	Week 2	Week 3	Week 4	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