Pathway and biomarker discovery in a posttraumatic stress disorder mouse model

Chi-Ya Kao

Dissertation der Graduate School of Systemic Neurosciences der Ludwig-Maximilians-Universität München

Munich, 8th July 2015
Pathway and biomarker discovery in a posttraumatic stress disorder mouse model

Dissertation of the Graduate School of Systemic Neurosciences at the Ludwig-Maximilians-University Munich

submitted by Chi-Ya Kao

Munich, 8th July 2015
First reviewer/supervisor: Prof. Dr. Christoph W. Turck

Second reviewer: Dr. Carsten T. Wotjak

Third reviewer: Prof. Dr. Anja Horn-Bochtler

Date of defense: 15th December, 2015
To my beloved parents
Abstract

Posttraumatic stress disorder (PTSD), a prevalent psychiatric disorder, is caused by exposure to a traumatic event. Individuals diagnosed for PTSD not only experience significant functional impairments but also have higher rates of physical morbidity and mortality. Despite intense research efforts, the neurobiological pathways affecting fear circuit brain regions in PTSD remain obscure and most of the previous studies were limited to characterization of specific markers in periphery or defined brain regions. In my PhD study, I employed proteomics, metabolomics and transcriptomics technologies interrogating a foot shock induced PTSD mouse model. In addition, I studied the effects of early intervention of chronic fluoxetine treatment. By in silico analyses, altered cellular pathways associated with PTSD were identified in stress-vulnerable brain regions, including prelimbic cortex (PrL), anterior cingulate cortex (ACC), basolateral amygdala (BLA), central nucleus of amygdala (CeA), nucleus accumbens (NAc) and CA1 of the dorsal hippocampus. With RNA sequencing, I compared the brain transcriptome between shocked and control mice, with and without fluoxetine treatment. Differentially expressed genes were identified and clustered, and I observed increased inflammation in ACC and decreased neurotransmitter signaling in both ACC and CA1. I applied in vivo $^{15}$N metabolic labeling combined with mass spectrometry to study alterations at proteome level in the brain. By integrating proteomics and metabolomics profiling analyses, I found decreased Citric Acid Cycle pathway in both NAc and ACC, and dysregulated cytoskeleton assembly and myelination pathways in BLA, CeA and CA1. In addition, chronic fluoxetine treatment 12 hours after foot shock prevented altered inflammatory gene expression in ACC, and Citric Acid Cycle in NAc and ACC, and ameliorated conditioned fear response in shocked mice. These results shed light on the role of
Abstract

immune response and energy metabolism in PTSD pathogenesis. Furthermore, I performed microdialysis in medial prefrontal cortex and hippocampus to measure the changes in extracellular norepinephrine and free corticosterone (CORT) in the shocked mouse and related them to PTSD-like symptoms, including hyperarousal and contextual fear response. I found that increased free CORT was related to immediate stress response, whereas norepinephrine level, in a brain region specific manner, predicted arousal and contextual fear response one month after trauma. I also applied metabolomics analysis to investigate molecular changes in prefrontal microdialysates of shocked mice. Citric Acid Cycle, Glyoxylate and Dicarboxylate metabolism and Alanine, Aspartate and Glutamate metabolism pathways were found to be involved in foot shock induced hyperarousal. Taken together, my study provides novel insights into PTSD pathogenesis and suggests potential therapeutic applications targeting dysregulated pathways.
Table of contents

Abstract........................................................................................................................................... i

Table of contents.............................................................................................................................. iii

Abbreviations.................................................................................................................................... v

1. INTRODUCTION .......................................................................................................................... 1

1.1. History, epidemiology and diagnosis of PTSD ................................................................. 1

1.2. Neurobiology of PTSD........................................................................................................... 3

1.2.1. Alterations in the neuroendocrine system ................................................................. 3

1.2.2. Neurotransmitter system in PTSD .............................................................................. 6

1.2.3. Alterations of the immune system .............................................................................. 9

1.2.4. Pathoanatomical alterations associated with PTSD .............................................. 10

1.2.4.1. Prefrontal cortex ....................................................................................................... 12

1.2.4.2. Hippocampus .......................................................................................................... 13

1.2.4.3. Amygdala ................................................................................................................. 14

1.2.4.4. Nucleus accumbens ................................................................................................. 15

1.3. Risk factors and biomarkers of PTSD ............................................................................. 16

1.3.1. Risk factors ...................................................................................................................... 16

1.3.2. Biomarkers ...................................................................................................................... 19

1.4. Pharmaco- and psychological therapy of PTSD ............................................................ 20

1.5. Animal models of PTSD ................................................................................................... 22

1.5.1. PTSD animal model ....................................................................................................... 23

1.6. Hypothesis-free –omics: quantitative proteomics and metabolomics, and RNA-seq
transcriptomics ............................................................................................................................. 25

1.6.1. Quantitative proteomics ............................................................................................... 26

1.6.1.1. Mass spectrometry based proteomics ....................................................................... 27

1.6.1.2. Quantitative proteomics using stable isotope labeling ........................................ 28

1.6.2. Quantitative metabolomics ........................................................................................... 29

1.6.3. Quantitative transcriptomics .......................................................................................... 31

1.6.4. Computational biology/Systems biology ..................................................................... 32

1.7. Microdialysis .......................................................................................................................... 33
Table of contents

1.8. Aims of the thesis ............................................................................................................ 35

2. RESEARCH ARTICLES .................................................................................................... 37
2.1. Fluoxetine treatment rescues energy metabolism pathway and myelin sheath protein alterations in a posttraumatic stress disorder mouse model ................................................................. 37
2.2. Transcriptomic profiling reveals affected inflammatory pathway in a mouse model of posttraumatic stress disorder ......................................................................................................................... 79
2.3. Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice ......................................................................................................................... 119
2.4. NextGen Brain Microdialysis – applying modern metabolomics technology to the analysis of extracellular fluids ......................................................................................................................... 130

3. DISCUSSION AND FUTURE PERSPECTIVES ................................................................ 139
3.1. Energy metabolism ........................................................................................................ 139
  3.1.1. Altered energy metabolism in psychiatric diseases ...................................................... 139
  3.1.2. Interaction between energy metabolism, inflammatory response and neuroendocrine function ................................................................................................................................. 141
  3.1.3. Role of energy metabolism in different neurons ........................................................ 142
3.2. Systemic view of PTSD brain circuitry .......................................................................... 143
3.3. Fluoxetine treatment ..................................................................................................... 145
3.4. Future perspectives ....................................................................................................... 146

Bibliography ....................................................................................................................... 148

Acknowledgements ............................................................................................................. 183

Curriculum vitae and publications ..................................................................................... 185

List of author contributions ............................................................................................... 187

Affidavit ............................................................................................................................... 188
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HTTLPR</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>API</td>
<td>atmospheric pressure ionization</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
</tr>
<tr>
<td>CA1</td>
<td>cornu ammonis 1</td>
</tr>
<tr>
<td>CAGE</td>
<td>cap analysis of gene expression</td>
</tr>
<tr>
<td>CeA</td>
<td>central nucleus of amygdala</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRHR1</td>
<td>CRH receptor 1</td>
</tr>
<tr>
<td>CR-PTSD</td>
<td>combat-related PTSD</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>ETD</td>
<td>electron transfer dissociation</td>
</tr>
<tr>
<td>FKBP5</td>
<td>FK506 binding protein 5</td>
</tr>
<tr>
<td>FS</td>
<td>foot shock</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GLT-1</td>
<td>glutamate transporter-1</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptors</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome-wide association studies</td>
</tr>
<tr>
<td>HCD</td>
<td>high energy collision dissociation</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICAT</td>
<td>isotope-coded affinity tag</td>
</tr>
<tr>
<td>ICPL</td>
<td>isotope-coded protein label</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL</td>
<td>infralimbic cortex</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>isobaric tag for relative and absolute quantitation</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>LTQ</td>
<td>linear ion trap</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted-laser-desorption ionization</td>
</tr>
<tr>
<td>mPFC</td>
<td>medial prefrontal cortex</td>
</tr>
<tr>
<td>MPSS</td>
<td>massively parallel signature sequencing</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptors</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple-reaction-monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
</tr>
<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>NCS</td>
<td>National Comorbidity Survey</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κ B</td>
</tr>
<tr>
<td>NGFI-A</td>
<td>nerve growth factor-induced protein A</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating peptide</td>
</tr>
<tr>
<td>PGC</td>
<td>Psychiatric Genomics Consortium</td>
</tr>
<tr>
<td>PrL</td>
<td>prelimbic cortex</td>
</tr>
<tr>
<td>PTSD</td>
<td>posttraumatic stress disorder</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>Q</td>
<td>quadrupole</td>
</tr>
<tr>
<td>QqQ</td>
<td>triple-quadrupole</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>quadrupole-time-of-flight</td>
</tr>
<tr>
<td>RNA-seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxidative species</td>
</tr>
<tr>
<td>SAGE</td>
<td>serial analysis of gene expression</td>
</tr>
<tr>
<td>SAM</td>
<td>sympathetic-adrenal-medullary</td>
</tr>
<tr>
<td>SCZ</td>
<td>schizophrenia</td>
</tr>
<tr>
<td>SILAC</td>
<td>stable isotope labeling using amino acids in cell culture</td>
</tr>
<tr>
<td>SILAM</td>
<td>stable isotope labeling of mammals</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SPS</td>
<td>single prolonged stress</td>
</tr>
<tr>
<td>SRM</td>
<td>selected-reaction-monitoring</td>
</tr>
<tr>
<td>SSRIs</td>
<td>selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>vmPFC</td>
<td>ventral medial prefrontal cortex</td>
</tr>
</tbody>
</table>
Abbreviations

VTA  ventral tegmental area
1. INTRODUCTION

1.1. History, epidemiology and diagnosis of PTSD

The term Trauma was originally used to describe physical wound or injury (Spiers and Harrington, 2001). Later in medicine and psychiatry literature, trauma was referred to a wound inflicted upon the mind rather than the body (Caruth, 1996). During the 19th century and into the mid-20th century, there was an ongoing debate whether traumatic disorder was psychological or physiological. Not until post-World War II was the concept of interaction between psychology and neurophysiology for traumatic disorders identified and recognized (Abram, 1970; Selye, 1974).

What we call PTSD nowadays was first classified as “Stress Response Syndrome” caused by tremendous stress reaction under the category of transient situational personality disorder in the first edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-I) published by the American Psychiatric Association (APA, 1952). In 1980, PTSD was first officially mentioned in DSM-III (APA, 1980). The revised DSM-III (APA, 1987) then separated the cause of PTSD from ordinary stressors. In the current DSM-5 (APA, 2013) PTSD was officially removed from anxiety disorder to a new category of trauma- and stress-related disorder with more distinct diagnoses based on behavioral symptoms.

The epidemiology of PTSD was not recognized until 1980 in DSM-III (APA, 1980). Described by the National Comorbidity Survey (NCS), the life-time prevalence of PTSD was 10.4 % in females and 5.0 % in males of the U.S. population (Kessler et al, 1995), while in Germany was reported to be 2.2 % in females and less than 1 % in males (Perkonigg et al, 2000). The definition of PTSD starts with an exposure to trauma, a stressor that is not in the range of normal human experience. Among those individuals who were exposed to severe trauma, about 10-20% will develop PTSD. The risk for developing PTSD depends on the
1. INTRODUCTION

stress dose and coping strategy of the affected individual. Other risk factors including childhood stress/trauma, domestic violence, gender or pre-existing anxiety/depression also impact the onset of PTSD (Foa et al, 2006; Yehuda et al, 2011a).

The development of PTSD is often preceded by acute stress disorder. The symptoms usually begin within the first three months after encountering the trauma but may also initiate more than six months after the stressor (Ballenger et al, 2000; Brunello et al, 2001; Kessler, 2000).

To date, the diagnosis of PTSD relies much on self-reports, clinical expert interviews and clinical behavioral monitoring. According to DSM-5, five criterions should be met for the diagnosis of PTSD:

Criterion A – The patient was exposed to a traumatic event, such as death threat, serious injury or sexual violence or has witnessed another person being exposed to such tragic events, with the response of intense fear.

Criterion B – Intrusion (re-experiencing) symptoms which include intrusive memories, flashbacks as well as nightmares of the traumatic event. Re-experiencing symptoms are usually triggered by environmental cues that remind the patients of the tragic experience.

Criterion C – Symptoms which involve avoiding thoughts, feelings, places, activities or people associated with the traumatic event.

Criterion D – Emotional numbing, decreased interest and symptoms of negative alterations in cognition and mood related to the traumatic experience.

Criterion E – Hyperarousal symptoms, including sleep difficulties, hypervigilance, exaggerated startle response, aggressiveness, loss of concentration or self-destructive behavior.
1. INTRODUCTION

1.2. Neurobiology of PTSD

1.2.1. Alterations in the neuroendocrine system

Upon stressor stimulation, glucocorticoids (GC) and catecholamines play a major role in regulating stress response and result in brain metabolism and behavior changes. Immediate release of catecholamines induces an acute response to the stressors and is regulated by sympathetic-adrenal-medullary (SAM) axis. GC secretion, on the other hand, affects long-term adaption processes and is regulated by hypothalamic-pituitary-adrenal (HPA) axis.

HPA axis is composed of the paraventricular nucleus (PVN) of hypothalamus, the anterior pituitary and the cortex of the adrenal gland. Stressor first simulates corticotropin-releasing hormone (CRH) release from parvocellular secretory neurons which project from the hypothalamic PVN to the median eminence of the hypothalamic-hypophyseal portal circulation. CRH is then transported to anterior pituitary via the blood vessel system and stimulates the secretion of adrenocorticotropic hormone (ACTH). Subsequently, ACTH is transported to the adrenal cortex, where it rapidly stimulates biosynthesis and release of GC (cortisol for human and corticosterone for rodents) (Papadimitriou and Priftis, 2009) as well as the mineralocorticoids (Funder, 2010). Under physiological conditions, HPA axis activation is regulated by two negative feedback loops, inhibitory effects of cortisol on the ACTH-secreting cells in the anterior pituitary and the CRH-secreting neurons in the hypothalamus. In addition, limbic brain regions involved in emotional response to stress modulate HPA axis activity, with frontal cortex and hippocampus inhibiting while amygdala increasing HPA axis activity (Herman et al, 2005).

In addition to HPA axis, stress exposure activates SAM axis immediately, which leads to production and release of the catecholamines (norepinephrine, epinephrine) from adrenal medulla. Meanwhile, stressors induce norepinephrine release from locus coerules (LC)
neurons in the forebrain regions, including frontal cortex, hippocampus and amygdala (Robertson et al, 2013). Increased GC and catecholamines evoke physical stress response, the “fight-or flight” response, by increasing heart rate, blood pressure and pupil dilation while inhibiting digestion, urination or reproductive functions (Carrasco and Van de Kar, 2003) to prepare the organism to survive and deal with stressors.

Many studies have indicated HPA and SAM axes activity dysregulations in PTSD patients. However, in clinical studies, inconsistent findings of cortisol levels were observed in PTSD patients. Increased cortisol reactivity in response to psychosocial stress has been observed in sexual and physical childhood abuse patients (Heim et al, 2000). On the other hand, lower cortisol secretion has been reported in patients with PTSD (Morris et al, 2012; Yehuda and Seckl, 2011b). Yet, Wingenfeld et al. (Wingenfeld et al, 2015) found lower cortisol levels only in patients with lifetime PTSD, but not in patients with current PTSD. This phenomenon may underlie the biological significance reflecting dose-dependent and time course effects of cortisol release. In addition to PTSD, abnormalities in cortisol secretion and HPA axis activity were also reported in other depressive and anxiety-related disorders. Hypercortisolism was found in major depression patients, with hypersecretion of cortisol at baseline and in the dexamethasone suppression test (Parker et al, 2003). Abelson and Curtis (Abelson and Curtis, 1996) observed that alterations in HPA system in panic disorder patients are modulated by illness severity and treatment seeking behavior. The shared dysregulation of HPA axis in major depression, PTSD and other stress-related disorders might in part explain the high comorbidity of these psychiatric disorders.

Release of GC induces massive changes throughout the whole body, primarily activating adaptation mechanisms. Chronic stimulation of GC release by stress may initiate differential regulation of its downstream targets, i.e. GC receptors. Two types of receptors mediate GC
actions in the brain. Mineralocorticoid receptors (MR) bind cortisol with nearly ten-fold higher affinity than glucocorticoid receptors (GR) (Veldhuis et al, 1982). At basal or during mild stressful conditions cortisol mostly binds to MR to enhance synaptic strength (LTP). During cortisol level elevation, GRs become fully occupied and LTP induction is impaired (de Kloet et al, 2005; Joëls et al, 2006). This dual system may work in opposing directions regulating the behavioral responses (Pavlides et al, 1995). Chronic GC hypersecretion results in MR and GR downregulation. In a rat model of single-prolonged-stress PTSD, MR and GR levels were found decreased (Zhe et al, 2008). In addition, GC sensitivity correlates with symptom changes in combat veteran PTSD patients and GC receptor hypersensitivity has been shown to develop after childhood trauma and (later) PTSD, indicating the important roles of MR and GR in the clinical state (McGowan et al, 2009; Yehuda et al, 2014). Moreover, GC regulates the HPA system via a negative feedback loop by inhibiting ACTH and CRH secretions. Elevated CRH levels are associated with increased fear-potentiated startle response (Keen-Rhinehart et al, 2009; Pelton et al, 1997) and enhanced fear conditioning (Roozendaal et al, 2002). Higher concentrations of CRH in the cerebrospinal fluid were observed in PTSD patients (Baker et al, 1999).

Catecholamine (norepinephrine) not only regulates sleep and arousal, but also attention, learning and memory. Activation of the norepinephrinergic system mediates consolidation of memories associated with emotional events (Barsegyan et al, 2014; Soeter and Kindt, 2011). Dysregulated norepinephrine signaling has been linked to PTSD symptom severity, with chronic PTSD patients showing greater CNS norepinephrinergic activity than healthy subjects (Geracioti et al, 2001). In another study, facilitation of norepinephrine signaling by using the α2 selective antagonist yohimbine resulted in increased anxiety in PTSD patients (Bremner et al, 1997a; Southwick et al, 1997). However, administration of the β receptor antagonist propranolol showed mixed results both in clinical trials and in animal models of
1. INTRODUCTION

PTSD (Brunet et al, 2008; Cohen et al, 2011; Dębiec et al, 2011; Pitman et al, 2002; Stein et al, 2007). These studies indicate a dual role of norepinephrine, both adaptive and deteriorating, in the mediation of the post-stress response (Jett and Morilak, 2013; Lapiz and Morilak, 2006). In addition to regulation of stress responses, norepinephrine and GC interact with each other and mediate downstream events. Several studies indicated that GC affects α1-adrenergic receptor function and modulates α- and β-adrenergic receptor-coupled signaling (Duman et al, 1989; Stone et al, 1987). Moreover, local administration of β-adrenergic receptor antagonist propranolol in the amygdala prevented GC-induced memory enhancement from emotional arousal-induced norepinephrine activation (Quirarte et al, 1997; Roozendaal et al, 2006). In PTSD patients, interaction between norepinephrine and cortisol was shown to be predictive for negative intrusive memories in PTSD (Nicholson et al, 2014).

1.2.2. Neurotransmitter system in PTSD

Central neurotransmitter imbalance has been linked to the pathophysiological mechanisms of PTSD. Traumatic experiences activate certain neuronal circuits and induce long-lasting changes in neurotransmitter systems. In addition to the abnormalities in norepinephrine and GC signaling described in chapter 1.2.1, monoaminergic neurotransmitters, including serotonin and dopamine, were found dysregulated in PTSD. Clinical studies showed that combat-related PTSD (CR-PTSD) is associated with enhanced norepinephrinergic activity and diminished serotonin activity (Spivak et al, 1999). In addition, hypofunction of serotonergic system was associated with PTSD symptoms, i.e. increased startle response and impulsive aggression in chronic CR-PTSD. Treatment of CR-PTSD patients with SSRIs showed improvements in symptoms including arousal, intrusion, irritability and avoidance (Marmar et al, 1996). Increased central serotonin function has been shown to play an
important role in stress adaptation in animal studies (Joseph and Kennett, 1983). A significantly lower binding affinity of amygdala serotonin transporter was found in PTSD patients compared to healthy subjects (Murrough et al, 2011). A meta-analysis revealed an association between SS genotype of the serotonin transporter (5-HTTLPR) polymorphism and PTSD in high trauma-exposed patients, implicating the role of 5-HTTLPR polymorphism as a risk factor for developing PTSD (Gressier et al, 2013). Taken together, serotonin deficit is an important factor for the development and persistence of PTSD symptoms.

The mesolimbic dopaminergic pathway also plays an important role in fear and anxiety. It was shown that over-activation of dopamine transmission resulted in increased fear response (Pezze and Feldon, 2004). Dysfunction of dopamine in frontal cortex is associated with intrusive thoughts and insufficient extinction of trauma-related memory, which are core features of PTSD (Morrow et al, 1999; Seamans and Yang, 2004). A clinical study showed that the decline of dopamine metabolite homovanillic acid levels was associated with laboratory-induced symptoms in chronic PTSD patients (Geracioti et al, 2013). On the other hand, higher urinary excretions of dopamine, norepinephrine and epinephrine were observed in PTSD outpatients, and dopamine and norepinephrine levels were correlated with the severity of PTSD (Yehuda et al, 1992). Similar to serotonergic transporters, dopamine transporter single nucleotide polymorphisms are associated with the occurrence of PTSD among trauma survivors (Segman et al, 2002). Meanwhile, cross talk between norepinephrine, serotonin and dopamine has been reported such that norepinephrine stimulates serotonin and dopamine release while serotonin release from norepinephrine neurons reduces norepinephrine release (Fink and Göthert, 2007). These studies implicate the importance of balanced monoaminergic neurotransmitter signaling for PTSD underlying mechanisms.

Besides dysfunctional monoaminergic pathways, alterations in amino acid neurotransmitter signaling have been implicated in PTSD. Higher levels of gamma-aminobutyric acid (GABA)
1. INTRODUCTION

in dorsolateral prefrontal cortex and anterior cingulate cortex were identified in PTSD patients (Michels et al, 2014). However, in human plasma, low GABA levels after encountering a traumatic event may predict subsequent PTSD development (Vaiva et al, 2004). In addition to GABA itself, imbalance between GABA and glutamate is associated with hippocampal neuronal apoptosis in the PTSD animal model (Gao et al, 2014). Glutamatergic neurotransmission is known to be involved in the stress response and anxiety-related disorders (Popoli et al, 2012). The glutamatergic system regulates acquisition and extinction of fear conditioning. Stress-induced glutamate release facilitates memory consolidation by inducing long-term potentiation (Anwyl, 2009). Treatment with the anticonvulsivant drug, phenytoin, which may reduce glutamate neurotransmission, has demonstrated significant efficacy in combat PTSD by reducing avoidance and arousal symptoms (Bremner et al, 2005a). Furthermore, blocking N-methyl-D-aspartate (NMDA) receptors prior to exposure to a predator scent stress prevented anxiety-like behaviour in rodents (Blundell et al, 2005). Thoreinger et al. showed that AMPA receptor GluR1 signaling in dentate gyrus is involved in consolidation of remote fear memories via CRH receptor type 1 pathway (Thoeringer et al, 2012). These studies provide evidence for the involvement of glutamatergic signaling in PTSD pathogenesis. Interestingly, recent findings suggest that polyamine modulates ionotropic glutamate receptors and the metabolism was shown to be disrupted in anxiety and depression (Fiori and Turecki, 2008; Vaquero-Lorenzo et al, 2009). Finally, neuropeptides, e.g. neuropeptide Y (NPY), has been shown to be involved in PTSD development (Sah and Geracioti, 2013). Recent studies suggest a protective effect of NPY for PTSD via its function in regulating fear conditioning and extinction and suppressing startle responses (Fendt et al, 2009; Karl et al, 2010b). Another neuropeptide, PACAP, which stimulates CRH secretion in the hypothalamus (Grinevich et al, 1997), has been shown to be associated with female PTSD (Ressler et al, 2011).
1.2.3. Alterations of the immune system

As mentioned in chapter 1.2.1, physical and psychological stress induces the release of stress hormone. Stress hormones alter the status of the immune system (Glaser and Kiecolt-Glaser, 2005), and in turn, immune system disturbance affects CNS function via humoral and cellular pathways conveying signals to the brain (Maier and Watkins, 1998). Recent studies have indicated an altered inflammatory response in various psychiatric disorders, including schizophrenia (de Baumont et al, 2015; Neelamekam et al, 2014), major depression (Maes, 1995, 2008), and anxiety-related disorders (Ogłodek et al, 2015; Solati et al, 2015). It is known that chronic stress exposure affects sympathetic and steroid hormone pathways, and results in alterations of lymphocyte migration and numbers (Silberman et al, 2004). Upon stressors, the expression of cytokine and chemokine genes for mobilizing the immune cells is regulated so that the tissue function can be restored upon intrusions (Black and Garbutt, 2002). Depending on the nature, intensity, and duration of the stressor, different types of immune response are challenged and stress effects on the immune system differ. Exposure to prolonged stressors induces immunosuppression and results in anti-inflammatory response that makes the subject more susceptible to diseases (Cohen et al, 2012). On the other hand, repeated defeat stress (e.g., repeated social defeat) prevents the GC-induced suppression of inflammation via inhibition of nuclear factor κ B (NFκB) pathway and results in an enhancement of the immune response (Barnes and Adcock, 2009; Chrousos et al, 1996).

Recent studies have shed light on the role of the immune response in anxiety-related disorders. Studies on adult male Sprague-Dawley rats revealed that chronic unpredictable restraint stress exposure selectively increases the number of microglial cells and results in a transition of microglia from a ramified-resting state to a non-resting state in certain stress-vulnerable brain regions, including anterior cingulate cortex, infralimic medial prefrontal
1. INTRODUCTION

cortex, nucleus accumbens, medial amygdala, dorsal bed nucleus of the stria terminalis, CA3 region of the hippocampus and periaqueductal gray (Tynan et al., 2010). These finding indicate that microglial activation might play an important role in the regulation and/or adaptation to stress. In addition, women with PTSD due to childhood sexual or physical abuse showed enhancement in delayed-type hypersensitivity skin test which suggests higher cell-mediated inflammatory reactions (Altemus et al., 2003). In the psychogenic stress-exposed rats, treatment of minocycline, an anti-inflammatory, anti-apoptotic and neuroprotective tetracycline agent, reduced local levels of the cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) in the hippocampus, frontal cortex and hypothalamus, and attenuated anxious-like behaviors (Levkovitz et al., 2015). Taken together, dysregulations in immunological processes upon psychological and physiological stress may disturb the adaptation/resilience to stress and therefore results in PTSD development.

1.2.4. Pathoanatomical alterations associated with PTSD

Development of PTSD involves memory- and stress-related processes (Siegmund and Wotjak, 2006). Maintenance of strong memory of an aversive encounter due to resistance to extinction, memory reinstatement, too fragile inhibitory mechanisms, overgeneralization or disturbance of declarative memory is a potent cause for PTSD (Bremner et al., 1992; Charney et al., 1993; McFarlane et al., 2002; Solomon et al., 1987). Different brain structures are associated with the memory process in PTSD. Brain imaging studies in PTSD patients during fear conditioning and in response to trauma-related stimuli showed an increased activity in amygdala, the brain region responsible for expression of conditioned fear, and decreased activity in prefrontal cortex, the brain region related to extinction (Bremner, 2002; Bremner et al., 2005b). In addition, the size of the hippocampus (a brain structure for contextual fear memory formation) seems to have an inverse correlation with the susceptibility of developing
PTSD (Gilbertson et al., 2002). Traumatic events not only result in the formation of associative memories but also sensitize individuals in a non-associative manner, increasing the general responsiveness to potentially harmful stimuli. The hyper-responsiveness in amygdala and medial prefrontal cortex observed in PTSD patients is in accordance with slower habituating activity in response to trauma-related stimuli (Shin et al., 2005). Meanwhile, studies with rats and mice have demonstrated that a single exposure to an extreme stressor may cause long-lasting changes in neurochemistry (Martí et al., 2001; van Dijken et al., 1993), startle response (Balogh et al., 2002) and electrical excitability of the fear circuit (Adamec et al., 2005; Adamec et al., 2001).

In addition to an investigation of the role of individual brain region, recent studies have aimed to establish a functional neuronal network in which local circuits interact to transfer signals across different brain regions for the generation of behavioral responses that are relevant for mental disorders (Bielczyk et al., 2015; Robinson et al., 2014). The amygdala is a key brain region regulating acquired and innate fear and anxiety-related behaviors (Krettek and Price, 1978; Maren and Quirk, 2004). Basolateral amygdala (BLA), which exhibits cortex-like function, receives sensory afferents from somatosensory cortex as well as thalamus (Pape and Pare, 2010; Tovote et al., 2015). Strong inputs from the ventral hippocampus and the medial prefrontal cortex (mPFC), with the prelimbic cortex contribute to sustained fear. The infralimbic cortex is critical for fear memory extinction (Quirk and Mueller, 2008) and projects to both BLA and intercalated cells between BLA and central nucleus of amygdala (CeA). Activated intercalated cells in turn directly or indirectly inhibit CeA output neurons (Pare and Duvarci, 2012). On the other hand, BLA also sends projections to forebrain structures, including the ventral hippocampus and mPFC (Pitkänen et al., 2000) to regulate fear acquisition/extinction and anxiety-related behaviors. mPFC and amygdala send excitatory afferents to the ventral tegmental area (VTA), and dopamine
neurons from VTA project to nucleus accumbens (NAc) as well as to the mPFC (Fields et al., 2007). Synaptic connections between mPFC, amygdala, hippocampus, VTA and NAc reflect a neural circuit that underlies pathological behavior responses in anxiety-related disorders (Figure 1).

**Figure 1. Brain circuits involved in fear perception.** Simplified scheme depicting different projections from distinct brain regions for regulation of fear response. Red, blue and yellow arrows represent DA, glutamate, and GABA projections, respectively. Adapted from Lüthi and Lüscher (Lüthi and Lüscher, 2014).

**1.2.4.1. Prefrontal cortex**

The prefrontal cortex integrates sensory information received from various brain regions within the limbic system, regulates emotions and initiates goal-oriented behaviour (Gray et al., 2002; Miller et al., 2002a). Preclinical studies have shown that medial prefrontal cortex (mPFC) plays a crucial role in both the acquisition and extinction of fear response (Markham *et al*, 2012; Morgan *et al*, 1993). The mPFC is composed of anterior cingulate (ACC),
prelimbic (PrL) and infralimbic (IL) cortices. PrL is associated with increased fear expression while IL has inhibitory functions for the fear response (Corcoran and Quirk, 2007; Fenton et al, 2014; Laurent and Westbrook, 2009). PTSD is associated with diminished responsivity in the adjacent ventral mPFC (vmPFC) (Shin and Liberzon, 2010). Functional neural imaging studies of PTSD revealed a failure or decreased activation of ACC during the presentation of trauma-related stimuli (Hou et al, 2007; Yang et al, 2004) and negative, non-traumatic stimuli (Kim et al, 2008; Williams et al, 2006). In addition, activation of ACC appears to be inversely related to PTSD symptom severity (Kim et al., 2008; Williams et al., 2006) and positively associated with symptomatic improvement following treatment (Felmingham et al, 2007; Peres et al, 2007). Furthermore, decreases in vmPFC (Shin et al, 2010) and ACC (Corbo et al, 2005) volumes were found in PTSD patients and smaller ACC volumes have been linked with greater PTSD symptom severity (Woodward et al, 2006). Studies with rats exposed to chronic retrain stress demonstrated reductions in prefrontocortical (ACC and prelimbic area) dendrite lengths (Radley et al, 2004) which might underlie the mechanism for PFC volume loss and cognitive impairments in stress-related disorders.

1.2.4.2. Hippocampus

The hippocampus is involved in formation and maintenance of contextual and declarative memory, as well as in memory consolidation for generating long-term memory (Elzinga and Bremner, 2002). Hypoactivation of hippocampus was found in PTSD patients and inversely correlated with symptom severity (Shin and Liberzon, 2010). Other studies, however, have reported increased activation in the hippocampus in PTSD (Geuze et al, 2007; Werner et al, 2009).
1. INTRODUCTION

Some, but not all studies reported a reduction in hippocampal volume in PTSD patients (Bossini et al., 2008; Golier et al., 2005; Karl et al., 2006; Pederson et al., 2004). Studies with twins demonstrated decreased hippocampal volumes may be a familial risk factor for developing PTSD following traumatic events (Gilbertson et al., 2002). Moreover, N-acetylaspartate (NAA), a marker for neuronal integrity, has been associated with dysfunctional hippocampal neuronal networks in PTSD patients (Schuff et al., 2006), a finding also observed in the PTSD mouse model applied in the current study (Siegmund et al., 2009). The mechanisms underlying stress-induced hippocampal shrinkage still remain elusive. GC has been proposed to be involved in the development of hippocampal volume loss. Chronic exposure to GC has neurotoxic effects (You et al., 2009), partially mediated by GC-induced increase in hippocampal glutamate levels and subsequent excitotoxic effects (McEwen, 1997). Studies in PTSD rat models suggested that stress-stimulated alterations in hippocampal expression levels of GC receptors (Kohda et al., 2007) and NMDA receptors (Yamamoto et al., 2010) might aggravate the neurotoxic effects of increased GC and glutamate levels. Furthermore, Golub et al. found a long-lasting reduced hippocampal volume possibly due to shrinkage of axonal protrusions in an electric foot shock-induced PTSD mouse model (Golub et al., 2011).

1.2.4.3. Amygdala

Hyperactivation of the amygdala has been repeatedly shown in PTSD patients (Bremner et al., 2005b; Morey et al., 2009; Vermetten et al., 2007). In addition, some studies have demonstrated that the activation of amygdala correlated positively with PTSD symptom severity (Dickie et al., 2008; Rauch et al., 2000). As hippocampus and PFC exert inhibitory actions on the amygdala, hypoactivity of these two brain regions might further result in
enhancement of amygdala hyperresponsivity which in turns deteriorates emotional processing upon stress (Rauch et al., 2006). Moreover, PTSD resilience was associated with reduced amygdala activation (Osuch et al., 2008) and amygdala lesions may decrease the occurrence of PTSD (Koenigs et al., 2008).

Several studies reported shrinkage of amygdala volume in PTSD patients (Karl et al., 2006; Rogers et al., 2009). Findings from animal studies suggested that apoptosis in the amygdala contributes extensively to the volume loss (Ding et al., 2010; Liu et al., 2011). Increases in dendritic arborisation and spine density were found in animal models of PTSD and were proposed to be involved in hyperactivation of the amygdala (Adamec et al., 2012; Mitra et al., 2005). Furthermore, the abnormalities in the dendritic spine density are thought to affect the long-term maintenance of fear memories, which is found disturbed in PTSD (Mitra et al., 2005).

1.2.4.4. Nucleus accumbens

Nucleus accumbens (NAc) is the brain structure involved in motivation and addiction. Upon acute stressor (Wang et al., 2005) or arousing environmental stimuli (Merali et al., 2004), CRH is released from paraventricular nucleus of hypothalamus and acts on the NAc. Subsequently, dopamine release is increased through co-activation of CRH R1 and R2 in the NAc, and facilitates cue-elicited motivation (Peciña et al., 2006) and social bonding (Lim et al., 2007). In a mouse study, severe stress exposure abolished CRH’s capacity to regulate dopamine release in the NAc and switch the positive-affective state of CRH-dopamine interaction to an aversive state (Lemos et al., 2012). These studies demonstrated that severe stress produces a persistent dysregulation of CRH-dopamine interactions in the NAc and results in depression-like phenotype in the mouse. Krishnan et al. observed increased BDNF
1. INTRODUCTION

protein levels in NAc of social defeat susceptible mice. Furthermore, reducing BDNF release from ventral tegmental area (VTA) to NAc enhances resistance to stress (Krishnan et al, 2007).

In both animal and human studies, increased alcohol consumption and drug addiction are closely associated with traumatic event encounter (Bremner et al, 1996; Cross et al, 2015; Volpicelli and Ulm, 1990). Studies on women exposed to childhood rape report turning to alcohol to reduce PTSD symptoms (Epstein et al, 1998). Furthermore, 40% of inpatients receiving treatment for substance abuse also met the criteria or PTSD (Dansky et al, 1997). It is known that drug addiction acts on the mesocorticolimbic system, which consists of the VTA and the brain regions that are innervated by projections from VTA, such as NAc, PFC and amygdala (Swanson, 1982). Studies have shown that addictive drug use induces synaptic potentiation in the VTA and subsequently triggers synaptic alteration in downstream brain structures (NAc and PFC), with further drug exposure (van Huijstee and Mansvelder, 2014). These findings indicate that abnormal neuronal signaling in the NAc, which is part of the mesocorticolimbic system, plays an important role in the comorbidity of alcohol and drug addiction in PTSD patients.

1.3. Risk factors and biomarkers for PTSD

1.3.1. Risk factors

The term risk factor refers to factors that preclude a causal involvement in disease. On the other hand a biomarker serves as an indicator for disease development and therapy response. As mentioned in chapter 1.1, only 10-20% of individual exposed to severe trauma will develop PTSD. To date, several important risk factors both externally (e.g. lack of social support) and internally (e.g. innate versus acquired) have been identified for PTSD.
development. A prior exposure to trauma or chronic stress increases dramatically the occurrence of PTSD (Davidson et al., 1991). In addition, the type and exposure time of the trauma are potent factors, with prior assaults at a young age upon subsequent traumatization raises the prevalence of PTSD significantly (Bremner et al., 1993; Breslau et al., 1999). Social and demographic factors, such as family instability, lower education and income, and being widowed or divorced are also associated with PTSD development (King et al., 1996). A past history of psychological problems or psychiatric disorder increases the risk for the occurrence of PTSD (McFarlane, 1989). Furthermore, epidemiologic studies showed that antisocial (Schnurr and Vielhauer, 1999) or neurotic personalities (Breslau et al., 1998) prior to the traumatic event predicted the development of PTSD.

Abnormalities in cortisol secretion and HPA axis activity underlie the pathophysiological mechanism for PTSD. Lower basal cortisol has been proposed to be a pre-existing risk factor for PTSD (Zoladz and Diamond, 2013). Studies showed that individuals with lower cortisol levels immediately after trauma have a greater risk for developing PTSD (Delahanty et al., 2005; Ehring et al., 2008; McFarlane et al., 2011). It is possible that lower cortisol leads to chronic enhancement of glucocorticoid receptor hypersensitivity which in turn results in the development of later PTSD after trauma (van Zuiden et al., 2013; Wingenfeld and Wolf, 2011).

Additional risk factors including genetic and epigenetic ones are considered to contribute to an individual’s predisposition to develop PTSD. Studies on trauma-exposed twin pairs revealed that approximately 30% of the PTSD symptoms are heritable and comorbidity of PTSD with other disorders may be partly due to shared environmental and genetic influences (Afifi et al., 2010). Investigations on the candidate genes for association of Single Nucleotide Polymorphisms (SNP) with PTSD symptoms have aimed to identify specific genetic regions involved in PTSD (Cornelis et al., 2010). Identification of candidate genes mainly focused on
1. INTRODUCTION

searching for genetic variations associated with the HPA axis. SNPs in the CRH receptor 1 (CRHR1) gene (Amstadter et al, 2011) and in the FK506 binding protein 5 (FKBP5) gene (Binder et al, 2008) were found to be associated with PTSD symptoms. FKBP5 regulates the cortisol-binding affinity of the glucocorticoid receptor and it is hypothesized that genetic variations in the CRHR1 and FKBP5 genes lead to the dysregulation of the HPA axis upon stress exposure and result in PTSD development. Besides genetic variations, epigenetic modifications caused by DNA methylation or histone modification also contribute to the risk for PTSD occurrence. Environmental factors like early-life stress can modify gene activity via epigenetic mechanisms. A study on holocaust survivors showed that maternal PTSD increases the risk for offspring to develop PTSD. This finding illustrates the transgenerational transmission of potential trauma-induced epigenetic modifications (Yehuda et al, 2008). In a study of survivors of the Rwandan genocide, increased DNA methylation at the nerve growth factor-induced protein A (NGFI-A) binding site of the GR gene was found to be related to less intrusive memory of the traumatic event and reduced PTSD risk in male but not female survivors. These findings indicate that epigenetic modification of GRs is associated with gender-specific PTSD risk (Vukojevic et al, 2014). A recent study showed that a SNP in the PAC1 receptor, which is involved in stress response by stimulating CRH gene expression, is associated with PTSD in female patients. Furthermore, methylation of the PAC1 receptor gene has been correlated with PTSD symptom severity (Ressler et al, 2011). Taken together, the findings on genetic and epigenetic variations of HPA axis associated genes underscore the importance of the neuroendocrine stress response system for PTSD pathogenesis and vulnerability.
1.3.2. Biomarkers

The term biomarker was defined by the Biomarker Definitions Working Group at National Institutes of Health as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). In addition to providing a diagnostic tool in the clinical setting, the search for new biomarkers aims to further elucidate the mechanisms underlying pathogenic processes. A wide variety of indicators can serve as biomarkers: genomic alterations such as single nucleotide polymorphisms (SNPs), alterations in gene or protein expression, or changes in metabolite profile. Genomic alterations or mutations are trait biomarkers that can predict the likelihood of developing a disease and indicate disease susceptibility. On the other hand, proteins and metabolites represent dynamic biomarkers, able to reflect the disease state. Besides molecular markers, altered brain activity evaluated by fMRI or PET imaging and other physiological parameters such as blood pressure or heart rate can also serve as biomarkers (Hampel et al, 2010; Zhang et al, 2009).

Biomarkers for PTSD comprise imaging, psychological, endocrine, and molecular measures. Due to the complex etiology and polygenic character of psychiatric disorders including PTSD, biosignatures rather than a single biomarker are expected to reflect more accurately PTSD pathophysiology.

HPA-axis dysregulation has been frequently found to be associated with PTSD. Elevated levels of CRH were detected in cerebrospinal fluid (CSF) from PTSD patients (Baker et al, 1999; Bremner et al, 1997b). However, another study reported decreased CSF CRH concentrations during exposure to a trauma-related audio-visual stimulus (Geracioti et al, 2008). These clinical findings indicate the strong need for systemic analyses of HPA-axis
function and reactivity in larger cohorts of PTSD patients to clarify the inconsistencies. Hyperactivity of the SAM was found in PTSD patients (Pitman et al., 2012; Strawn and Geracioti, 2008), which is reflected by elevated urine and plasma norepinephrine levels (Kosten et al., 1987; Yehuda et al., 1998). In addition, adrenoreceptor blockers were reported to improve PTSD symptoms, but not in all studies. Norepinephrinergic hyperactivity has been associated with several symptoms of PTSD, including night mare and exaggerated startle response (Dierks et al., 2007). Besides the association with SAM hyperactivity, startle response was found to be positively correlated with cortisol levels (Grillon et al., 2006) and cortisol suppression by dexamethasone reduces exaggerated startle responses in PTSD patients (Jovanovic et al., 2011). Brain region specific structural and functional abnormalities were reported in PTSD patients and animal models of PTSD. Reductions in the volume of the amygdala, the hippocampus and the anterior cingulate cortex have been described (Karl et al., 2006). Furthermore, lower levels of NAA, a marker for neuronal density, have been reported in the hippocampus of PTSD patients (Karl and Werner, 2010a; Schuff et al., 2008). NAA levels not only serve as a risk factor for PTSD susceptibility as described in chapter 1.3.1, but also as a diagnostic marker for PTSD.

Protein biomarkers for PTSD are mostly immunological factors. The cytokines IL-2, IL-6 and IL-8 have been reported to be increased in PTSD patients (Baker et al., 2001; Maes et al., 1999; Song et al., 2007). On the other hand, proteins involved in the immune response, such as C-reactive protein, serum amyloid, and neopterin were found to have decreased levels in PTSD patients (Atmaca et al., 2002; Söndergaard et al., 2004; von Känel et al., 2010).

### 1.4. Pharmaco- and psychological therapy of PTSD

The earliest pharmacotherapy for psychological trauma-related psychopathology dates back
to Weir Mitchell in 1885 (Mitchell, 1885). Following exposure to trauma, he self-administrated and described ‘the mischievous tale of bromides, opium, chloral and brandy’. Due to the complex etiology and symptomatology of PTSD, not all patients respond to treatment and at present there is no drug that acts on all the PTSD symptoms mentioned in chapter 1.1. The main goals of PTSD pharmacotherapy can be summarized as follows: (1) to reduce core PTSD symptoms; (2) to improve resilience to stress; (3) to reduce comorbidity, and (4) to prevent relapse over follow-up (Brunello et al, 2001). Serotonergic agents, such as selective serotonin reuptake inhibitors (SSRIs), are currently the most commonly used pharmaco-treatment options. SSRIs increase serotonin functioning by inhibiting its uptake from the synaptic cleft into pre-synapse. Treatment with SSRIs increased tolerance to aversion and decreased stress-fear response. Currently, SSRIs including fluoxetine, paroxetine, sertraline and fluvoxamine have been officially approved by the U.S. food and Drug Administration (FDA) for treatment of PTSD (Ravindran and Stein, 2009). In addition to SSRIs, noradrenergic agents, such as the α2-adrenoreceptor agonist clonidine and β-adrenoreceptor antagonist propranolol, have been proven to be effective for the treatment of PTSD associated hyperarousal symptoms by correcting dysregulated norepinephrine signaling (Ravindran and Stein, 2009). However, other clinical trials have shown mixed results (Pitman et al, 2002; Stein et al, 2007). Meanwhile, dysregulation of HPA axis activity was found in PTSD patients. It is known that GCs regulate memory formation, inhibit unrelated information and enhance the consolidation of extinction memory (de Quervain et al, 2009; Joëls et al, 2006; Karst et al, 2005) which makes targeting glucocorticoids a possible option for treating PTSD. In a double-blind study, one-month of low-dose cortisol treatment of chronic PTSD patients inhibited memory retrieval of traumatic events and reduced symptoms even beyond the treatment period (de Quervain, 2008). Besides the above mentioned antidepressants, neuropeptides and a combination of anti-inflammatory
1. INTRODUCTION

Compounds are potential treatment options for PTSD (Aga-Mizrachi et al, 2014; Steckler and Risbrough, 2012). Currently, a combination of trauma-psychotherapy and pharmacotherapy, mainly employing SSRIs, is considered as the golden standard for PTSD treatment (Berger et al, 2009). Cognitive-behavioral therapy, the dominant psychological treatment for PTSD, applies the approaches of exposure and cognitive therapies. Exposure therapy aims to reduce emotional association with traumatic memories. It helps PTSD patients to learn effective regulation of stress with relaxation training and stress coping techniques while they are being progressively exposed to the fearful stimulus (Smith et al, 2015). The cognitive therapy aims to modify the inaccurate thoughts and emotions attributed to the traumatic memories of the patients. Studies from Resick and Schnicke (1992) showed that cognitive therapy improved PTSD and depression measures and the improvements were maintained for 6 months in rape victims.

1.5. Animal models of PTSD

To study the underlying mechanisms of psychiatric disorders and potential pharmaceutical treatments, animal models provide researchers a nice tool to investigate the affected pathways. However, modeling psychiatric disorders in animals is challenging since diagnosis of the symptoms are not objectively measureable and it is impossible to model the complex etiology and expression of human psychiatric disorders. Therefore, animal research focusses more on the basic emotional process which is shared by humans and other mammals (LeDoux, 2000; Ohman and Mineka, 2001). The aim for animal research is to model selected endophenotypes proposed to reflect features of the respective psychiatric disorder.

A valid animal model should fulfil the following three criteria (Chadman et al, 2009; Siegmund et al, 2006):

- **Face validity** describes how closely core symptoms of the human disorder are reflected by
the animal model. In the case of PTSD, the key to differentiate it from other anxiety disorders is the encounter of a traumatic event (see chapter 1.5.1). The stressor should be intense but of short duration and the severity of symptoms increase with the intensity of the stressors. The core symptoms for PTSD need to include behavioral phenotypes of hyper-responding (exaggerated fear responses to trauma-related cues), hyperarousal and hypo-responding (emotional numbing). In addition, behavioral alterations are expected to last for a long time from weeks to months.

**Construct validity** represents the underlying pathophysiological mechanisms for disorders which are similar or correlate with humans with the animal model employed. In the animal model of PTSD, studies have suggested hippocampal hypotrophy and hyperactivity of the amygdala or the CRH system which correlates with the observations in PTSD patients (Bremner, 2002; Thoeringer *et al.*, 2012).

**Predictive validity** represents the treatment options human disorders are applicable to and can be verified in animal research. Although there is no golden standard for treatment of PTSD, SSRIs have shown to be effective in PTSD patients (Albucher and Liberzon, 2002). Therefore, application of SSRIs can be verified for the predictive validity in animal models.

### 1.5.1. PTSD animal model

PTSD animal models can be generated by applying physical, psychosocial, psychogenic or early life stressors. Restraint stress is induced by placing a mouse or rat in restraining tubes for 2-6 hours which results in increased anxiety behaviour and changes in neuronal morphology within brain regions involved in fear and anxiety (Miller and McEwen, 2006; Vyas *et al.*, 2002). Another physical stressor model, single prolonged stress (SPS), combines the administration of different stressors (restrain stress, forced swim and ether exposure). SPS is widely used for PTSD animal studies because the combination of complex stressors...
1. INTRODUCTION

mimics the situation for PTSD development in humans (Liberzon et al, 1997; Pitman et al, 2012; Takei et al, 2011). In another model animals are subjected to a variety of psychosocial stressors, such as social defeat and social isolation. When exposing to chronic social instability, animals exhibit long-lasting changes in anxiety-like behaviors resembling human PTSD symptoms (Zoladz et al, 2008). In another commonly used PTSD animal model a predator is used as a psychogenic stressor (Dielenberg and McGregor, 2001). In this model, animals receive threat but no pain. The exposure to species-relevant predators or odor/scent lead to long-lasting manifestations of anxiety and acoustic startle response (Hebb et al, 2003; Roseboom et al, 2007; Zoladz et al, 2012). In another model, animals were re-exposed to a trauma reminder which allows the study of underlying neural mechanisms for persistent re-experiencing and intrusive memories, the core symptoms of PTSD (Ritov et al, 2014). In humans, trauma exposure during development leads to long-term HPA axis abnormality and increases the risk of developing PTSD later in life (Delahanty and Nugent, 2006). Similarly, in the early life stress induced PTSD mouse model, maternal separation results in enhanced stress and anxiety responses upon exposure to severe stress later in life (Diehl et al, 2012; Tsoory et al, 2007).

The PTSD model used in the current study fulfils all the above mentioned criteria (face, construct and predictive validities) (Siegmund and Wotjak, 2007). Two inescapable electric foot shocks (FS) with 1.5mA amplitude and 2 seconds duration were given to the mice as traumatic encounters. After 28 days of incubation, the shocked mice were assessed for behavioral phenotypes of PTSD-like symptoms. In the shocked mice, both the conditioned fear and generalized fear responses are increased. The conditioned fear response is evaluated by the freezing behavior in the shock context and the generalized fear response is assessed by measuring the freezing response in a completely neutral context or in a context resembling the dominant feature (the metal grid) of the shock context (Siegmund and Wotjak, 2007). In
addition, shocked mice show a hyperarousal phenotype which can be evaluated by the intensity of the acoustic startle response when presenting the mice different intensities of neutral tone (Siegmund and Wotjak, 2007) or to startle-response provoking white noise (Golub et al. 2009). For the treatment of PTSD, early intervention and chronic administration of fluoxetine in shocked mice has been shown to ameliorate PTSD-like symptoms (Siegmund and Wotjak, 2007). Until now, the pathophysiological mechanisms and affected molecular pathways underlying PTSD development still remain elusive. Shrinkage of hippocampus volume and dysregulations of CRH system were observed in the PTSD model we applied, which resembles the clinical findings in PTSD patients (Golub et al, 2011; Thoeringer et al, 2012). Taken together, the face validity (traumatic foot shock, increased contextual fear and hyperarousal phenotype), predictive validity (fluoxetine treatment) and constructive validity (sharing similar biological alterations) are met in our PTSD mouse model.

1.6. **Hypothesis-free –omics: quantitative proteomics and metabolomics, and RNA-seq transcriptomics**

PTSD is a complex and polygenic disease. Therefore data integration from a variety of approaches including -omics-based systems biology is critical for a better understanding of disease pertinent molecular pathways (Barabási and Oltvai, 2004; Kitano, 2002). Functional genomics, including transcriptomics, proteomics and metabolomics add an important dimension to the study of psychiatric disorders for the analysis of spatial and temporal alterations (Geschwind and Konopka, 2009; Turck et al, 2005). Both transcriptomics and proteomics approaches have been applied to study the underlying mechanisms of PTSD (Henningsen et al, 2012; O'Donovan et al, 2011; Tylee et al, 2015). Transcriptomics analysis examines mRNA expression which reflects the genes that are actively expressed at any given time. To date, RNA-seq technology is used to study the transcriptome (Szabo, 2014). In
addition to transcriptomics, the proteomics approach facilitates organelle specific analyses, e.g. synapse specific alterations and is able to detect protein post-translational modifications (PTM) which play an important role in protein function and activity. Since mRNA levels do not always reflect protein expression levels complementary proteomics analyses have merit.

The metabolome represents the final product of interactions between gene expression, protein expression, gene and protein interaction and the cellular environment (Kaddurah-Daouk and Krishnan, 2009). With the advance of instrumentation and data analysis strategies, metabolomics technology is able to complement transcriptomics and proteomics investigations in the field of systems biology and pathway analysis. The integration of transcriptomics, proteomics and metabolomics analyses can help delineate dysregulated molecular pathways in disease. The following chapters describe proteomics (1.6.1) metabolomics (1.6.2) and transcriptomics (1.6.3) technologies with a focus on the methods that were used over the course of the thesis project.

1.6.1. Quantitative proteomics

Traditional proteomics strategies mainly exploited two-dimensional gel electrophoresis for high resolution protein separation and relative protein quantification. Complex protein mixtures are first separated based on their isoelectric point in the first dimension and by molecular weight in the second dimension. Staining of the proteins allows the detection of both quantitative (expression level) and qualitative (post-translational modification) protein differences. Differentially expressed proteins between disease/treatment vs. control can then be identified using mass spectrometry (O'Farrell, 1975). Today quantitative proteomics is carried out with mass spectrometry (MS) due to its high sensitivity and reproducibility. By
utilizing sensitive MS in combination with multidimensional protein and peptide separation techniques thousands of proteins can be identified and quantified in a sample.

1.6.1.1. **Mass spectrometry based proteomics**

The main components of a mass spectrometer are an ionization source, a mass analyzer and an ion detector. A variety of ionization methods are available for introducing the ions into the mass analyzer. For proteins and peptides the preferred methods are matrix-assisted-laser-desorption ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Whitehouse *et al*, 1985; Wilm and Mann, 1996a; Wilm *et al*, 1996b). Depending on the analytical question, different kinds of mass analyzers exhibiting distinct strengths can be utilized: linear ion trap (LTQ), Orbitrap, time-of-flight (TOF), quadrupole (Q) mass filter, magnetic sector and combinations thereof like LTQ-Orbitrap, Q-TOF or triple-quadrupole (QqQ) instruments.

MS measures the mass-to-charge (m/z) ratio of ions. The mass spectrum is the final readout of the mass spectrometer and represents the signal intensity of the ions and their m/z ratios (Steen and Mann, 2004). Precursor ions can be fragmented to product ions via methods such as collision-induced dissociation (CID), electron transfer dissociation (ETD) or high energy collision dissociation (HCD). Parent and fragment masses are compared against a database for protein identification (Eng *et al*, 1994; Perkins *et al*, 1999).

With its high mass accuracy (<5 ppm) and high sensitivity (sub-femtomol range for peptides) the LTQ-Orbitrap hybrid mass spectrometer fulfills the requirements for sensitive and comprehensive proteomics analysis. In addition, the LTQ-Orbitrap has a high dynamic range (>10³) and high mass resolution (up to 100.000 at m/z 400) (Yates *et al*, 2006).

1.6.1.2. **Quantitative proteomics using stable isotope labeling**
1. INTRODUCTION

According to the isotope dilution theory (de Leenheer and Thienpont, 1992), peptides that differ only in isotopic composition behave identical during an MS experiment. As a result relative protein amounts are reflected by the unlabeled and labeled peptide signal ratios of the extracted ion chromatograms (Steen and Mann, 2004). A great variety of methods are available for relative protein quantification with stable isotopes ($^2$H, $^{13}$C, $^{18}$O and $^{15}$N). These include post-synthesis labeling such as isotope-coded protein label (ICPL) (Schmidt, 2005), isotope-coded affinity tag (ICAT) (Gygi et al, 1999) and isobaric tag for relative and absolute quantitation (iTRAQ) (Ross et al, 2004). ICPL is based on adding a stable isotope tag to the free amino groups of intact proteins (N-terminus and lysine side chains) with the help of specific reagents. Protein mixtures are first subjected to reduction and alklylation. For a comparison of four samples the free amino groups are then derivatized with the ICPL_0, ICPL_4 (4 Deuteriums), ICPL_6 (6 $^{13}$C) and ICPL_10 (4 Deuteriums + 6 $^{13}$C) reagents. The samples are then combined for further separation and enzymatic digestion. After enzymatic cleavage, the relative abundance of identical peptides, which due to their different tags differ in their mass, can be quantified according to their signal intensities (Lottspeich and Kellermann, 2011; Schmidt et al, 2005). The chemical probes used for the ICAT method are composed of three elements: a reactive group that is able to label a specific amino acid side chain, an isotopically coded linker and a tag (e.g. biotin) for the affinity isolation of labeled peptides/proteins. For quantitative analysis of two protein samples, one sample is labeled with isotopically light ($d_0$) tag while the other sample is labeled with the isotopically heavy ($d_8$) tag. Both samples are subsequently mixed and subjected to enzyme (mostly trypsin) digestion, followed by an avidin affinity chromatography to isolate peptides labeled with isotope-coded tagging reagents. Relative protein levels of the two samples are then calculated based on the signal intensity ratios of differentially mass-tagged peptide pairs (Adam et al, 2002). iTRAQ is based on covalent labeling of the N-terminus and lysine side chain amino
groups using tags of different masses. The derivatized peptides are isobaric and yield reporter ions following CID which can then be used to identify and quantify relative peptide abundance (Shadforth et al, 2005).

Alternatively, stable isotopes are introduced into proteins via metabolic labeling with either ‘stable isotope labeling using amino acids in cell culture’ (SILAC) (Ong et al, 2002) or ‘stable isotope labeling of mammals’ (SILAM) (Oda et al, 1999) methods.

For SILAC, one cell sample is cultured in media with standard essential amino acids, while the other cell sample is grown in media supplemented with the heavy stable isotope form of amino acids (e.g. $^{13}$C-lysine). Proteins are harvested from the two experimental cell samples and combined. After extraction and fractionation proteins are digested and peptides subjected to MS analysis. Unlabeled and labeled peptide pair peak intensity ratios reflect the relative abundance of the two proteins in the cell samples (Ong et al, 2002). SILAM has been applied to investigate proteome changes in animals. Animal proteomes can be uniformly labeled with $^{15}$N through feeding with a protein-based, $^{15}$N-labeled diet (Filiou et al, 2011). Labeled tissues can then be used as an internal standard when mixed with tissues of interest from an animal model. For SILAC and SILAM unlabeled and labeled samples are combined at the very beginning of sample preparation which results in high quantitation accuracy because potential experimental biases affect both labeled and unlabeled proteins in the same manner (Bantscheff et al, 2012). Therefore SILAC and SILAM represent the current gold standard for accurate relative protein quantification.

1.6.2. Quantitative metabolomics
1. INTRODUCTION

Quantitative metabolomics complements other –omics approaches and has been applied to psychiatric research (Dai et al., 2010; Su et al., 2011). The major challenges for metabolomics analyses are the large dynamic range of metabolites (>10⁶) and the absence of reliable automatic identification tools (Moco et al., 2007). To achieve a comprehensive and hypothesis-free global analysis, diverse complementary analytical methods need to be applied. Depending on the biological question, sub-metabolome or targeted analyses reveal significant insights into the system or particular pathways of interest. To date, most commonly used platforms are based on liquid chromatography-MS (LC-MS), gas chromatography-MS (GC-MS) and nuclear magnetic resonance (NMR) (Moco et al., 2007; Theodoridis et al., 2012).

LC-MS has become an important method in the field of quantitative metabolomics. Metabolites are first separated using chromatography and then introduced into the ionization source. Metabolite ions are subsequently generated by ESI in positive or negative modes or atmospheric pressure ionization (API). Ions are then measured by triple quadrupole or quadrupole-time-of-flight (Q-TOF) mass analyzers. Ion analyzers like FT-Orbitrap or FT-ICR-MS of high mass resolution (100.000-1.000.000) and mass accuracy (>2 ppm) are used for metabolite identification. Metabolite identification is based on individual characteristic parameters, including metabolite mass, isotopic distribution, fragmentation pattern and retention time (Moco et al., 2007).

Metabolomics can be performed by targeted analysis to assess and quantitate a selected list of metabolites or untargeted for comprehensive metabolite profiling. When configured in Selected-Reaction-Monitoring (SRM) mode molecules can be identified with high confidence. In addition, Multiple-Reaction-Monitoring (MRM) on Triple-Quadrupole mass spectrometers allows accurate quantification of metabolite precursor and product ions (Lu et al., 2008). Recently, a metabolomics platform using LC-MRM-MS was introduced. It allows the reliable
1. INTRODUCTION

qualitative and quantitative determination of a wide range of metabolites (>300 metabolites) targeting major metabolic pathways, including Krebs cycle, glycolysis and metabolism of amino acids (Yuan et al, 2012).

1.6.3. Quantitative transcriptomics

Transcriptomics technology allows to catalogue all transcript species, to determine the transcriptional structure of genes and to quantify the expression levels of each transcript under different conditions. Various technologies have been developed to quantify and deduce the transcriptome, including hybridization- or sequence-based approaches. Hybridization-based approaches involve incubating fluorescently labeled cDNA with custom-made or commercial high-density oligo microarrays. The sequence-based approach on the other hand directly determines the cDNA sequence by a tag-based sequencing approach, such as serial analysis of gene expression (SAGE) (Harbers and Carninci, 2005; Velculescu et al, 1995), cap analysis of gene expression (CAGE) (Kodzius et al, 2006; Shiraki et al, 2003) and massively parallel signature sequencing (MPSS) (Brenner et al, 2000; Reinartz et al, 2002). The tag-based sequencing approaches allow high throughput analyses and provide precise gene expression levels. However, only a portion of the transcript can be analyzed and isoforms are generally indistinguishable from each other.

RNA sequencing (RNA-Seq) has clear advantages over existing approaches and has been introduced as a high-throughput sequencing method for both mapping and quantifying transcriptomes. A population of extracted RNA from sample tissue is converted to a library of cDNA fragments with adaptors attached to one or both ends. Subsequently, each molecule is sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). Following sequencing, the genomes are
1. INTRODUCTION

aligned to a reference genome or transcript to produce a genome-scale transcription map that reflects the transcriptional structure and/or level of expression for each gene (Wang et al, 2009).

RNA-seq has been used to accurately monitor gene expression during yeast vegetative growth (Nagalakshmi et al, 2008), mouse embryonic stem-cell differentiation (Cloonan et al, 2008) or brain development (Dillman and Cookson, 2014). With RNA-seq, RNA expression levels in a cell population can be determined accurately. Furthermore, differences in gene expression levels between disease/treatment vs. control can be compared directly to delineate dysregulated genomic pathways.

1.6.4. Computational biology/Systems biology

For the past decade, studies on complex interactions within a biological system have increased significantly and this biology-based interdisciplinary field of study requires data interpretation by computational biologists. Computational biology involves the application of large-scale data set analysis, mathematical modeling and computational simulation techniques to study biological systems (Biomedical Information Science and Technology Initiative, 2000). In systems biology complex data sets from multiple experimental sources are integrated and analyzed using interdisciplinary tools. The typical experimental platforms include transcriptomics, genomics, epigenomics, metabolomics, lipidomics, glycomics, proteomics and phenomics. The study of a single component of a biological system is not enough to reflect the mechanisms that give rise to the function and behavior of one particular system. The aim of systems biology studies is to model and discover the properties of cells, tissues and organisms and how they function as a system (Bu and Callaway, 2011). With the
1. INTRODUCTION

aid of computational biology techniques, algorithms and relations among various biological systems can be established. Computational and systems biology have been applied to the Human Genome Project and other genetic studies (Collins and Galas, 1993; Imam et al, 2015).

Computational and systems biology are also powerful tools to investigate neuronal networks and to come up with accurate models for delineating pathophysiological mechanisms. For psychiatric disorders, interactions between different transmitter systems are important to understand their complex etiology and polygenic characters. Neurocomputational models based on recurrent excitation within cell assemblies, synfire chains and single-cell bistability have been established to study the contributions of different synaptic and voltage-gated conductances and neuromodulation to the formation of working memory and their relevance for schizophrenia (Durstewitz et al, 2000). Only a limited number of modeling algorithms for classification was applied in PTSD studies (Glatt et al, 2013; Yang et al, 2013). In a study of male PTSD soldiers, a logistic regression analysis model based on factors of predeployment GR number, messenger RNA expression of GR target genes, including FKBP5, GILZ and SGK1, plasma cortisol and childhood trauma, was applied to predict PTSD symptoms development (van Zuiden et al, 2012). To date, there are no studies integrating heterogeneous data analysis for investigating the interaction networks of PTSD.

1.7. Microdialysis

Microdialysis can be applied to interstitial cell fluid of selected tissues (Rittenhouse and Pollack, 2000) and body fluids (Chan et al, 2002; Tsai et al, 2001) for dynamically monitoring endogenous substances or drugs and metabolites. A microdialysis system consists of a micordialysis probe, a micordialysis pump and a microvial in which the samples are
1. INTRODUCTION

collected. During the process of microdialysis the probe is implanted into targeted tissue, e.g. specific brain region, and perfused with artificial cerebrospinal fluid (aCSF). aCSF perfusion of identical pH, osmolarity and ion composition as the brain extracellular fluid with an implanted microdialysis probe will not disturb tissue homeostasis, therefore making microdialysis a truly physiological method for the purpose of in vivo sampling. Commonly utilized methods for the analysis of micordialysates include UV, fluorescence and electrochemical detection equipped with high performance liquid chromatography (HPLC), as well as ELISA, radioimmunoassay and spectrophotometric biochemical assays (Guihen and O’Connor, 2009).

Microdialysis is frequently used for the investigation of brain neurotransmission, neurochemistry and metabolism (Anderzhanova and Wotjak, 2013; Ungerstedt and Pycock, 1974). Furthermore, by acquiring pharmacokinetic and pharmacodynamic data from living animals, microdialysis continues to be the method of choice for experimental neuropharmacology studies (Westerhout et al, 2012). Sampling of specific brain regions like the medial prefrontal cortex (Jalkanen et al, 2014; Tanda et al, 2015), hippocampus (Maki et al, 2014; Yau et al, 2015), paraventricular nucleus (Czell et al, 2012; Tachi et al, 2014), and amygdala (Hambrecht-Wiedbusch et al, 2014; Su et al, 2015) during behavioral experiments in freely moving rodents can be carried out with potent spatial and temporal resolution of millimetres and minutes, respectively, reflecting the actual physiological state of the animal under investigation. By targeting different brain regions, microdialysis enables assessment of release and metabolism of neuroactive endogenous substances which provides important insights into affected brain circuits underlying the physiological/pathological mechanisms.

1.8. Aims of the thesis
The thesis project aimed to delineate affected molecular pathways in brain circuits involved in fear and stress response and the fluoxetine treatment effect in a mouse model of PTSD with the help of transcriptomic, proteomic and metabolomic technologies. Another aim was to investigate the effects of stress on neurochemical and neuroendocrine changes and their relations to PTSD-like symptoms.

Specific aims of the individual manuscripts:

Chapter 2.1.:

Fluoxetine treatment rescues energy metabolism pathway and myelin sheath protein alterations in a posttraumatic stress disorder mouse model

- To delineate affected molecular pathways in the brain circuit involved in fear and stress response in a mouse model of PTSD at the proteome and metabolome levels.
- To identify potential targets upon fluoxetine treatment that may modulate these pathways.
- To identify plasma metabolite biomarkers.

Chapter 2.2.:

Transcriptomic profiling reveals affected inflammatory pathway in a mouse model of posttraumatic stress disorder

- To identify affected pathways in the brain circuit involved in fear and stress response in a mouse model of PTSD at the transcriptome level.
1. INTRODUCTION

- To identify potential targets upon fluoxetine treatment that may modulate these pathways.

Chapter 2.3.: Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice.

- To analyze early post-traumatic brain extracellular norepinephrine and free corticosterone (CORT) levels in the medial prefrontal cortex (mPFC) and hippocampus in a mouse model of PTSD.
- To relate the levels of stress-induced norepinephrine and free CORT release in the mPFC and hippocampus to PTSD-like symptoms, including hyperarousal and fear memory retention.

Chapter 2.4.: NextGen Brain Microdialysis – applying modern metabolomics technology to the analysis of extracellular fluids

- To apply modern LC-MS metabolomics technology to the analysis of prefrontal microdialysates in a foot shock-induced PTSD mouse model for hypothesis free metabolite profiling.
- To correlate prefrontal metabolite levels to foot shock-induced arousal change.
- To identify foot shock-induced molecular pathway alterations in the prefrontal cortex of a PTSD mouse model.
2. RESEARCH ARTICLES

2.1. Fluoxetine treatment rescues energy metabolism pathway and myelin sheath protein alterations in a posttraumatic stress disorder mouse model

Chi-Ya Kao, Zhisong He, Kathrin Henes, John M. Asara, Christian Webhofer, Michaela D. Filiou, Philipp Khaitovich, Carsten T. Wotjak, Christoph W. Turck

Declaration of contribution: Chi-Ya Kao designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with coauthors.

- Study design and plan: in collaboration with ZH, CW and CT
- Conducting the experiments: in collaboration with KH and JA
- Data analysis: in collaboration with ZH, PK and CT
- Manuscript preparation: in collaboration with ZH, CW and CT

Supplemental material is available on enclosed DVD and upon request.
Fluoxetine treatment rescues energy metabolism pathway and myelin sheath protein alterations in a posttraumatic stress disorder mouse model

Chi-Ya Kao\(^{1,2,*}\), Zhisong He\(^{3,*}\), Kathrin Henes\(^{4}\), John M. Asara\(^{5}\), Christian Webhofer\(^{1}\), Michaela D. Filiou\(^{1}\), Philipp Khaitovich\(^{3}\), Carsten T. Wotjak\(^{2,4}\), Christoph W. Turck\(^{1,2,*}\)

\(^{1}\)Max Planck Institute of Psychiatry, Department of Translational Research in Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany

\(^{2}\)Graduate School of Systemic Neurosciences, Ludwig-Maximilians-University Munich, Grosshadernerstr. 2, 82152 Planegg-Martinsried, Germany

\(^{3}\)CAS-MPG Partner Institute for Computational Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai, The People’s Republic of China

\(^{4}\)Max Planck Institute of Psychiatry, Department of Stress Neurobiology and Neurogenetics, Kraepelinstrasse 2-10, 80804 Munich, Germany

\(^{5}\)Division of Signal Transduction, Beth Israel Deaconess Medical Center, and Department of Medicine, Harvard Medical School, Boston, MA, USA

\(^{*}\)These authors contributed equally to this work

\(*\)Correspondence:
Christoph W. Turck
Max Planck Institute of Psychiatry
Department of Translational Research in Psychiatry
Kraepelinstrasse 2-10
Running title: PTSD mouse model fluoxetine treatment

Funding and Disclosure: This work was supported by the Max Planck Society. All authors report no biomedical financial interests or potential conflicts of interest.
2. RESEARCH ARTICLES

**Abbreviations**

**PTSD** posttraumatic stress disorder

**PrL** prelimbic cortex

**ACC** anterior cingulate cortex

**BLA** basolateral amygdala

**CeA** central nucleus of amygdala

**CA1** cornu ammonis 1

**NAc** nucleus accumbens

**SSRIs** selective serotonin reuptake inhibitors

**FS** foot shock

**FDR** false discovery rate

**DE** differentially expressed

**KS** Kolmogorov-Smirnov

**GSEA** gene set enrichment analysis

**HMDB** Human Metabolome Database

**MSEA** metabolite set enrichment analysis

**ATP** adenosine triphosphate

**CB1** cannabinoid type 1
NAA N-acetylaspartate

Summary

Posttraumatic stress disorder (PTSD) is a prevalent psychiatric disorder. Several studies have attempted to characterize molecular alterations associated with PTSD, but most findings were limited to the investigation of specific cellular markers in the periphery or defined brain regions. In the current study, we aimed to unravel affected molecular pathways/mechanisms in the fear circuitry associated with PTSD. We interrogated a foot shock induced PTSD mouse model by integrating proteomics and metabolomics profiling data. Alterations at the proteome level were analyzed using in vivo $^{15}$N metabolic labeling combined with mass spectrometry in prelimbic cortex (PrL), anterior cingulate cortex (ACC), basolateral amygdala (BLA), central nucleus of amygdala (CeA) and CA1 of hippocampus between shocked and non-shocked (control) mice, with and without fluoxetine treatment. In silico pathway analyses revealed an upregulation of Citric Acid Cycle pathway in PrL, and downregulation in ACC and NAc. Dysregulated cytoskeleton assembly and myelination pathways were identified in BLA, CeA and CA1. Chronic fluoxetine treatment prevented decreased Citric Acid Cycle activity in NAc and ACC and ameliorated conditioned fear response in shocked mice. Our results shed light on the role of energy metabolism in PTSD pathogenesis and suggest potential therapy through mitochondrial targeting.
2. RESEARCH ARTICLES

Introduction

Life threatening events, such as combat, sexual abuse or natural disasters are traumatic stressors that can lead to the development of posttraumatic stress disorder (PTSD). The core symptoms of PTSD include hyperarousal/hypervigilance, avoidance of trauma-related cues and excessive recall of traumatic memories (1). PTSD does not develop until weeks to months after trauma exposure. Hence, the long-lasting effect of molecular changes in brain regions involved in stress and traumatic memory (fear circuitry) play an important role in the pathogenesis of PTSD.

Although clinical studies on PTSD have identified dysregulations in endocrine signaling (2, 3), neurotransmitter system (4), as well as genetic and epigenetic risk factors (5, 6) there is still a lack of global investigations on altered cellular and molecular pathways and biomarkers of fear circuitry involved in PTSD development. Selective serotonin reuptake inhibitors (SSRIs) antidepressants, such as fluoxetine, have been widely used to improve PTSD symptoms in patients (7) and have shown therapeutic efficacy in animal studies (8). However, the underlying molecular mechanisms affected by fluoxetine in distinct brain regions still remain elusive. A global investigation of molecular pathways associated with fear circuitry with the help of PTSD animal models subjected to fluoxetine treatment can provide useful insights into molecular pathology and treatment efficacy.

For PTSD, a complex and polygenic psychiatric disorder, the integration of different –omics-based methods and systems biology is necessary for a better understanding of disease related molecular pathways (9, 10). These include non-hypothesis driven proteomics, metabolomics and transcriptomics methods for the identification of affected pathways and biomarkers.
In the present study, we investigated a previously published PTSD mouse model generated by inescapable foot shocks as traumatic stressors (8). Shocked mice showed PTSD-like symptoms, including conditioned fear response, which was ameliorated upon chronic fluoxetine treatment. To identify dysregulated pathways and therapeutic targets of fluoxetine, we applied proteomics and metabolomics for the analyses of specific mouse model brain regions believed to be involved in PTSD. We focused on brain regions involved in fear circuitry, including prelimbic cortex (PrL), anterior cingulate cortex (ACC), nucleus accumbens (NAc), basolateral amygdala (BLA), central nucleus of amygdala (CeA) and CA1 region of hippocampus (11-13). For proteomics analysis, $^{15}$N metabolically labeled reference material was used for quantitative mass spectrometry (14). We first identified altered molecular pathways upon foot shock exposure followed up by an investigation of the fluoxetine rescue effect on the proteome level. We then subjected PrL, ACC, NAc, BLA, CeA and CA1 punched tissue to targeted polar metabolomics profiling analysis to further corroborate our proteomics findings (15). Proteomics and metabolomics data were integrated to delineate affected pathways contributing to PTSD-like pathogenesis and fluoxetine targets.

### Experimental Procedures

#### Animals

Male C57BL/6NCrl mice, 7-8 weeks old (Charles River GmbH, Sulzfeld, Germany), were housed in groups of four in Makrolon type II cages (23×16.5×14 cm$^3$) under standard conditions (inverse 12:12 h light-dark cycle, light off at 7:00, room temperature 23°C±2°C, humidity 60%) with food and water *ad libitum*. All experiments were carried out according to the European Community Council Directive 2010/63/EEC and approved by the local
government of Upper Bavaria (55.2.1.54-2532-41-09 and 55.2.1.54-2532-141-12). Laboratory animal care and experiments were conducted according to the regulations of the current version of The German Animal Welfare Act.

**PTSD mouse model and fluoxetine treatment**

A previously published PTSD mouse model (8, 16) was applied by performing fear conditioning (administration of an unsignaled foot shock (FS) in the shock chamber with house light on) during the active phase of the circadian cycle. Mice were conditioned in a plexiglas cage (16×16×32 cm\(^3\)) with a grid harness package (ENV-407, ENV-307A, MED Associates, 7 St. Albans, VT, USA) connected to a shock generator (Shocker/Scrambler: ENV-414, MED Associates). After 198 sec of habituation, animals underwent two electric FSs (1.5 mA, 2 sec of length) at moderate illumination (40 Lux) with a 60 sec interval in between. Animals remained in the shock chamber for another 60 sec before being returned to their home cages. Non-shocked (control) mice went through the same procedure, but without receiving FSs.

Fluoxetine (20mg/kg/day; Ratiopharm GmbH, Ulm, Germany) or vehicle (tap water) treatment was applied to shocked mice 12 h after FSs for 28 days, followed by a 28-day wash-out period. To assess fluoxetine treatment efficacy, sensitized fear response was evaluated by exposing mice to a neutral tone (60 sec of length, 80 dB, 9 kHz) in a neutral cylindrical context on day 28 after FSs. Long-lasting effect of shock application and fluoxetine treatment was assessed after wash-out period, by placing mice into the neutral (cylindrical and hexagonal shaped) and shock chambers for contextual fear test for 3 min. In the cylindrical grid chamber, mice were additionally exposed to a neutral tone for 3 min to test sensitized fear (Supplemental Fig. S1). Freezing behavior was defined as immobility except for respiration movements and was video-taped for off-line rating.
Brain punch sampling

Mice were euthanized by an overdose of isoflurane (Forene, Abbott, Wiesbaden, Germany) and sacrificed. Brains were harvested after decapitation and immediately fixed with ice-cold 2-methylbutane (Merck, Darmstadt, Germany), snap frozen in liquid nitrogen and stored at -80°C. For brain punch, brains were dissected with a cryostat (Microm, Walldorf, Germany) up to the appearance of target sub-regions. Punch specimens were isolated using cylindrical punchers (Fine Science Tools, Heidelberg, Germany) essentially as described (17). The location and length of the punches were selected based on a stereotaxic atlas (18) described as follows (in mm: starting point rostral-caudal to bregma, punch diameter, punch length): for prelimbic cortex (PrL) (2.3 mm posterior to bregma, 1.0 mm in diameter, 0.9 mm in length), anterior cingulate cortex (ACC) (0.86, 0.5, 1.0), nucleus accumbens (NAc) (1.7, 0.5, 1.0), basolateral amygdala (BLA) (-0.8, 0.35, 1.0), central nucleus of amygdala (CeA) (-0.9, 0.5, 0.8), cornu ammonis 1 (CA1) region of the dorsal hippocampus (-1.5, 0.5, 0.8). The dissection site was verified by Nissl histological staining with a stereomicroscope.

Experimental design and Statistical Rational

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative proteomics analysis

Tissue pools (n=5 for non-shock-vehicle group, n=5 for shock-vehicle group, n=3 for shock-fluoxetine group) from each bilaterally punched brain region (PrL, ACC, NAc, BLA, CeA, CA1) were subjected to extraction of cytosolic and membrane-associated proteins (19). To all samples, equal protein amounts of $^{15}$N-labeled internal reference standard were added (14). $^{14}$N/$^{15}$N protein mixtures representing 50-60 μg were separated by SDS polyacrylamide gel electrophoresis, fixed and stained with Coomassie Brilliant Blue R-250 (Biorad, Hercules,
CA, USA). The gel was destained and each gel lane was cut into 2.5 mm slices (20-22 slices per sample) for tryptic in-gel digestion and peptide extraction as described previously (14). Tryptic peptide extracts were dissolved in 0.1 % formic acid and subjected to a nanoflow HPLC-2D system (Eksigent, Dublin, CA, USA) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Prior to peptide separation, samples were on-line desalted for 10 min with 0.1% formic acid at a flow rate of 3 μl/min (Zorbax-C18 (5μm) guard column, 300 μm x 5 mm, Agilent Technologies, Santa Clara, CA, USA). RP-C18 (3μm). Desalted peptides were then separated by chromatography (in-house packed Pico-frit column, 75 μm x 15 cm, New Objective, Woburn, MA, USA). A gradient of 95% acetonitrile/0.1% formic acid from 10% to 45% over 93 min at a flow rate of 200 nl/min was applied for peptide elution. Column effluents were directly injected into the mass spectrometer via a nanoelectrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in positive mode applying a data-dependent scan switch between MS and MS/MS acquisition. Full scans were recorded in the Orbitrap mass analyzer (profile mode, m/z 380-1600, resolution R=60000 at m/z 400). The top 5 most intense peaks in each scan were fragmented and recorded in the LTQ with a target value of 10000 ions in centroid mode. Other MS parameters were set as described previously (14, 20).

**Protein identification and quantitation**

Peptides were identified by $^{14}\text{N}$ and $^{15}\text{N}$ database searches using Sequest (v28, implemented in Bioworks v3.3.1, Thermo Fisher Scientific) against a decoy Uniprot mouse protein database (release 2010_02) containing 110,128 entries (including forward and reverse sequences). Enzyme specificity was set to trypsin. Mass accuracy settings were 10 ppm and 1 Da for MS and MS/MS, respectively. Two missed cleavages were allowed, and cysteine carboxyamidomethylation and methionine oxidation were set as fixed and variable
modifications, respectively. Filtering parameters for peptide identifications were minimum Delta Cn: 0.08 and Xcorr: 1.90 ($z=1+$), 2.7 ($z=2+$), 3.50 ($z=3+$) and 3.00 ($z\geq 4+$). $^{15}$N peptide identification was assessed by a variable modification of -0.99970 Da for lysine and arginine for the frequent shift from $^{15}$N monoisotopic to the most intense $^{15}$N isotopomer as described previously (21). False Discovery Rate (FDR) of 0.1% was set as filtering criteria for peptide hits using PeptideProphet, $^{14}$N and $^{15}$N database searches were combined via iProphet and protein groups were identified by ProteinProphet (22). Keratin and proteins with one peptide identified were excluded. Relative protein quantification was performed with the ProRata software (v1.0) with default parameter settings (23). The identified peptides for every $^{14}$N/$^{15}$N were filtered and quantified with a minimum signal-to-noise ratio cutoff of 2 and ambiguous peptides were excluded for further analysis. To correct potential mixing errors during sample preparation, quantification results were normalized by subtracting the median from all log$_2$ ratios of the quantified proteins.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (24) via the PRIDE partner repository with the dataset identifiers PXD002231, PXD002271, PXD002272.

LC-MS/MS quantitative metabolomics analysis

Punched tissues from 15 mice for each brain region (PrL, ACC, NAc, BLA, CeA, CA1) were pooled into 5 biological replicates (three pooled mice tissue per analysis) and homogenized (2 min $\times$ 1200 min$^{-1}$, homogenizer PotterS, Sartorius, Göttingen, Germany) in 30-fold (W/V) ice-cold 80% methanol (Merck, Darmstadt, Germany). Homogenates were centrifuged (14,000 g, 10 min) at 4 °C and supernatants were incubated on dry ice for 2 h. Tissue pellets were further disrupted and homogenized with 6-fold (w/v) ice-cold 80 % methanol and
combined with previous supernatants. Extracts were lyophilized and stored at -80 °C for further analysis.

Samples were re-suspended in 20 μl LC-MS grade water. Ten μl were injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX, Framingham, MA, USA) coupled to a Prominence UFLC HPLC system (Shimadzu, Columbia, MD, USA). Samples were delivered to the mass spectrometer via normal phase chromatography using a 4.6 mm i.d × 10 cm Amide Xbridge HILIC column (Waters Corp., Milford, MA, USA) at 350 μl/min. HPLC running buffers and gradients were set as described previously (Webhofer et al., 2013). Some metabolites were targeted in both positive and negative ion modes for a total of 320 SRM transitions using positive/negative polarity switching with previously described mass spectrometer parameter settings (Webhofer et al., 2013). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/Sciex).

**Statistics and data analysis**

**Identification of differentially expressed (DE) proteins**

To compare protein expression levels between two experimental groups, profile likelihoods of both groups were combined via cross-correlation to get a probabilistic estimate of the indirect abundance ratio between two groups. For each protein, a p-value for the null hypothesis was derived from the profile likelihood by means of a likelihood ratio test to identify whether the estimated log₂ ratio is significantly different from 0. All p-values were adjusted using the "Benjamini-Hochberg" method.

**In silico pathway analysis**
To identify altered biological pathways and processes between different mouse groups in distinct brain regions, DE proteins ($p < 0.05$) were uploaded to DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/tools.jsp) with all quantified proteins as background. Functional enrichment of KEGG pathway and Gene ontology was acquired and pathways were considered enriched with p-value < 0.05 after Benjamini-Hochberg correction.

One-sided Kolmogorov-Smirnov test based Gene Set Enrichment Analysis (GSEA) and Metabolite Set Enrichment Analysis (MSEA)

GSEA and MSEA are commonly used statistical methods to identify enriched gene sets or metabolite sets by considering the genome-wide expression profiles of samples from two different groups (25). The one-sided KS test based on GSEA and MSEA is a variation of the typical GSEA/MSEA and uses random walk with permutation to estimate significance of enrichment. The one-sided KS test based GSEA/MSEA uses one-sided KS test to estimate whether genes or metabolites involved in the set distribute unevenly in the ranked list (Supplemental Fig. S2).

Genes or metabolites are first ranked based on the Pearson’s correlation coefficients between their expression profiles and the group distinction pattern, i.e. the pattern using 0 and 1 to distinguish samples from the two different groups. Given *a priori* defined sets of genes or metabolites, e.g. the genes or metabolites involved in a given metabolic pathway, the distribution of ranks of these genes or metabolites is compared with the uniform distribution across the list using one-sided KS test, by trying two different null hypotheses: 1) the rank distribution of the defined gene set is not less than the uniform distribution; 2) the rank distribution of the defined gene set is not greater than the uniform distribution. The significance and the direction of the changes are determined based on the p-values for the two null hypotheses.
2. RESEARCH ARTICLES

In this study, the metabolic pathway information, including genes and metabolites that are part of the pathways was obtained from the Small Molecule Pathway Database (SMPDB) (http://smpdb.ca/) and Human Metabolome Database (HMDB) (http://www.hmdb.ca/).

Results

Alterations on the proteome level in foot shock-induced PTSD mouse model.

We applied a previously published PTSD mouse model to investigate the molecular changes in the brain (8). Adult C57BL/6 mice were subjected to two non-escapable electric foot shocks (FS) (1.5 mA. 2 sec), followed by a 28-day incubation to develop PTSD-like symptoms. Shocked mice showed significantly higher conditioned fear response when being exposed to neutral contexts (cylindrical and hexagonal shaped plexiglas chambers), neutral tone and shock context (cubic chamber with metal grid) compared to control (non-shocked) mice (Fig. 1).

A quantitative proteomics platform based on $^{15}$N metabolic labeling and LC-MS/MS analysis was used to investigate FS-induced proteome changes in different brain regions involved in fear circuitry (14, 21). $^{15}$N-labeled proteins were used as internal standard for the indirect comparison between control and shocked mice. We punched brain subregions that are involved in fear circuitry, including PrL, ACC, NAc, BLA, CeA and CA1 for further analysis. Due to the limited tissue amount punched brain tissues from 3-5 mice were pooled. Pooled brain tissues were then extracted for cytosolic and membrane-associated fractions. Non-redundant protein groups were quantified for each brain region by direct comparison between test sample and reference material (Supplemental Table S1). Indirect comparison between control and shocked mice revealed significantly differentially expressed (DE) proteins ($p < 0.05$) after multiple testing correction in both cytosolic and membrane-associated fractions.
ACC and NAc showed the strongest alterations in protein abundance, with 154 and 549 DE proteins in ACC, and 223 and 310 DE proteins in NAc in cytosolic and membrane-associated fractions, respectively.

We next investigated enriched cellular processes in different brain regions as described in 'Materials and Methods' (Table 1). Cytoskeleton proteins (e.g. microtubule), possibly linked to axon growth, were significantly enriched in the cytosolic fraction of all brain regions investigated. Interestingly, the membrane-associated fraction of ACC, NAc and PrL showed a significant enrichment of proteins involved in the generation of precursor metabolites and energy metabolism including Citric Acid Cycle (Table 1). We therefore decided to further interrogate FS-induced metabolic pathway changes.

**Integration of proteomics and metabolomics profiling data in shocked mice reveals altered energy metabolisms in nucleus accumbens and anterior cingulate cortex.**

We applied the one-sided Kolmogorov-Smirnov (KS) test based Gene Set Enrichment Analysis (GSEA) to cytosolic and membrane-associated fraction proteomics data of the different brain regions to enrich affected metabolic pathways. Multiple metabolic pathways were enriched in the shocked mice in distinct brain regions (Supplemental Table S2). We found a decrease of Citric Acid Cycle enzyme abundance in the membrane-associated fraction of both ACC and NAc, as well increased enzyme levels in PrL. These findings were consistent with the DAVID analysis results.

For further verification of these metabolic pathway alterations we subjected distinct brain region extracts (PrL, ACC, NAc, BLA, CeA and CA1) from control and shocked mice to metabolomics analyses. A total of 320 metabolites were quantified of which 268 were assigned to Human Metabolome Database (HMDB) annotated metabolites (Supplemental Table S3). Focusing on the brain regions PrL, ACC and NAc that had shown metabolic
2. RESEARCH ARTICLES

pathway alterations based on the proteomics data we applied the one-sided KS test based Metabolite Set Enrichment Analysis (MSEA) to identify significantly altered metabolic pathways in shocked mice (Supplemental Table S4). We next employed the permutation-based test by calculating Euclidean distance ($D_e$) for rank similarity between GSEA and MSEA analysis. The similarity test showed consistent output results between MSEA and GSEA in the membrane-associated fraction analysis of ACC and NAc (Benjamini & Hochberg corrected FDR < 0.1), suggesting that the proteomics and metabolomics data both implicate metabolic pathway alterations in these two brain regions.

We found a trend for a down-regulated Citric Acid Cycle pathway in NAc in shocked mice from MSEA analysis ($p = 0.077$). Decreased levels of Citric Acid Cycle intermediates include oxalacetic acid, citric acid, aconitic acid, isocitric acid, and succinic acid (Fig. 2A). This was consistent with the proteomics data from DAVID and GSEA analyses that also support down-regulation of Citric Acid Cycle in NAc in shocked mice. In addition, MSEA analysis also revealed a decreased Citric Acid Cycle pathway activity in ACC in shocked mice, though with a weak trend ($p = 0.27$). The weak MSEA signal can be in part explained by the increased abundance of certain metabolites such as NAD and NADH, which in addition to Citric Acid Cycle are members of several other metabolic pathways. The majority of metabolites involved in Citric Acid Cycle including pyruvic acid, citric acid, aconitic acid and isocitric acid were found at lower levels, while only few intermediates (oxoalutaric acid, succinic acid and oxalacetic acid) were found with small increases in ACC of shocked mice (Fig. 2B). We then applied the permutation-based test for the longest path of components with continuous decrease of expression in shocked mice, taking into consideration both the quantified enzymes and metabolites involved in Citric Acid Cycle in ACC. In the shocked mice, the observation that quantified enzymes and metabolites not only tended to have decreased abundance, but also tended to link with each other in one pathway (permutation...
P=0.0367) supports the notion that Citric Acid Cycle in ACC was down-regulated in the shocked mice.

**Chronic fluoxetine treatment prevents FS-induced Citric Acid Cycle proteome alterations.**

Previously published results from our PTSD mouse model showed therapeutic efficacy of chronic fluoxetine treatment (8). Mice were treated with fluoxetine (20mg/kg/day) or vehicle for 28 days, 12 hours after the FS, followed by a 28-day wash-out period. Conditioned fear response, one of the key PTSD-like symptoms, was tested after the fluoxetine wash-out, and was ameliorated after chronic fluoxetine treatment in shocked mice (Fig. 1). We compared brain proteomes between shocked mice treated with fluoxetine and vehicle to investigate molecular changes associated with the fluoxetine rescue. Focusing on the proteins that were affected in the shocked mice, proteomic analysis revealed a rescue effect of fluoxetine treatment for FS-induced altered pathways. With chronic fluoxetine treatment we found most of the significant FS-induced alterations rescued in the membrane-associated fraction of ACC and CA1 (Table 2). Furthermore, fluoxetine treatment increased the expression level of proteins involved in the GO pathway ‘generation of precursor metabolites and energy’ (aconitate hydratase, isocitrate dehydrogenase and succinyl-CoA ligase) which were found decreased in ACC in shocked mice (Fig. 3B). In CA1 of the hippocampus, myelination pathway was found downregulated in the shocked mice (Table 1), while fluoxetine treatment rescued myelin sheath protein levels (Table 2), including myelin oligodendrocyte protein, cyclic nucleotide phosphodiesterase 1 and 2’,3’-cyclic-nucleotide 3’-phosphodiesterase. In addition, upon fluoxetine treatment, FS-induced down-regulation of most enzymes involved in Citric Acid Cycle in NAc, including fumarate hydratase, malate dehydrogenase, pyruvate dehydrogenase, citrate synthase, aconitate hydratase, isocitrate dehydrogenase and
2. RESEARCH ARTICLES

oxoglutarate dehydrogenase, was rescued (Fig. 3A, Table 2). These findings suggest that fluoxetine treatment affects energy metabolism and myelination pathways in NAc, ACC and CA1 associated with FS-induced PTSD-like symptoms.

Discussion

In the present study we have for the first time combined proteomics and metabolomics data of a PTSD mouse model to unravel affected molecular pathways in brain regions believed to be involved in fear circuitry. Our data implicate energy metabolism alterations in nucleus accumbens (NAc) and anterior cingulate cortex (ACC) in the shocked mice. Chronic fluoxetine treatment prevents energy metabolism dysregulation in the same brain areas and ameliorates PTSD-like symptoms in shocked mice.

We applied a foot shock (FS) induced PTSD model using inbred C57BL/6NCrl mice, a strain that is susceptible to PTSD-like symptoms development (16, 17, 26-28) Using a standardized proteomics platform based on $^{15}$N metabolic labeling and mass spectrometry we accurately quantified and identified significantly altered protein expression levels in affected brain regions and enriched altered cellular pathways by in silico pathway analyses. Proteomics findings were further corroborated by targeted metabolite profiling. By integrating proteome and metabolome data we were able to unravel altered molecular pathways associated with PTSD pathogenesis as well as potential therapeutic targets for fluoxetine treatment.

Mitochondria are involved in intracellular processes regulating neuronal plasticity, survival and signal transduction (29-31). Dysregulated mitochondrial function has been implicated in psychiatric and neurodegenerative diseases. Filiou et al. showed that divergent mitochondrial
mechanisms, including energy metabolism, mitochondrial transport and oxidative stress contribute to anxiety-related behaviors in mice (14). Upon psychological trauma exposure, cellular metabolic responses are activated and chronic inflammation is produced in neurons (32, 33). Persistent dysregulated energy metabolism ultimately leads to bioenergetic impairment and chronic low-grade inflammation, and may result in chronic diseases such as PTSD (32) and schizophrenia (34). In this regard Flaquer et al. identified two mitochondrial variants located in the ATP synthase subunit 8 and NADH subunit 5 which were significantly associated with PTSD (35).

Citric Acid Cycle produces NADH as precursor for oxidative phosphorylation and chemical energy in the form of adenosine triphosphate (ATP) (36). In our study, we observed an increased expression of proteins involved in Citric Acid Cycle in PrL in shocked mice (Table 1, Supplemental Table S1). Persistent PrL activation was reported to associate with enhanced learned fear expression in female rats (37). Another study suggested that sustained conditioned responses in PrL correlated with fear extinction failure (38). Endocannabinoids have been shown to regulate PrL activity by activating cannabinoid type 1 (CB1) receptors and anxiety-like behaviors in rats (39). Furthermore, CB1 receptors are part of neuronal mitochondria membranes where they regulate respiration and energy metabolism (40). Persistent increased energy metabolism that we have observed in PrL may result in neuronal activity and signaling alterations causing exaggerated fear expression in the shocked mouse.

ACC belongs to the prefrontal cortex and interacts with PrL to regulate top-down attention and stimulus-guided action (41) and is involved in emotional control and cognitive functions which are dysregulated in PTSD (42, 43). Previous studies indicate that ACC exhibits diminished activation in response to threat-related events in PTSD (44). In addition, increased activation in the ventral ACC correlates with symptom improvement after successful
treatment of PTSD by cognitive behavioral therapy (45). Another study reported reduced N-acetylaspartate (NAA) levels in the right ACC of PTSD patients (46). NAA facilitates energy metabolism in neuronal mitochondria, and decreased NAA levels are associated with reversible neuronal or mitochondrial dysfunction (47, 48). In accordance with previous findings, our proteomics and metabolomics data revealed a down-regulation of Citric Acid Cycle pathway (Table 1, Fig. 2B), implying decreased mitochondrial/neuronal activity in the ACC of shocked mice. In another study with the same PTSD mouse model we have identified an increased inflammatory response in ACC in shocked mice (Kao et al., submitted). Pro-inflammatory cytokines, including TNF-α and IL-1α, were shown to induce mitochondrial damage through suppression of pyruvate dehydrogenase activities (49), which is in accordance with our finding of decreased pyruvate dehydrogenase expression levels in the ACC in shocked mice (Supplemental Table S1). The dysregulated energy metabolism found in the present study in combination with an inflammatory response may cause perturbed neuronal function in ACC and underlie the deficits in emotional control and cognitive function in PTSD.

NAc is associated with cognitive processing, such as motivation and reward (50, 51) and plays an important role in anxiety-like behaviors (52, 53), emotion (54) and depression (55, 56). Emotional numbing and negative alterations in cognition are among the core PTSD symptoms (1). Previous studies showed a lower activation of the NAc in PTSD patients which may be related to decreased motivation and altered reward processing (57). Similar to our findings in ACC, we observed a decreased expression of proteins and metabolites associated with Citric Acid Cycle in NAc in shocked mice (Table 1, Fig. 2A). Proteomics analysis revealed that rats reared with control condition exhibited decreased expression of proteins involved in Citric Acid Cycle upon acute stress exposure while rats reared with
environmental enrichment, which produced a protective antidepressant-like phenotype, showed increased protein expression in NAc (58). Taken together, upon exposure to a stressor, persistent mitochondrial dysfunction results in downstream cellular metabolic changes (e.g. decreased Citric Acid Cycle activity) in NAc and may contribute to the pathogenesis of PTSD.

In addition to energy metabolism perturbation, we also observed altered expression levels of cytoskeleton/microtubule and myelin sheath proteins in BLA and CeA (Table 1, Supplemental Table S1). Amygdala encompasses several nuclei with distinct functions, such as BLA, CeA and intercalated cell cluster, and plays an important role in emotional reactions and fear response (59, 60). BLA receives and processes sensory information for emotional events and projects to CeA for expression of fear-related behavior, such as freezing response (61-63). A recent study in rats has shown that repeated restraint stress elicits glutamatergic deficits in CeA and dendritic atrophy in pyramidal neurons in BLA (64, 65). Our observation of a long-term reduction of microtubule assembly pathway protein levels in BLA of shocked mice further supports these findings.

Decreased cytoskeleton and myelin sheath protein levels were also found in CA1 of hippocampus in our PTSD mouse model (Table 1, Supplemental Table S1). It has been suggested that during contextual fear memory recall, inhibition of CA1 interferes with the perception of the context during testing, rather than the recognition of the context from earlier training (66). A previous study showed that chronic stress induced hippocampal volume loss which is associated with neuronal loss and dendritic atrophy in CA1 and reduced neurogenesis in DG (67). Taken together, stress-induced structural and synaptic organizational alterations in CA1 may contribute to generalized fear response (neutral context) in PTSD.
2. RESEARCH ARTICLES

Fluoxetine is an SSRI antidepressant commonly used for treatment of major depressive disorder, obsessive-compulsive disorder, panic disorder, as well as PTSD (7, 68, 69). Psychotherapeutic actions of fluoxetine treatment are reported to be involved in cortical neurofilament alteration, increased synaptic remodeling and neurogenesis (70, 71). Furthermore, previous studies revealed an anti-inflammatory effect of fluoxetine treatment in vitro (72, 73), which was also observed in ACC in one of our studies applying the same PTSD mouse model (Kao et al., submitted). Early intervention of fluoxetine treatment improved long-term conditioned fear response and rescued downregulation of Citric Acid Cycle pathway in ACC and NAc in shocked mice after drug washout (Fig. 3), indicating a long-lasting effect of fluoxetine treatment on regulating mitochondrial functionality. Apart from serotonergic neurotransmission, several studies also indicated modulations of mitochondrial functionality after fluoxetine treatment. Upon norfluoxetine (active metabolite of fluoxetine) or other antidepressant treatment, a decreased transmembrane electrochemical gradient generated by the mitochondrial electron transport chain was observed in isolated rat heart mitochondria (74). Treatment of rats with paroxetine, another SSRI antidepressant, increased mitochondrial respiratory chain in prefrontal cortex, hippocampus, striatum and cerebral cortex (75). In line with previous animal studies, Zhao et al. demonstrated that fluoxetine and imipramine treatments regulate energy metabolism, amino acid metabolism and neurotransmitters in the hippocampus and showed anti-depressive effects in a chronic mild stress mouse model of depression (76). Furthermore, the anti-inflammatory effect of fluoxetine may improve stress-induced mitochondrial dysfunction and prevent neurons from cellular metabolism perturbations (77). Taken together, mitochondria appear to be the target of fluoxetine treatment by its ability to alter energy metabolism and production of amine. The modulation of neuronal energy metabolism might constitute a venue for treating PTSD.
In CA1 we also observed a fluoxetine treatment rescue effect on myelin sheath protein loss in shocked mice (Table 2). Activated microglia produce proinflammatory cytokines, reactive oxygen species and proteases which lead to apoptosis in oligodendrocytes and neurons (78, 79). Decreased myelin oligodendrocyte glycoprotein expression and myelin loss in mice is blocked by fluoxetine by inhibiting microglial activation after spinal cord injury (80). Another study revealed that fluoxetine activates the sterol-regulatory element-binding protein transcription factor with subsequent upregulation of the downstream lipogenesis-related genes, which are important for myelin component synthesis (81). In summary, fluoxetine exerts different functions on cellular pathways which may counteract myelin protein loss in CA1 associated with PTSD.

In conclusion, our proteomics and metabolomics data of a FS-induced PTSD mouse model have shown altered energy metabolism in PrL, ACC and NAc, dysregulated cytoskeleton protein expressions in BLA and CeA as well as myelin protein loss in CA1. Furthermore, fluoxetine treatment of PTSD mice rescued protein expression alterations associated with Citric Acid Cycle in ACC and NAc, and myelination in CA1. Through an integration of proteomics and metabolomics data we were able to provide novel insights into molecular pathways involved in PTSD and the potential for therapeutic mitochondrial targeting, which most recently is also considered for cancer (82) and neurodegenerative disorders treatments (83). Mitochondrial involvement has also been suggested for other psychiatric diseases (84, 85). Further studies are needed to find out whether mitochondrial-related alterations are specific for different classes of neurons and their role in the pathogenesis of distinct mental disorders.
2. RESEARCH ARTICLES

Funding and Disclosure

This work was supported by the Max Planck Society. All authors report no biomedical financial interests or potential conflicts of interest.

Acknowledgement

We would like to thank Dr. Judith Reichel for assistance with PTSD mouse model, and Stefan Reckow for help with data processing. Chi-Ya Kao was supported by the Graduate School of Systemic Neurosciences (GSN-LMU).
References


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES


52. Gray, J. A. (1995) Dopamine release in the nucleus accumbens: the perspective from aberrations of consciousness in schizophrenia. *Neuropsychologia* 33, 1143-1153


2. RESEARCH ARTICLES

the nucleus accumbens and mesial prefrontal cortex of patients with posttraumatic stress disorder. *Neuropsychologia* 46, 2836-2844


2. RESEARCH ARTICLES


### Table 1. Enriched molecular pathways in shocked mice.

<table>
<thead>
<tr>
<th>Region</th>
<th>Cellular Function</th>
<th>Pathway (Gene Ontology/KEGG)</th>
<th>Fold Enrichment</th>
<th>P-value</th>
<th>Bonferroni Corrected</th>
<th>ROR Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic islets</td>
<td>Exocrine cells</td>
<td>0048630—phylogenetic 5-phosphatase</td>
<td>2.330</td>
<td>1.37E-03</td>
<td>1.5E-03</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Exocrine cells</td>
<td>0048640—exocrine cells</td>
<td>2.332</td>
<td>1.37E-03</td>
<td>1.5E-03</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Exocrine cells</td>
<td>0005490—pancreatic islet</td>
<td>2.250</td>
<td>1.37E-03</td>
<td>1.5E-03</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Exocrine cells</td>
<td>0005490—pancreatic islet</td>
<td>2.250</td>
<td>1.37E-03</td>
<td>1.5E-03</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: ROR Function indicates the direction of the enriched pathway.
### Table 2. Rescued pathways upon chronic fluoxetine treatment in shocked mice.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>DAVID terms</th>
<th>(P_{\text{rescued}}) &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>transit peptide, acetylation, G0:0005525-GTP binding, gtp-binding, G0:0006091-generation of precursor metabolites and energy, iron</td>
<td>1.14E-13, 4.93E-13, 6.67E-11, 1.90E-10, 1.36E-08, 2.86E-06</td>
</tr>
<tr>
<td>CA1</td>
<td>transmembrane protein, G0:0007017-microtubule-based process, G0:0043209-myelin sheath, microtubule, myelin, PIRSF002867tubulin</td>
<td>1.53E-05, 0.001953125, 0.015625, 0.015625, 0.03125</td>
</tr>
<tr>
<td>NAc</td>
<td>mmu00020:Citrate cycle (TCA cycle)</td>
<td>0.009605408</td>
</tr>
</tbody>
</table>

\(P_{\text{rescued}}\) - P value before multiple test correction
Figure legends

Fig. 1. **Shock application and chronic fluoxetine treatment effects on contextual fear response in mice.** Mice receiving electric foot shocks showed significantly higher expression of freezing response when exposed to neutral context (cylinder chamber) and grid context (hexagon Plexiglas chamber), neutral tone and shock context (cubic chamber with metal grids for shock application) compared to control (non-shocked) mice. Contextual fear response in shocked mice was ameliorated upon chronic fluoxetine treatment. All comparisons were assessed by one way ANOVA analysis with Bonferroni’s Multiple Comparison test. N = 5 for control-vehicle group, N = 5 for shock-vehicle group and N = 3 for shock fluoxetine group. Data are presented as mean ± SEM (*p < 0.05, **p < 0.01, ns- not significant).

Fig. 2. **Altered enzyme and metabolite levels of Citric Acid Cycle in nucleus accumbens (NAc) and anterior cingulate cortex (ACC) in shocked mice.** In NAc (A) and ACC (B), enzymes and metabolites involved in Citric Acid Cycle were downregulated in the shocked mice. Oval- and rectangle-shaped boxes represent enzymes and metabolites of the Citric Acid Cycle, respectively. Red and blue boxes indicate increased and decreased levels, respectively, in the shocked mice compared to control mice. Red and blue box shade intensities represent the level of fold change expressed by log2 ratio.

Fig. 3. **Chronic fluoxetine treatment effect on the enzyme and metabolite levels of Citric Acid Cycle in nucleus accumbens (NAc) and anterior cingulate cortex (ACC) in shocked mice.** Proteome comparison of shocked mice with vehicle or fluoxetine treatments revealed lower enzyme levels of Citric Acid Cycle in both NAc (A) and ACC (B). The results indicated that chronic fluoxetine treatment rescued decreased enzyme expressions of the Citric Acid Cycle in NAc (A) and ACC (B) in shocked mice. Red and blue boxes indicate
increased and decreased levels, respectively, in the shocked mice compared to control mice. Red and blue box shade intensities represent the level of fold change expressed by log2 ratio.
Figure 1
Figure 2
2.2. Transcriptomic profiling reveals affected inflammatory pathway in a mouse model of posttraumatic stress disorder

Chi-Ya Kao, Zhisong He, Oliver Hahn, Carsten T. Wotjak, Philipp Khaitovich, Christoph W. Turck

Declaration of contribution: Chi-Ya Kao designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with coauthors.

- Study design and plan: in collaboration with ZH, CW, PK and CT
- Conducting the experiments: in collaboration with ZH and OH
- Data analysis: in collaboration with ZH, OH, PK and CT
- Manuscript preparation: in collaboration with ZH and CT

Supplemental material is available on enclosed DVD and upon request.
Transcriptomic profiling reveals affected inflammatory pathway in a mouse model of posttraumatic stress disorder

Chi-Ya Kao¹,²,†, Zhisong He³,†, Oliver Hahn³, Carsten T. Wotjak⁴, Philipp Khaitovich³,†, Christoph W. Turck¹,†*

¹Max Planck Institute of Psychiatry, Department of Translational Research in Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany
²Graduate School of Systemic Neurosciences, Ludwig-Maximilians-University Munich, Grosshadernerstr. 2, 82152 Planegg-Martinsried, Germany
³CAS-MPG Partner Institute for Computational Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai, The People’s Republic of China
⁴Max Planck Institute of Psychiatry, Department of Stress Neurobiology and Neurogenetics, Kraepelinstrasse 2-10, 80804 Munich, Germany

† These authors contributed equally to this work

*Correspondence:
Christoph W. Turck
Max Planck Institute of Psychiatry
Department of Translational Research in Psychiatry
Kraepelinstrasse 2-10
80804 Munich, Germany
Phone: +49-89-30622317, Fax: +49-89-30622610, E-mail: turck@psych.mpg.de

Keywords: Transcriptomics, inflammation, anterior cingulate cortex, fluoxetine, posttraumatic stress disorder, animal model

Running title: Brain transcriptomic profiling of a PTSD mouse model
Abstract

Background: Despite intense research efforts the molecular mechanisms affecting stress-vulnerable brain regions in posttraumatic stress disorder (PTSD) remain elusive. In the current study we have applied global transcriptomic profiling to a PTSD mouse model induced by foot shock fear conditioning.

Methods: We compared the transcriptomes of prelimbic cortex, anterior cingulate cortex (ACC), basolateral amygdala, central nucleus of amygdala and CA1 of the dorsal hippocampus between shocked and non-shocked (control) mice, with and without fluoxetine treatment by RNA sequencing. Differentially expressed (DE) genes were identified and clustered for in silico pathway analysis.

Results: DE genes belonging to 11 clusters were identified and include decreased neurotransmitter signaling in both ACC and CA1, and increased inflammatory response in ACC in shocked mice. Chronic fluoxetine treatment initiated in the aftermath of the trauma prevented inflammatory gene expression alterations in ACC and ameliorated PTSD-like symptoms, implying an important role of the immune response in PTSD pathobiology.

Conclusion: Our results provide novel insights into molecular mechanisms affected in PTSD and suggest therapeutic applications with anti-inflammatory agents.
2. RESEARCH ARTICLES

Introduction

According to *DSM-5*, posttraumatic stress disorder (PTSD), is classified as a trauma- and stress-related psychiatric disorder (1). PTSD develops upon encountering traumatic events such as natural disasters, sexual/childhood abuse or war combat. Persistent re-experiencing flashbacks as well as nightmares of the traumatic events (intrusion symptoms), avoiding thoughts and activities associated with the trauma (avoidance symptoms), decreased interest and negative alterations in cognition (emotional numbing symptom) and hypervigilance and aggressive behavior (hyperarousal symptoms) are the core features of PTSD (1).

PTSD symptoms develop days to months following trauma exposure, indicating long-lasting molecular/signaling sequelae of traumatic stressor central for the underlying pathophysiological mechanism. Dysregulations in hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes activities (2-5), and serotonin (6, 7) and dopamine (8, 9) neurotransmitter signaling have been observed in PTSD patients. In addition, genetic factors are considered to contribute to PTSD pathogenesis. Previous studies have shown single nucleotide polymorphisms (SNP) in the corticotropin-releasing hormone receptor 1 (*CRHR1*) gene (10) and in the FK506 binding protein 5 (*FKBP5*) gene (11) to be associated with PTSD symptoms. Furthermore, a SNP in the pituitary adenylate cyclase-activating polypeptide type I (*PAC1*) receptor stimulates CRH gene expression which regulates stress response in female PTSD patients (12, 13).

Memory- and stress- related processes regulated by brain structures involved in fear response circuitry such as amygdala, prefrontal cortex, hippocampus and nucleus accumbens, play important roles in the development of PTSD (13). Amygdala, the brain structure associated with heightened arousal, learning and fear has an increased activity in response to trauma-related stimuli in PTSD patients (14, 15). Clinical studies with PTSD patients have shown...
decreased activity in the prefrontal cortex, the brain region related to cognition and fear memory extinction (15). Furthermore, hippocampus size has been reported to correlate inversely with PTSD susceptibility (16). These data indicate that molecular dysregulations in the activity and functional connectivity of the fear circuitry result in abnormal perception to the traumatic events which may underlie the pathological mechanism of PTSD. Although brain imaging has advanced functional studies it is unclear how cellular alterations in specific brain regions contribute to PTSD development.

Animal models can be used to carry out targeted –omics analyses of brain regions believed to be part of the neural circuit relevant for the PTSD phenotype. In this study, we used a previously published electric foot shock-induced PTSD mouse model (17). This model is characterized by long-term maintenance of generalized fear, avoidance and hyperarousal (18, 19) as well as altered hippocampus volume (20). Our goal was to correlate PTSD-like symptoms and response to treatment with the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine, reported to effectively ameliorate PTSD-like syndrome in mice (17), with molecular changes in brain regions critical for fear memory and response. We performed global transcriptome profiling to identify transcripts and associated pathways underlying PTSD pathobiology. We were able to demonstrate that fluoxetine treatment effectively rescued transcripts and pathway alterations in the PTSD mouse model.

Methods and Materials

Animals

Male C57BL/6NCrl mice, 7-8 weeks old (Charles River GmbH, Sulzfeld, Germany) were housed in groups of four in Makrolon type II cages (23×16.5×14 cm³) under an inverse 12:12
h light-dark cycle (light off at 7:00) with food and water ad libitum. All experiments were carried out according to the European Community Council Directive 2010/63/EEC and approved by the local government of Upper Bavaria (55.2.1.54-2532-41-09 and 55.2.1.54-2532-141-12).

**PTSD model (Fear conditioning)**

For the PTSD mouse model, we performed foreground contextual fear conditioning (administration of the foot shock (FS) in the shock chamber with house light on) during the active phase according to a previously published protocol (17). The outline of the PTSD mouse model experiment is presented in Supplementary Figure S1. In brief, mice were conditioned in a plexiglas cage (16×16×32 cm³) with a grid harness package (ENV-407, ENV-307A, MED Associates, 7 St. Albans, VT, USA) connected to a shock generator (Shocker/Scrambler: ENV-414, MED Associates). After 198 sec of habituation, animals underwent one electric FS (1.5 mA, 2 sec of length) at moderate illumination (40 Lux). Animals remained in the shock chamber for another 60 sec before being returned to their home cages. Non-shocked (control) mice went through the same procedure, but without receiving FSs. To test intensity and specificity of contextual fear, animals were exposed to neutral environment, grid or shock context. 56 days after FS, animals were placed into the neutral test chamber (cylindrical shaped). After a 3 min neutral tone (80 dB, 9 kHz) presentation animals remained in the test chamber for another 60 sec before they were returned to their home cages. 57 days after FS, animals were exposed to the hexagonal chamber with grid floor and shock chamber with house light on for 3 min without further shock application. Freezing behavior was defined as immobility except for respiration movements.
Fluoxetine treatment

Fluoxetine-ratiopharm TM solution (Ratiopharm GmbH, Ulm, Germany; produced by Merckle GmbH, Blaubeuren, Germany) was dissolved in water and applied via lightproof drinking bottles in doses of 20 mg/kg/day. 12 hours after FS application, non-shocked and shocked mice received either fluoxetine or tap water (vehicle) for 28 days, followed by a 28-day wash-out period. Fluoxetine treatment efficacy was assessed by evaluating sensitized fear response during a presentation of 60 sec neutral tone (80 dB, 9 kHz) in a neutral context (cylinder) on day 28 after FS. Conditioned and sensitized fear was evaluated on days 56 to 57 as described above (Supplementary Figure S1) to inspect long-lasting effect of fluoxetine treatment.

Brain sampling

On day 64 after FS, i.e. one week after development of PTSD-like symptoms, mice were euthanized by an overdose of isoflurane (Forene, Abbott, Wiesbaden, Germany) and sacrificed. Mice were decapitated and brains were harvested and immediately fixed with ice-cold 2-methylbutane (Merck, Darmstadt, Germany), snap frozen in liquid nitrogen and stored at -80°C. For brain punch, brains were dissected with a cryostat (Microm, Walldorf, Germany) up to the appearance of target sub-regions. Punch specimens were isolated using cylindrical punchers (Fine Science Tools, Heidelberg, Germany) essentially as described (21). The location and length of the punches were selected based on a stereotaxic atlas (22) described as follows (in mm: starting point posterior to bregma, punch diameter, punch length): for prelimbic cortex (PrL) (2.3 mm rostral-caudal to bregma, 1.0 mm in diameter, 0.9 mm in length), anterior cingulate cortex (ACC) (0.86, 0.5, 1.0), nucleus accumbens (NAc) (1.7, 0.5,
2. RESEARCH ARTICLES

1.0), basolateral amygdala (BLA) (-0.8, 0.35, 1.0), central nucleus of amygdala (CeA) (-0.9, 0.5, 0.8), cornu ammonis 1 (CA1) region of the dorsal hippocampus (-1.5, 0.5, 0.8). Punches were stored at -80°C for RNA extraction. The dissection site was verified by histological staining with a stereomicroscope.

RNA extraction and RNA sequencing

RNA was isolated from brain sub-region cryo-punches using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and treated with RNase free DNase I (Ambion, Austin, TX) at 37°C for 30 min. The cDNA libraries were prepared by adding random hexamer primers (Invitrogen) to individual sample containing 300 μg total RNA according to manufacturer’s instructions. The Illumina sequencing libraries were prepared according to the single-end sample preparation protocol (HiSeq 2000, http://www.illumina.com), and were purified with Qiaquick PCR purification kit (Qiagen, Valencia, CA). The libraries (12 single-indexed samples per pool) were sequenced using the Illumina Genome Analyzer II system with sequenced read-length (starting after the index) of 100 bp per read. All sequence data including quality scores are deposited in the NCBI’s Short Read Archive, with SRA accession number SRP057486 and BioProject ID PRJNA281134.
Results

Transcriptome characteristics of brain regions involved in fear circuit

A posttraumatic stress disorder (PTSD) mouse model was generated by exposing adult C57BL/6 mice to two non-escapable electric foot shocks (FS) (1.5 mA, 2 sec) (17). After 56 days of incubation, shocked mice developed PTSD-like symptoms, apparent by a conditioned fear response. The beneficial effect of fluoxetine persisted after offset of treatment until days 56/57 (Figure 1). To investigate molecular changes at the transcriptome level in the shocked mice, RNA-seq analysis was performed for the brain regions involved in the fear circuit, including PrL, ACC, BLA, CeA, CA1 and NAc (Supplementary Methods and Materials). Three biological replicates were used for each brain region per group. RNA-seq resulted in 336.1 million and 336.6 million reads with 100 nucleotide (nt) length in shocked and control mice, respectively. In addition, we generated RNA-seq data for mice treated with fluoxetine which resulted in 352.8 million and 349.4 million RNA-seq reads with 100 nt length for shocked and control mice, respectively (Supplementary Table S1).

To quantify and compare gene expression, we used the STAR mapping program with default parameters to map all the RNA-seq reads to the mouse genome mm10 (Supplementary Methods and Materials). This resulted in 292.9 million reads (87.6%) in shocked mice with vehicle treatment, 294.8 million reads (87.6%) in control mice with vehicle treatment, 310.9 million reads (88.1%) in shocked mice with fluoxetine treatment and 306.7 million reads (87.8%) in control mice with fluoxetine treatment, uniquely mapped to the genome. We used GENCODE M2 annotation to define gene structure. Gene expression was first quantified as the number of reads per kilobase per million of total mapped reads (RPKM). Only the protein-coding genes with maximum RPKM higher than 1 were considered. DESeq normalization method implemented in DESeq2 Bioconductor package was then applied to the
Identification and characterization of PTSD-related expressed genes

To identify the protein-coding genes with expression level change between shocked and control mice, we applied ANOVA with generalized linear model (GLM) to the gene expression levels of the 14591 protein-coding genes in all the 72 samples, covering the six brain regions from four mouse groups (shocked with vehicle treatment, control with vehicle treatment, shocked with fluoxetine treatment and control with fluoxetine treatment) (Supplementary Methods and Materials). With Benjamini & Hochberg corrected FDR < 10 % as cutoff, 741 protein-coding genes were identified as PTSD-related expressed genes, that is, genes with significant expression level changes between shocked and control mice.

We next aimed to characterize the biological significance of the expression changes of these 741 PTSD-related expressed genes. With the assumption that the biologically related genes tended to be co-expressed to collaborate, we used hierarchical clustering with 1-r, where r is the Pearson’s correlation coefficient calculated using the samples from vehicle-treated control and shocked mice, as the distance to identify the co-expressed clusters. We obtained 11 co-expressed clusters with reasonable size (> 20 genes) (Supplementary Table S2). Each cluster was characterized by its distinct expression pattern across the six brain regions in control and shocked mice (Supplementary Figure S2). DAVID analysis (23, 24) featured three of the 11 clusters with their distinct enriched functional categories (Supplementary Table S3). Cluster C4 with 86 PTSD-related expressed genes is characterized by its functional enrichment, among others, of synapse, neurotransmitter secretion and regulation of neurotransmitter
levels. Cluster C5 showed functional enrichment of glycoprotein, intrinsic to membrane and integral to membrane. Cluster C8 exhibited functional enrichment of chemotaxis, inflammation and other pathways which are related to inflammation, such as drug metabolism and cytokine.

Alterations in the expression levels of the PTSD-related genes in clusters C4, C5 and C8 may indicate different roles for pathophysiological changes in distinct brain regions. To better characterize the changes in gene cluster expression, Wilcoxon’s test was applied to each brain region to investigate the clusters which showed alteration in shocked mice compared to control mice (Figure 2). Interestingly, we found significant decreases of C4 and C5 gene expression levels in ACC and CA1, and an increase in C8 genes in ACC. Taken together, these results indicate that FS-induced dysregulated synaptic function and inflammatory response in ACC are involved in the development of PTSD-like symptoms.

**Regulation of PTSD-related genes**

Synchronized expression of genes within each cluster implies the co-regulation by shared regulators such as transcription factors (TFs). To test this, we estimated the enrichment of TF binding sites (TFBSs) in the promoter regions of genes within each cluster (Supplementary Methods and Materials). We observed significant excess of TFBSs in cluster C8 (permutation test, \( p < 0.001 \), Figure 3A). In addition, the TFs with enriched TFBSs in C8 showed significantly better correlation with genes in the C8 clusters compared to other clusters (one-sided Wilcoxon test, Benjamini & Hochberg correction, FDR < 0.05) (Figure 3B, Supplementary Table S4). These TFs also showed massive crosstalk in terms of protein-protein interaction or regulatory relationships (Supplementary Figure S3), whose density of
connections between TFs was significantly higher than that of the random TF networks with the same number of TFs ($p < 0.001$), forming the regulatory network which might be responsible for the expression alteration of the PTSD-related genes. Furthermore, the enriched TFs, e.g. $NFκB1$ and $NFκB2$, regulate inflammatory response which is the enriched pathway in cluster C8 genes. These results imply that the PTSD-related gene alterations are in part driven by transcription factors, particularly for genes in cluster C8.

Fluoxetine rescue effect suggests an important role of ACC in PTSD-like symptoms development

Previous studies have shown the efficacy of fluoxetine treatment in human PTSD (25). In addition, results from our PTSD mouse model showed therapeutic potency of chronic fluoxetine treatment (17). Therefore we were interested whether chronic fluoxetine treatment is able to rescue differential brain gene expression in the shocked mice. Shocked mice were treated with vehicle or fluoxetine (20mg/kg/day) starting 12 hours after the FS for 28 days, followed by a 4-week wash-out period. This wash-out period allowed us to investigate long-lasting effects of fluoxetine treatment while excluding acute effects of chronic fluoxetine treatment on fear expression. As expected, shocked mice that underwent fluoxetine treatment showed a significant decrease in conditioned fear response compared to shocked mice with vehicle treatment on day 57 after FS (Figure 1).

To investigate the underlying mechanism of fluoxetine treatment with regard to gene expression, we calculated the log2-transformed fold change of gene expression level for each brain region by comparing 1) shocked-vehicle and control-vehicle ($\log2FC_{PTSD}$); and 2) shocked-fluoxetine and control-vehicle ($\log2FC_{rescued}$). Defining fluoxetine rescue as
\[|\log_{2} \text{FC}_{\text{rescued}}| < |\log_{2} \text{FC}_{\text{PTSD}}|, \] we used paired Wilcoxon’s test to check whether genes in each cluster tended to be rescued by fluoxetine treatment in each brain region. Most of the effective gene expression rescue upon fluoxetine treatment occurred in the ACC, including changes associated with C4, C5 and C8 clusters (Figure 4, Supplementary Figure S4). This observation again suggests that ACC is a critical brain region for the development of PTSD and the molecular alterations are causal for PTSD-like symptoms.

**Discussion**

In the present study, we applied global transcriptomics profiling to unravel altered neurocellular pathways in affected brain regions of a foot shock (FS) induced-PTSD mouse model. Chronic treatment with fluoxetine, an antidepressant drug used for PTSD patients, resulted in rescue of some of the affected pathways at transcriptome level in specific brain regions of the shocked mice. We identified PTSD-related genes by an ANOVA with generalized linear model (GLM) analysis and hierarchical clustering. Among the brain regions involved in fear circuits, we found anterior cingulate cortex (ACC) and CA1 region of the hippocampus to be affected the most in the shocked mice. Gene clusters enriched for neurotransmitter secretion and membrane integrity pathways were decreased in both ACC and CA1 and gene cluster enriched for inflammation pathway was increased in ACC. Further analyses showed that chronic fluoxetine treatment ameliorated the dysregulated expression of gene clusters in ACC in the shocked mice along with improved PTSD-like symptoms which were assessed by conditioned fear response.
PTSD, like other anxiety disorders, is associated with abnormalities in brain regions involved in the fear circuitry, including medial prefrontal cortex (mPFC), amygdala, hippocampus and NAc. Alterations in the functional connectivity and activation of these brain regions result in dysregulated perception and interpretation of traumatic events (26, 27). The mPFC regulates emotional control, cognition and fear extinction (28); the hippocampus is involved in contextual fear memory, traumatic memory consolidation and retrieval; the amygdala is associated with cue conditioning, hyperarousal and expressing fear response (29, 30); and the NAc is related to reward-related behavioral abnormalities (31). Altered emotion-cognition interaction plays an important role in dysregulated brain activity in PTSD (32). ACC is involved in generalized emotion- and cognitive-processing network, such as decision making (33, 34), emotion (35, 36) and impulse control (37). ACC integrates attentional and emotional processing and has been reported to be affected in PTSD. Neuroimaging studies revealed significant reduction in ACC volume (38) which might be related to attentional deficits and the inability to regulate emotions observed for PTSD patients. Furthermore, successful cognitive behavioral therapy leads to an increased activity of the right ACC (39) and larger rostral ACC was associated with PTSD symptom reduction (40). Another study reported reduced N-acetylaspartate (NAA) levels in the right ACC of PTSD patients, indicating neural integrity deficits (41).

Recent clinical studies implicate an important role of the immune system in PTSD pathogenesis (42, 43). Lindqvist et al. showed that PTSD-afflicted male soldiers who had been exposed to combat have higher pro-inflammatory cytokine levels in the blood (44). Pro-inflammatory cytokine injections into the brains of mice exposed to stress induce anxiogenic effects (45). Furthermore, increased microglial numbers were found in the ACC of adult male Sprague-Dawley rats exposed to chronic unpredictable restraint stress (46). Supporting these findings, in the present study we found increased expression of Cluster C8 genes, which are
involved in inflammatory and chemotaxis pathways, in the ACC of shocked mice (Supplementary Tables S2, S3). Chemokine ligand 5 (Ccl5), identified in cluster C8, is chemotactic and recruits leukocytes to inflammatory sites, induces proliferation of natural killer cells and activates an inflammatory response (47, 48). Increased neuroinflammation leads to demyelination and neurodegeneration which might be the cause for ACC volume loss in PTSD. In addition, immune response modulators have been implicated in functional synaptic plasticity (49). Several proinflammatory cytokines, including interleukin- (IL-) 6 (50, 51), IL-1β (52, 53), IL-8 (54) and interferon-α and β (55, 56) regulate long-term potentiation in the hippocampus and improve long-term memory formation (57). In another study enhanced synaptic transmission via increased AMPA-receptor-mediated response in ACC neurons in rats with inflammation was observed (58). Taken together, it seems that inflammation induces perturbations in synaptic plasticity afflicting neurons associated with abnormal ACC activity, a phenomenon that has also been reported in PTSD patients when processing neutral and emotional information (59, 60). In summary, our findings reveal a long-lasting effect of inflammation in ACC which appears to result in structural and functional abnormalities in neurons. PTSD symptoms develop from days to weeks upon trauma exposure, indicating that chronic and propagated inflammation may be the underlying mechanisms for cognitive deficits and the inability of emotional control when confronted with trauma-related information is critical for PTSD development.

Elevated inflammation has also been observed in dorsal ACC white matter of depressed suicidal individuals. Increased neuroinflammation involving macrophage recruitment and microglial priming in dorsal ACC may be associated with increased circulating pro-inflammatory cytokines that have been reported for depressed patients (61). Setiawan et al. have provided further evidence that microglial activation is associated with major depressive
episodes. In addition, greater distribution volume of translocator protein density in ACC, a marker for neuroinflammation, correlated with greater depression severity (62). The results from our study on a PTSD mouse model provide further support for the notion that inflammation-induced perturbation in ACC plays an important role in the pathogenesis of psychiatric disorders.

In the current study we found synchronized expression of genes within each cluster with cluster C4, C5, and C8 genes exhibiting significant alterations in the shocked mice (Figure 2). We therefore hypothesized that cluster C4, C5 and C8 genes are co-regulated by upstream transcription factors (TF). With the help of transcription factor binding site (TFBS) enrichment analysis we identified TFs for cluster C8 genes (Figure 3, Supplementary Table S4) which form a closely interconnected network (Supplementary Figure S3). Among the identified TFs, Spi1 regulates the majority of cluster C8 genes (Supplementary Table S5), enriched for inflammatory and chemotaxis pathways. SPI1 encodes PU.1 regulator and is involved in microglia activation and B-lymphoid cell development (63). Clinical and animal studies have shown an upregulation of immune response genes and regulatory regions, which are targeted by PU.1 in Alzheimer’s disease (AD) (64), suggesting its role in inflammation-induced neurodegeneration in AD. Another study of a PTSD rat model from Daskalakis et al. (65) showed that Spi1 together with other TFs regulates stress-exposure-associated differentially expressed genes in the blood that are part of the glucocorticoid receptor signaling pathway. PU.1 regulator contains an ETS domain which interacts with Ets1 (66). Similar to SPI1, ETS1 regulates immune cell differentiation and cytokine expression (67). Furthermore, it was shown that upon cytokine activation Ets1 and NFkB work synergistically to transactivate downstream targets associated with inflammation (68). NFkB is the master transcriptional regulator of the immune response and is associated with neurogenesis (69). NFkB function can be manipulated through the activities of Toll-like-receptors (TLRs) (70,
In this regard Zimmermann et al. were able to show that peripheral administration of specific oligonucleotide activators of the innate immune receptor TLR9 can prevent posttraumatic symptoms in stressed mice (72). These findings implicate TLR9 as a key regulator of the pro-inflammatory NFKB pathway in posttraumatic anxiety. In accordance with previous studies, our findings support an important role for NFKB, Ets1, Spi1 as well as other TFs in the regulation of inflammatory gene expression associated with stress-related disorders. Our findings of an enrichment of inflammatory genes and their upstream TFs which have been reported to be associated with stress-related disorders lend further support to the hypothesis that the inflammatory response in ACC is involved in PTSD pathogenesis.

A previous study reported a role of IL-1ß in the hippocampus after surgery in mice with postoperative cognitive dysfunction (73). Furthermore, elevated inflammation perturbs neurogenesis and is implicated in hippocampal volume loss and higher PTSD symptom severity in Gulf War veterans (74). Together with the current study, these findings indicate a possible role of inflammation in perturbed interconnection between ACC and hippocampus, which leads to dysregulated fear extinction learning signals and PTSD pathogenesis.

Traumatic events induce lost-lasting changes in neurotransmitter signaling and synaptic plasticity in stress-vulnerable brain regions and have been implicated in the pathophysiological mechanisms of PTSD (75, 76). In this regard it has been suggested that an imbalance of gamma-aminobutyric acid (GABA) and glutamate is associated with hippocampal neuronal apoptosis in a PTSD animal model (77). In addition, clinical studies revealed lower glutamate levels in ACC in PTSD patients with alcoholism comorbidity (78). In our study we found a downregulation of genes that are enriched for neurotransmitter secretion, synapse and membrane integration pathways in CA1 and ACC of shocked mice (Clusters C4 and C5, Figure 2, Supplementary Table S3). This suggests that imbalanced
neurotransmitter secretion due to dysregulated synaptic vesicle function may be associated with abnormal CA1 and ACC activities and may contribute to deficits of fear extinction and cognition in shocked mice. Unlike for the inflammatory genes of Cluster C8 we were unable to find an enrichment of TFs for genes in clusters C4 and C5. We therefore submit that whereas an increased inflammation in ACC is mediated through upstream regulators, dysregulated neurotransmitter signaling may be a secondary phenomenon involved in PTSD development. Interestingly, we found that enriched PTSD-related genes of clusters C4, C5 and C8 in the ACC of our mouse model resemble the expression pattern of a transcriptomics study on prefrontal cortex of autistic patients (79). Further investigation revealed that our PTSD mouse model- and autism-related alterations have a significant overlap in 14 genes (one-sided Fisher’s exact test $p = 0.0067$, odds ratio = 2.23). Furthermore, 13 of the 14 PTSD mouse model- and autism-related genes also exhibit consistent direction of change (one-sided Binomial test $p = 0.0004$), hinting that the underlying pathophysiological mechanisms of dysregulated inflammatory response and synaptic function are shared between PTSD and autism.

Fluoxetine is a well-known SSRI antidepressant and has been officially approved for treatment of PTSD (84). Fluoxetine’s mode of action affects neurotransmission, neurofilament pathways, synaptic remodeling and neurogenesis (85-87). Previous studies with the same PTSD mouse model have demonstrated that fluoxetine treatment counteracts synaptic protein loss in hippocampus (88). In our study, chronic fluoxetine treatment ameliorates long-lasting conditioned fear response in the shocked mice (Figure 1) and rescues dysregulated inflammatory gene expression in ACC (Figure 4). Previous in vitro studies have demonstrated that fluoxetine suppresses production or expression of pro-inflammatory cytokines, such as TNF-$\alpha$, IL-1$\beta$ and IL-6 by microglial cells (89-91). Furthermore, Obychowicz et al. showed that fluoxetine exerts anti-inflammatory properties in the CNS by
silencing overactivated glia upon lipopolysaccharide stimulation (92). In an animal of PTSD, rats were exposed to psychogenic stress and treated 1 hour later with minocycline, a tetracycline with anti-inflammatory and neuroprotective capacities (93). Minocycline attenuated anxious-like behavior and decreased IL-6 levels in frontal cortex, hippocampus and hypothalamus in stressed rats. In accordance with these previous findings our study showed that chronic fluoxetine treatment initiated 12 hours after shock application resulted in an anti-inflammatory effect in ACC and improved PTSD-like symptoms in shocked mice. Importantly, these effects were observed after discontinuation of treatment. Thus, early intervention with fluoxetine seems to prevent physiological and behavioral alterations resulting from stress exposure via its anti-inflammatory capacity in the CNS.

In the current study, we identified gene clusters with significant changes, applied functional enrichment analysis and characterized their alterations in each brain region. We were able to detect significant alterations at the transcriptome level that are related to synapse and inflammatory pathways in ACC and CA1. Another recently published transcriptomics study with a PTSD mouse model has found alterations in neuronal signaling pathways important for neurogenesis, cognition and extinction of fear memory in other brain structures involved in the fear response circuitry (94). The genome-wide analysis performed here significantly extends previous findings on the dysregulated inflammatory response and synaptic signaling in PTSD (42, 43, 76) by providing an unbiased systemic investigation of transcriptional alterations without acute prior challenge (i.e. tonic rather than phasic changes). We were also able to demonstrate that early intervention by fluoxetine treatment counteracts inflammatory gene expression and improves PTSD-like symptoms. Our findings provide novel insights on the role of the immune response in the CNS relevant for the pathogenesis of PTSD and suggest potential therapeutic mechanism that can be exploited with anti-inflammatory agents.
2. RESEARCH ARTICLES

The knowledge of affected CNS inflammatory pathways is an important step towards clinical translation for improved diagnosis and treatment of PTSD.

Acknowledgement

We would like to thank the CAS-MPG Partner Institute for Computational Biology Omics Core HiSeq platform for assistance with RNA-sequencing. Chi-Ya Kao was supported by the Graduate School of Systemic Neurosciences (GSN-LMU).

Financial Disclosures

This work was supported by the National Natural Science Foundation of China (Nr. 91331203 and 31420103920), the 1000 Plan for Foreign Experts (WR20123100078) and the Max Planck Society. All authors report no biomedical financial interests or potential conflicts of interest.
2. RESEARCH ARTICLES

Reference


15. Bremner JD, Vermetten E, Schmahl C, Vaccarino V, Vythilingam M, Afzal N, et al. (2005): Positron emission tomographic imaging of neural correlates of a fear acquisition and


2. RESEARCH ARTICLES


102


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES


2. RESEARCH ARTICLES

00760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res.* 1002:1-10.


2. RESEARCH ARTICLES

Figure Legends

Figure 1. Chronic fluoxetine treatment ameliorates conditioned fear response in shocked mice. Mice were exposed to neutral context, including cylindrically and hexagonally shaped plexiglass and shock context, a cubic-shaped chamber with metal grid for shock application. Shocked mice with fluoxetine treatment showed significantly decreased conditioned fear response in both neutral and shock contexts. N = 3 for each group. Two-way ANOVA analysis was applied and data are presented as mean ± SEM (* p < 0.05, ** p < 0.01, **** p < 0.0001).

Figure 2. The gene expression level change of the 11 co-expressed clusters in the six brain regions. Upper panel: The tree represents the hierarchical clustering. Lower panel: For each brain region, one circle represents one cluster, with its radius proportional to the size of the cluster. Clusters with bold numbers represent gene clusters with significant expression level alterations. The X-axis represents the log10-transformed P value of Wilcoxon’s test, and the Y-axis represents the median of log2-transformed fold change of genes in the cluster between shocked mice and control mice samples. Clusters with significant gene expression level increase in the brain region are indicated in red, while clusters with significant gene expression level decrease are indicated in blue. ACC, anterior cingulate cortex; BLA, basolateral amygdala; CA1, cornu ammonis 1 of dorsal hippocampus; CeA, central nucleus of amygdala; NAc: nucleus accumbens; PrL, prelimbic cortex.

Figure 3. Regulation of the PTSD-related genes by TFs. (A) Number of transcription factor binding sites (TFBSs) enriched among genes within each of the 11 co-expressed clusters. The grey bars represent the observed number of enriched TFBSs in each cluster (one-sided Fisher’s exact test, p < 0.05). The hashed bars represent the mean number of enriched TFBSs expected by chance, calculated by 1000 random assignment of the PTSD-related genes into
the 11 clusters with the same size as observed. Cluster C8 had significantly more enriched TFBSs than random cluster of the same size (* $p < 0.05$). (B) Number of transcription factors (TFs) with TFBSs enriched and correlated with the targets in the cluster (one-sided Wilcoxon’s test, Benjamini & Hochberg corrected FDR < 0.05). The hashed bars represent the mean number of enriched TFBSs expected by chance, calculated by 1000 random assignment of the PTSD-related genes into the 11 clusters with the same size as observed. Expression level of Cluster C8 genes showed a significant positive correlation with the enriched TFs (* $p < 0.05$).

**Figure 4.** The rescue effect of fluoxetine in each brain region for each cluster. (A) The effective rescue for the co-expressed clusters. The cells representing the cluster with significant rescue effect in a certain brain region were marked with streaks. The red and blue cells represent the clusters with a significant increase and decrease expression alteration in the shocked mice compared to the control mice, respectively. (B) Gene expression patterns in co-expressed clusters C1, C4, C5 and C8. Gene expression alteration was rescued by fluoxetine treatment in ACC for control-vehicle (NS), shocked-vehicle (S) and shocked-fluoxetine (SF) mice.

**Supplementary Figure S1.** C57BL/6 mice were exposed to a single electric footshock and subsequently received treatment with either fluoxetine (20mg/kg/day) or vehicle (drinking water). On day 28, sensitized fear was measured by exposing the mice to neutral environment (cylinder plus tone displayed). After a 28-day drug-washout period, the intensity and specificity of conditioned fear response was investigated by exposing mice to cylinder plus tone on day 56, shock context (shocking chamber without shock application) and hexagonal context with grid floor as shock reminders on day 57. Mice were sacrificed one week after conditioned fear response evaluation.
2. RESEARCH ARTICLES

Supplementary Figure S2. The average expression pattern of the 11 co-expressed clusters for vehicle-treated mice. The white boxes represent control (NS) mice, and the black boxes represent shocked (S) mice.

Supplementary Figure S3. The crosstalk among transcription factors (TFs) with transcription factor binding sites (TFBSs) enriched in cluster C8. The network was generated using *EdgeExpressDB* in FANTOM 4.

Supplementary Figure S4. The average expression pattern of the 11 co-expressed clusters across control-vehicle (NS), shocked-vehicle (S), and shocked-fluoxetine (SF) mice.

Supplementary Table S1. Sample and read mapping information.

Supplementary Table S2. PTSD-related expressed genes and hierarchical clustering result.

Supplementary Table S3. The enriched functional terms of each co-expressed cluster output by DAVID.

Supplementary Table S4. The enriched TFBSs with the corresponding TFs that significantly correlated with genes in the respective cluster.

Supplementary Table S5. Transcription factor targeted genes in cluster C8.
Figure 1
A

Number of TFBSs

C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11

0 2 4 6 8

observed
expected

B

positive correlated

Number of TFs

C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11

4 2 0 2 4 6 8 10

negative correlated

*
A

<table>
<thead>
<tr>
<th></th>
<th>ACC</th>
<th>BLA</th>
<th>CA1</th>
<th>CeA</th>
<th>NAc</th>
<th>PrL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Increase in shocked

Decrease in shocked

Rescued upon fluoxetine treatment

B

![Box plots for ACC expression levels](image)

Expression Level (z-transformed)

Figure 5
2.3. Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice

Chi-Ya Kao, Guenter Stalla, Johanna Stalla, Carsten T. Wotjak, Elmira Anderzhanova

Declaration of contribution: Chi-Ya Kao designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with coauthors.

- Study design and plan: in collaboration with EA and CW
- Conducting the experiments: in collaboration with EA, GS and JS
- Data analysis: in collaboration with EA and CW
- Manuscript preparation: in collaboration with EA and CW
Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice

C.-Y. Kao,1,2 G. Stalla,3 J. Stalla,3 C. T. Wotjak4 and E. Anderzhanova4
1Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany
2Graduate School of Systemic Neurosciences, Ludwig-Maximilians-University Munich, Planegg-Martinsried, Germany
3Department of Clinical Research, Max Planck Institute of Psychiatry, Munich, Germany
4Department of Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, Kraepelinstrasse 10, 80804 Munich, Germany

Keywords: animal model, corticosterone, forebrain, norepinephrine, PTSD

Abstract
This study measured changes in brain extracellular norepinephrine (NE) and free corticosterone (CORT) levels in a mouse model of post-traumatic stress disorder and related them to hyperarousal and fear memory retention. To this end, microdialysis in the medial prefrontal cortex (mPFC) and the hippocampus (HPC) of male C57BL/6NCrl mice was performed during an acoustic startle response (ASR) and following an electric foot shock (FS), as well as during an ASR and recall of contextual fear (CF) 1 day later. Changes in ASR-stimulated NE levels in the mPFC corresponded to ASR 34 days after FS. Changes in basal and ASR-stimulated extracellular NE levels in the HPC, in contrast, were related to expression of early (day 2) and late (day 34) CF after FS. The increase in extracellular NE levels correlated in a U-shape manner with arousal levels and CF, thus suggesting a non-linear relationship. Stress of different modalities/strength (ASR, FS and CF) caused a similar relative increase in free CORT levels both in the mPFC and the HPC. One day after FS, ASR-induced increases in the CORT content in the mPFC tended to correlate with the FS-potentiated ASR in a U-shape manner. Taken together, these data show that the intracerebral increase in free CORT was likely related to an immediate response to stress, whereas NE neurotransmission in the forebrain predicted arousal and CF 1 month after trauma.

Introduction
Hallmark symptoms of post-traumatic stress disorder (PTSD) are the retention of trauma memory, which is expressed in a specific and contextual fear (CF), hyperarousal, and reactive depression (American Psychiatric Association, DSM-IV-TR, 2000). Dysregulation of hypothalamic–pituitary–adrenal (HPA) axis activity is critically involved in PTSD pathophysiology. Clinical studies have shown that patients with PTSD exhibit abnormal corticotrophin-releasing factor levels in cerebrospinal fluids and elevated cortisol levels in 24-h urine samples (Pitman & Orr, 1990; Bremner et al., 1997a; Baker et al., 1999). Under stressful conditions, an increase in norepinephrine (NE) signaling significantly contributes to HPA axis activation. However, it is still unclear to what extent NE-mediated cognitive effects are independent of its influence on stress hormones release (Ma & Morilak, 2005; Morilak et al., 2005).

Abnormality in adrenergic signaling has been linked to the severity of PTSD symptoms (Geracioti et al., 2001). Previous studies showed that facilitation of adrenergic signaling with the α2-selective antagonist yohimbine increased anxiety in patients with PTSD (Bremner et al., 1997b; Southwick et al., 1997). In addition, consolidation of memories associated with emotional events was mediated by activation of the norepinephrinergic system in animals (Soeter & Kindt, 2011; Barsegyan et al., 2014). Nonetheless, administration of the β-receptor antagonist propranolol showed mixed results both in clinical trials and in animal models of PTSD (Pitman et al., 2002; Stein et al., 2007; Cohen et al., 2011; Debic et al., 2011).

NE is involved in sleep, vigilance and arousal, as well as attention, learning and memory in a structure-dependent manner. In the medial prefrontal cortex (mPFC), increased NE neurotransmission facilitates cognitive performance. Because cognitive performance contributes to coping with stress, a dual role of NE, both adaptive and deteriorating, in the mediation of the post-stress endophenotype has recently been proposed and studied (Lapiz & Morilak, 2006; Jett & Morilak, 2013).

Other evidence points to a significant NE and cortisol interaction, but fails to show any independent effects of NE signaling and salivary cortisol levels in the prediction of negative intrusive memories in patients with PTSD (Nicholson et al., 2014). It was hypothesised that the assessment of brain NE and corticosterone (CORT) levels is able to predict distinct PTSD outcomes. In the present study, a
mouse model of electric foot shock (FS)-induced PTSD was employed (Siegmund & Wotjak, 2007) in C57Bl/6N mice particularly susceptible for developing PTSD-like symptoms (Golub et al., 2009; Pamplona et al., 2011; Herrmann et al., 2012), and the temporal profile of NE release and free CORT levels in the mPFC and the hippocampus (HPC) during and after acoustic startle response (ASR), FS and CF was evaluated. The effect of stress on NE release in relation to HPA axis activation was compared by performing dynamic measurements of free CORT levels. In addition, immediate in relation to HPA axis activation was compared by performing immediate and delayed changes in arousal and fear memory retention induced by FS were assessed.

Materials and methods

Animals
Male C57BL/6NCrl mice, 7–8 weeks old (66 animals; Charles River GmbH, Sulzfeld, Germany) were housed in groups of four in Makrolon type II cages (23 × 16.5 × 14 cm³) under an inverse 12 : 12 h light : dark cycle (light off at 07:00 h) with food and water ad libitum. Experimental procedures were approved by the Government of Upper Bavaria. Laboratory animal care and experiments were conducted according to the regulations of the current version of The German Animal Welfare Act.

PTSD model validation

The outline of the PTSD model validation experiment is presented in Fig. 1A. The level of arousal was assessed on day 30, and CF memory was measured in shocked mice (see the protocol in the respective section) on day 32 (ASR30 and CF32) after FS in contrast to the non-shocked control group.

Microdialysis/PTSD experiment

The outline of the microdialysis experiment is presented in Fig. 1B. On experimental day 1, mice were subjected to a 20-min ASR test (ASR1) in a sound-protected environment. Two hours later, mice were subjected to FS (see next section). On experimental day 2, mice were recurrently tested for ASR (ASR2) and re-exposed to the FS chamber for CF examination (CF2) 2 h later. The dialysate fractions corresponding to the periods of Basal 1 (samples 1–3), ASR1/recovery (samples 4–7) and FS1/recovery (samples 8–11) on experimental day 1, and Basal 2 (samples 1–3), ASR2/recovery (samples 4–7) and CF2/recovery (samples 8–11) on experimental day 2 were collected. The levels of arousal and CF memory were assessed on day 34 (ASR34 and CF34).

Fear conditioning and fear recall

Fear conditioning (administration of the FS in the shock chamber with house light on) was performed during the active phase according to a previously published protocol for the PTSD mouse model (Siegmund & Wotjak, 2007). Mice were conditioned in a Plexiglas cage (16 × 16 × 32 cm³) with a grid harness package (ENV-407, ENV-307A; MED Associates, St Albans, VT, USA) connected to a shock generator (Shocker/Scrambler: ENV-414; MED Associates). After 198 s of habituation, animals underwent two electric FSs (1.5 mA, 2 s long) at moderate illumination (40 Lux) with a 60-s interval inbetween. Animals remained in the shock chamber for another 60 s before being returned to their home cages. CF (shock chamber + light) memory was examined by evaluation of the freezing response in the FS chamber at the same illumination intensity.

ASR

To test for arousal levels, ASRs were measured automatically as described before (Golub et al., 2009; Yen et al., 2012). Mice were placed into a Plexiglas enclosure, acclimated to the apparatus for 5 min and then presented to 136 startle pulses with interpulse intervals (IPI) of 13–17 s. The intensities of white noise were 75, 90, 105 and 115 dB with 30 startle trials at each level in a pseudo-random order. Additionally, animals’ startle responses were recorded 16 times under background noise. To permit a continuous dialysis in the microdialysis experiments, a custom-made startle chamber equipped with a commercially available ASR apparatus was used (San Diego Instruments, San Diego, CA, USA). Response was expressed either in mV (PTSD validation experiment) or in arbitrary units (microdialysis experiment). At the end of each test, Plexiglas cylinders and chamber were cleaned thoroughly with soap water.

Brain microdialysis

Microdialysis was performed as described before (Anderzhanova et al., 2013). Guide cannulas were implanted into the right mPFC (coordinates: AP +2.20 mm, ML 0.35 mm and DV −1.50 mm) or the right HPC (coordinates: AP −3.00 mm, ML 3.00 mm and DV −1.80 mm) under isoflurane (Abbot, India)/Metacam® (Boehringer Ingelheim GmbH, Germany) anesthesia/analgesia, in accordance with Paxinos & Franklin (2001). Recovery in experimental Plexiglas home cages (16 × 16 × 32 cm³) lasted for 1 week and included Metacam® supplementation, 0.25 mg/100 mL with drinking water, for 3 days after surgery, with daily inspection of food intake.

One day before the experiment, mice were shortly anesthetised with isoflurane. Microdialysis probes (o.d. 0.2 mm, cuprophane membrane 2 mm long, MAB 4.15.2.Cu, Microbiotech/se AB, Sweden, for the mPFC; and o.d. 0.24 mm, cuprophane membrane 3 mm long, CMA 11, CMA Microdialysis, Sweden, for the HPC) were inserted and connected to the perfusion lines consisting of FEP tubing and low-volume liquid swivel TCS2-23 (Eicom, Japan). From the moment of insertion, probes were continuously perfused with sterile artificial cerebrospinal fluid (concentrations in mM: NaCl, 145; KCl, 2.7; CaCl₂, 1.2; MgCl₂, 1.0; Na2HPO₄, 2.0; pH 7.4) at a
flow rate of 0.3 μL/min. On experimental days 1 and 2, following a 1.5-h equilibration period, microdialysis fractions (30 min) were constantly collected into 300-μL microtubes (Microbiotech/se AB, Sweden) containing 3 μL of 0.4 M perchloric acid at a perfusion rate of 1.5 μL/min. During collection time, tubes were kept in a refrigerated Univentor 820 Microsampler (Univentor, Malta). The dead volume of the outlet line was compensated by a delay in fraction harvesting (7 min). The perfusion flow rate was reduced to 0.3 μL/min for ongoing overnight perfusion. Once collected, samples were split into two equal aliquots for monoamine and CORT quantification, and stored at −80 °C prior to analysis. Each microdialysis probe was removed from the mouse brain after finishing the sample collection.

NE analysis

NE content in the microdialysates was determined by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (UltiMate3000 HPLC system/Coulometric III, ThermoFischer, USA). All reagents used for the phosphate-citrate mobile phase (methanol 10%, pH 5.6) were of analytical grade (Carl Roth GmbH or MERCK KGaA, Germany). Monoamines were separated on an analytical column (C18, 150 × 3 mm, 3 μm; YMC Triart, YMC Europe GmbH, Germany) at a flow rate of 0.4 mL/min. The potentials of the working electrodes were set at −75 mV, +250 mV, and the guard cell potential was set at +350 mV. NE concentrations were calculated by external standard curve calibration using the peak area for quantification. Those NE data that were lower than the detection limit of 0.05 nm were excluded from further analysis, which may result from inefficient microdialysis probes.

CORT analysis

The microdialysis membrane strictly limits the passage of conjugated CORT into the probe. Therefore, the microdialysates’ content reflects only changes in the free CORT levels. Free CORT in the microdialysates was measured by radioimmunoassay (RIA kit, MP Biomedicals, USA). The detection limit was 7.7 ng/mL, and the coefficients of variation of inter-assay and intra-assay were 6.9% and 7.3%, respectively. Microdialysates were diluted 2.5-fold and CORT content was determined using a standard calibration curve.

Histology

The targeted regions of the mPFC and the HPC are depicted in the schematic representation of the respective brain coronal sections (Fig. 3A and E). Because the microdialysis probes only transiently remained in the brain whereas animals were kept 1 month after removal, the placement of the microdialysis membrane could not be reliably visualised due to tissue recovery. An approximate estimation of the microdialysis membrane placement was possible by looking at the guide cannula residual tracks in the histological sections stained with Cresyl violet.

Data analysis

The working hypothesis of this study was that basal and stimulated NE and CORT levels correspond to the long-term behavioral outcome. Median split, a simple and conservative criterion, was used to group animals in accordance with NE and CORT levels. Only data significantly different from median (out of median ± 5% range) were used to assign animals to low- and high-level cohorts.

Normalization of neurochemical data was performed as in Hinkelmann et al. (2010) with slight modifications. This algorithm employs a comparison of running values (x_i) and measure of the last 30 min of the baseline period (x_30), applying the following equation:

\[ x(%) = 100 \cdot \frac{x_30}{x_i^2 + x_30^2} \]

The data are expressed as mean ± SEM. Two-way ANOVA with Bonferroni post hoc test was used for the microdialysis data, with brain region (mPFC and HPC) or experimental day (day 1 and day 2) as the between-subjects factors, and time as the within-subjects factor. The ASR and CF data were analysed employing a two-tailed Student’s t-test. Fitting linear models approach was applied for the linear and polynomial (quadratic) regression correlation analysis, with neurochemical measure (NE/CORT) as the argument and behavioral readouts as the function (R package, version 3.1.0, http://www.r-project.org).

Results

Validation of PTSD model

To confirm the FS protocol, two behavioral parameters representing symptoms of PTSD-like endophenotype were examined, and non-shocked (NS, n = 16) were compared with shocked (FS, n = 16) mice who had not undergone surgery. FS (1.5 mA, 2 s, twice) evoked hyperarousal, which was manifested in the elevated arousal (ASR30) 1 month after FS [two-way ANOVA (shock × intensity) F_1,10 = 16.81, P = 0.0003; Fig. 2A]. Shocked mice showed a strong CF memory (CF32; twofold increase in the freezing response) in comparison to non-shocked mice when exposed to the shock context 32 days after FS [Student’s t-test (two-tailed): t_30 = 3.098, P < 0.01; Fig. 2B].

Stressors (ASR, FS and CF) transiently increase NE and CORT in the mouse forebrain

Prefrontal and hippocampal NE and CORT levels were evaluated by microdialysis in different cohorts of mice under basal condition, and upon ASR1 and FS1 or ASR2 and CF2 on days 1 and 2, respectively. Absolute mean basal extracellular CORT levels were 0.84 ± 0.06 nm and 0.16 ± 0.01 nm in the mPFC and the HPC, respectively. Absolute mean basal extracellular CORT levels were 286.2 ± 33.11 ng/mL in the mPFC and 581.8 ± 48.86 ng/mL in the HPC. ASR1 and FS1 as well as ASR2 and CF2 were accompanied by increased extracellular NE levels in the mPFC (Fig. 3B) and the HPC (Fig. 3F). Two-way ANOVA (time, day) showed significance of time for both the mPFC (F_1,10 = 2.93, P = 0.0023) and the HPC (F_1,10 = 2.69, P = 0.0054). Post hoc analysis revealed that the magnitude of stress-induced NE release in the mPFC on day 2 was significantly different from median (out of median ± 5% range) were used to assign animals to low- and high-level cohorts.

Normalizing the CORT levels revealed that the magnitude of stress-induced CORT release in the HPC on day 2 was significantly different from median (out of median ± 5% range) were used to assign animals to low- and high-level cohorts.

Fig. 2. PTSD mouse model validation. Foot shock (FS) resulted in facilitation of the acoustic startle response (ASR) (A) and retention of contextual fear (CF) memory (B) measured on days 30 and 32 in shocked (FS, closed circles) and non-shocked (NS, open circles) mice. **P < 0.01; ***P < 0.001. SPL, sound pressure level.
lower than on day 1 (microdialysis fractions 5 and 9, \( P < 0.05 \); fraction 6, \( P < 0.01 \)). The extracellular CORT levels similarly increased following ASR1 and FS1 on day 1 and ASR2 and CF2 on day 2 in the mPFC (time: \( F_{1,10} = 21.23, P < 0.0001 \); Fig. 3C) and the HPC (time: \( F_{1,10} = 58.95, P < 0.0001 \); Fig. 3G).

The dynamics of relative changes in the NE release and free CORT content in the mPFC and HPC were juxtaposed. Stressors of different modalities (ASR1 and FS1 on day 1, and ASR2 and CF2 on day 2), exhibiting significance of the time effect in two-way ANOVA, increased the extracellular NE and CORT levels in both the mPFC (day 1, \( F_{1,10} = 6.021, P < 0.0001 \); day 2, \( F_{1,10} = 19.41, P < 0.0001 \); Fig. 3D) and the HPC (day 1: time, \( F_{1,10} = 12.72, P < 0.0001 \); day 2: \( F_{1,10} = 5.676, P < 0.0001 \); Fig. 3H). Notably, the NE and CORT level peaks were not completely synchronous.

### Table

<table>
<thead>
<tr>
<th>Time</th>
<th>mPFC</th>
<th>HPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>NE</td>
<td>CORT</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>ASR</td>
<td>ASR</td>
</tr>
<tr>
<td></td>
<td>FS</td>
<td>CF</td>
</tr>
<tr>
<td>Day 2</td>
<td>NE</td>
<td>CORT</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>ASR</td>
<td>ASR</td>
</tr>
<tr>
<td></td>
<td>CF</td>
<td>CF</td>
</tr>
</tbody>
</table>

### Figures

**Fig. 3.** Microdialysis data. The schematic diagrams show the targeted areas for microdialysis probe implantation in the medial prefrontal cortex (mPFC) (A) and the hippocampus (HPC) (E). The computer-based atlas by Paxinos & Franklin (2001) was used to mark probe locations; numbers refer to distances from the bregma, mm. Stress [acoustic startle response (ASR) and foot shock (FS) on day 1, ASR and contextual fear (CF) on day 2] evoked extracellular norepinephrine (NE) release in both the mPFC (B) and the HPC (F) on day 1 (closed circles) and day 2 (open circles). Dynamics of NE release were comparable on days 1 and 2 in the HPC, but not in the mPFC. Asterisks depict a difference in the magnitude of the NE release in the mPFC revealed by post hoc test (*\( P < 0.05 \), **\( P < 0.01 \)). An increase of free corticosterone (CORT) levels concurred with the stress (ASR1, FS1, ASR2 and CF2) on day 1 (closed circles) and day 2 (open circles) both in the mPFC (C) and the HPC (G). Arrows point to the samples corresponding to the ASR, FS and CF procedures. Vertical dashed lines separate basal sample collection period and the epochs corresponding to the aforementioned stressful procedures. Stressors of different modalities, ASR1 and FS1 on day 1, and ASR2 and CF2 on day 2, significantly increased the extracellular NE (closed circles, solid lines corresponding to right y-axis) and CORT levels (open circles, dashed lines corresponding to left y-axis) in both the mPFC (D) and the HPC (H). However, NE release and CORT accumulation peaks were not synchronised. Arrows point to the samples corresponding to the ASR, FS and CF procedures. Vertical dashed lines separate basal sample collection periods and the epochs corresponding to the aforementioned stressful procedures. For statistical analysis, see the ‘Results’ section.
While NE levels peaked 30 min after a stressor onset and then declined, CORT levels reached at maximum values 60 min after stress and stayed elevated for a longer time (two-way ANOVA, day 2 in the mPFC: NE/CORT: $F_{1,10} = 0.74$, $P = 0.4041$; time × NE/CORT: $F_{1,10} = 13.45$, $P < 0.0001$; day 1 in the HPC: NE/CORT: $F_{1,10} = 2.19$, $P = 0.1564$; time × NE/CORT: $F_{1,10} = 7.087$, $P < 0.0001$; day 2 in the HPC: NE/CORT: $F_{1,10} = 6.861$, $P = 0.0174$; time × NE/CORT: $F_{1,10} = 5.923$, $P < 0.0001$). Only on day 1 did the courses in the NE and CORT levels in the mPFC overlap (two-way ANOVA, NE/CORT: $F_{1,10} = 0.06$, $P = 0.8130$; time × NE/CORT: $F_{1,10} = 2.872$, $P = 0.0034$). Importantly, the measurements were done with the same samples excluding a method-related shift.

Taken together, these data show that the NE system and the HPA axis readily respond to recurrent different environmental challenges with similar strength.

### Changes in cortical and hippocampal NE and CORT levels are differentially involved in the expression of hyperarousal and CF

The changes in NE (pg/mL) levels expressed as means of the area under the curve were considered corresponding to the following epochs: Basal 1 or Basal 2 (samples 1–3); ASR1 or ASR2 (samples 4–7); FS1 (day 1 samples 8–11) and CF2 (day 2 samples 8–11).

First, the animals were grouped into low- and high-ASR2 NE or ASR2 CORT cohorts by median split criterion (Fig. 4A2 and B2). In the fear-conditioned mice with higher ASR2 NE in the mPFC, the ASR34 was lower (Student’s t-test, $t_{10} = 2.481$, $P = 0.0325$), indicating a lower degree of fear-induced hyperarousal (Fig. 4A3). ASR2 NE in the HPC failed to show a similar relationship (Student’s t-test, $t_{6} = 0.6761$, $P = 0.5180$; Fig. 4A3). In contrast to NE groups, mice with higher CORT accumulation in the mPFC upon ASR2 tended to show higher arousal on day 34 (Student’s t-test, $t_{11} = 2.134$, $P = 0.0562$; Fig. 4B3).

Next, mice were grouped into low- and high-Basal 2 NE (Fig. 5A2) or ASR2 CF2 NE (Fig. 5B2). After fear conditioning, mice with higher Basal 2 NE in the HPC showed less CF both on day 2 (Student’s t-test: $t_{9} = 2.562$, $P = 0.0335$; Fig. 5A3) and on day 34 (Student’s t-test: $t_{6} = 2.366$, $P = 0.0455$; Fig. 5A4). This was not the case in the mPFC. In contrast, mice with high hippocampal ASR2 + CF2 NE showed elevated CF on day 2 (Student’s t-test: $t_{6} = 2.566$, $P = 0.0333$; Fig. 5B3) and a tendency to express higher CF on day 34 (Student’s t-test: $t_{6} = 1.997$, $P = 0.081$) compared with mice with low ASR2 + CF2 NE (Fig. 5B4).

### NE and CORT levels independently correspond to hyperarousal and CF in a U-shape manner

The correlations between the total NE release (ASR1 + FS1 NE or ASR2 + CF2 NE) and levels of arousal (ASR34) or CF (CF34) 1 month after FS were examined. It was observed that in the mPFC, NE release during stress in naïve mice (ASR1 + FS1) correlated with the hyperarousal in a U-shape manner (residual standard error = 333.5, adjusted $R^2 = 0.3133$, $P = 0.061$; Fig. 6A) on day 34. In the HPC, NE release upon repeated stressor presentations (ASR2 + CF2) correlated with the CF response on day 34 (CF34) in an inverted U-shape manner (residual standard error = 12.54, adjusted $R^2 = 0.5616$, $P = 0.023$; Fig. 6B).

It was found that CORT accumulation in the mPFC during ASR2 showed a strong tendency to correlate with FS-potentiated ASR (ASR2/ASR1, %) in a U-shape manner (residual standard error = 45.28, adjusted $R^2 = 0.344$, $P = 0.09$; Fig. 7A). No correlation between changes in CORT accumulation during ASR1 and ASR2 was found (data not shown). Interestingly, basal and stress-stimulated CORT levels did not correlate on day 1, while recurrent stress showed a linear correlation between basal and stimulated levels ($R^2 = 0.3958$, $P = 0.0022$; data not shown). ASR2 NE in the mPFC showed no correlation with FS-potentiated ASR (residual standard error = 52.46, adjusted $R^2 = -0.041$, $P = 0.498$; Fig. 7B).

### Discussion

Forebrain NE and CORT levels and long-term PTSD-like symptoms in mice

In the present study, it was initially assumed that the magnitude of the innate ASR might be a predictor for long-term PTSD-like symptom severity in mice. However, it was not possible to find any correlation between ASR1 and the PTSD-like symptom strength ($P = 0.1223$ for ASR34 and $P = 0.3662$ for CF34, data not shown). Based on recent reports (Salehi et al., 2010; Nicholson et al., 2014), it was hypothesised that level changes of stress mediators, specifically NE and CORT, serve as predictors for the PTSD endophenotype. Correlations between the stress-stimulated neurochemical changes or the concurrent hormonal output in the mouse brain and the acute/delayed arousal (ASR1, ASR2 and ASR34) or the strength of aversive memories (CF2 and CF34) induced by FS were evaluated. ASR2 NE levels in the mPFC on day 2 after FS were inversely related to the hyperarousal assessed on day 34. NE levels in the HPC upon challenges on day 2 were positively related to the CF on days 2 and 34 after FS. U-shape relationships between the NE levels during peri-FS stress and the long-term PTSD-like symptoms were also observed, and between the free CORT contents during post-FS stress and the acute changes in arousal.

NE signaling in the mPFC predicts emotional response to stress

All kinds of stressors induce NE release from locus coeruleus (LC) neurons in the upstream forebrain regions, including the PFC and the HPC (Robertson et al., 2013), structures that are tightly involved in the regulation of emotions and cognition.

In these experiments, ASR1, FS1, ASR2 and CF2 resulted in a moderate increase (up to 200%) of extracellular NE levels. A faster return of the mPFC NE levels to basal values in case of recurrent stress (ASR2) was observed on the second experimental day compared with the period of the first startle measurement (ASR1). The difference in the NE release on days 1 and 2 cannot directly result from acute changes in HPA axis activity, as the corresponding free CORT levels showed the same elevations in the mPFC as well as in the HPC (Fig. 3C and G). Nonetheless, under acute stress, NE neurons in the LC show neuroplasticity both at the levels of neuronal activity and of neurotransmitter release, which may differ from the effects induced by chronic stress (Hajós-Korcso et al., 2003; Jedema et al., 2008). Stress could induce a selective increase in $\alpha$2-adrenergic receptor activity in the mPFC and/or LC with respective stronger suppression of the NE release via a feedback mechanism. Reyes et al. (2012) showed the re-sensitization of $\alpha$2-adrenergic receptors due to interaction with CB1 cannabinoid receptors. Elevation in endocannabinoid levels induced by priming stress (Hillard, 2014) may therefore contribute to the decrease in NE release in the mPFC upon the recurrent stress.
Fig. 4. Predictability of low and high norepinephrine (NE) and corticosterone (CORT) levels for arousal. (A) NE release in the medial prefrontal cortex (mPFC), but not in the hippocampus (HPC), coinciding with acoustic startle response (ASR)2 (A1) predicts ASR34 response (A3). Gray and black bars correspond to low and high ASR2 NE cohorts, respectively, assigned in accordance with median (Me) split (A2). Open circles represent the data of the Me value, which was excluded from further analysis. (B) CORT levels in the mPFC coinciding with ASR2 (B1) show a tendency of correspondence with ASR34 (B3). Gray and black bars correspond to low and high ASR2 CORT cohorts, respectively, obtained by Me split (B2). Numbers inside the bars correspond to the sample size. *P < 0.05. CF, contextual fear; FS, foot shock.

Employing the low- and high-NE-level selection criterion, an inverted relationship between the magnitude of the NE release in the mPFC during repeated ASR and the arousal on day 34 was shown (Fig. 4A). It is known that stress-induced enhancement of NE neurotransmission bidirectionally modulates mPFC functions. An increase of NE signaling leads to a reduction in the cognitive set-shifting in the chronic unpredictable stress paradigm (Morilak et al., 2005; Jett & Morilak, 2013). On the other hand, an increase in NE neurotransmission favors stress resistance (Bondi et al., 2010). The negative relationship between NE levels and FS outcome found in this experiment is in line with these earlier results. Upon further analysis, a U-shape function of the prefrontal NE release on experimental day 1 and ASR34 was observed (Fig. 6A). The middle NE levels in the mPFC may therefore favor an adaptive response, as they coincide with minimal ASR facilitation on day 34. It was hypothesised that the extreme changes in NE neurotransmission in the mPFC either do not provide an optimal attentional lever to discriminate important and unimportant informational input or promote unspecific hypervigilance. This finding is in accordance with a previous report about an inverted U-shape relationship between the LC neuron activity and the performance of tasks that required focused attention (Aston-Jones & Cohen, 2005). Lapiz & Morilak (2006) showed that the systemic pretreatment with the α2-adrenergic receptor antagonist atipamezole significantly improved the performance of an attentional task that was blocked by the α1-blocker benoxathian microinjected in the mPFC. Moreover, the α1/α2-receptor-specific regulation of the emotional and habitual responses of the amygdala and the basal ganglia seems to depend on the extent of NE release in the mPFC (Arnsten et al., 2015). This suggests a fine balance between the activation of different subtypes of adrenergic receptors, which may underlie the U-shape NE function. It also points to a critical range of endogenous neurotransmitter release for the optimal task performance and the top-down control of emotional states.

Overall, these results suggest a bimodal role of NE neurotransmission in the mPFC that regulates the arousal level after FS.

**NE signaling in the HPC relates to CF memory**

The HPC receives dense projections from the LC (Schroeter et al., 2000). This anatomical connection supports the general role of NE in learning/memory (Barsegyan et al., 2014; Yan et al., 2014). Several studies have indicated the differential involvement of the HPC NE in fear memory acquisition, consolidation and retrieval (Fiorenza et al., 2012; Zhang et al., 2013). In this study, mice with higher NE release in the HPC during repeated ASR and re-exposure to FS context (ASR2 + CF2 NE) showed stronger acute and long-term CF (CF2 and CF34; Fig. 5B3 and B4). This corroborates previous reports showing that NE signaling in the HPC mediates the effects of arousal on memory (Urry et al., 2006;
Cerqueira et al., 2007), affects memory consolidation associated with emotional stimulation (Izquierdo & Medina, 1997), and interferes with CF memory mechanisms (Thomas & Palmiter, 1997; Murchison et al., 2004). Previous studies suggested the inverted U-shape dependence of early learning and memory in HPC-dependent tasks on the stressor intensity (Salehi et al., 2010). NE regulates fast neuromediator signaling [glutamate and \(\gamma\)-aminobutyric acid (GABA)-mediated], both increasing and decreasing its efficiency that depends on the receptors involved (Pralong et al., 2002). The innate difference in \(\beta\)-adrenergic receptor activity in the HPC might play a role mediating the U-shape relationship between fear memory and NE levels (Introini-Collison et al., 1994). In this study, the inverted U-shape relationship between stress (ASR2 + CF2)-stimulated NE level increase in the HPC and CF memory retrieval were observed (CF34; Fig. 5B), suggesting that intermediate NE levels promoted fear memory consolidation. Without considering the specifics of the memorized event, it is inappropriate to speculate about the adaptive vs. maladaptive impact of NE. Because the HPC is involved in all types of memory, particularly the contextual component of each event, the middle level increase in NE levels might be considered adaptive. The dysadaptive or adaptive character of the strong memory may be weighted only with respect to the memory modality. Moreover, the inverted U-shape relationship between conditioned fear expression and fear potentiated startle was shown in clinical PTSD studies, suggesting beneficial consequences of optimal fear level (Norrholm et al., 2014).

Taken together, these results suggest a defined range of NE neurotransmission facilitation (120–170%) that mediates stress-induced HPC-dependent CF memory.
Fig. 6. U-shape relationship between norepinephrine (NE) and behavior. (A) NE release in the medial prefrontal cortex (mPFC) upon stressors’ presentation on day 1 [acoustic startle response (ASR)1 + foot shock (FS)1 NE] showed a tendency to correlation with ASR on day 34 (ASR34) in a U-shape manner (adjusted \( R^2 = 0.3133, P = 0.061 \)). (B) NE release in the hippocampus (HPC) upon stress presentations on day 2 [ASR2 + conditioned fear (CF2 NE) after FS correlated with CF response on day 34 (CF34) in an inverted U-shape manner (adjusted \( R^2 = 0.5616, P < 0.05 \)). NE measures are expressed as area under the curve of relative changes in their levels for respective time intervals (ASR, FS and CF). The equations represent theoretical functions that fit the experimental data.

Fig. 7. U-shape relationship between corticosterone (CORT) and behavior. (A) In the medial prefrontal cortex (mPFC), free CORT accumulation during acoustic startle response (ASR) showed a U-shape relationship with FS-potentiated ASR (ASR2/ASR1, \%): adjusted \( R^2 = 0.344, P = 0.09 \). (B) Notably, no correlation was found between ASR2 norepinephrine (NE) release in the mPFC and immediate FS-potentiated ASR (adjusted \( R^2 = -0.041, P = 0.498 \)). Neurochemical measures are expressed as area under the curve of absolute (CORT) and relative (NE) level changes for the respective time interval (ASR2). The equations represent theoretical functions that fit the experimental data.

The role of CORT in the mPFC in mediation of hyperarousal after FS

In contrast to the expectations of this study, stressors of different modalities applied to either naive (ASR1, FS1) or stressed mice (ASR2, CF2) induced similar relative free CORT level increases in both mPFC and HPC. This does not support a differential modulation of the CORT level with respect to stressors, and neither suggests sensitization nor desensitization/habituation of the HPA axis.

The current analytical approach based on group assignment according to low and high values failed to demonstrate that free CORT levels (ASR2 CORT) are strong predictors of FS outcome. Higher ASR2 CORT levels in the mPFC only tended to correspond to stronger fear-potentiated ASR34 (Fig. 4B3). It is known that in the HPC, formation of memories and inhibition of unrelated information are associated with CORT-mediated long-term potentiation suppression and long-term depression facilitation (Kars et al., 2005; Joëls et al., 2006). In the amygdala, CORT gates the nucleus activity (Daskalakis et al., 2014). Elevation of CORT in the mPFC during ASR2 would specifically favor modulation of cortical activity to increase the next sensory input. This assumption goes along with clinical studies showing that glucocorticoid (GC) administration in doses corresponding to stress levels impaired delayed, but not immediate, recall of episodic task performance (Wolf et al., 2001; Buss et al., 2004).

In clinical studies, PTSD patient cortisol levels were found mostly unchanged, but also either increased or decreased (Pitman & Orr, 1990; Yehuda et al., 1990; Eckart et al., 2009). This phenomenon may have biological significance reflecting a dose-dependent effect of stress hormones. A U-shape relationship between the individual stimulated (ASR2) CORT levels and the FS-potentiated ASR (ASR2/ASR1) was observed in the mPFC, but not in the HPC (Fig. 7A). Even though brain CORT originates from the blood pool and its levels should be similar in each brain region, the free hormone’s content appears to be different between structures. Possible inter-individual differences in the kinetics of the CORT–GC receptors (GRs) interaction, in CORT metabolism as well as in physical properties of brain tissue matrix (Croit et al., 2008; Little et al., 2008), may contribute to a variation of free CORT levels between different regions and explain the U-shape relationship with behavioral readouts that was only found in the mPFC.

This observation agrees with the reported inverted U-shape relationship between stress levels and HPC-dependent learning (Salehi et al., 2010), with the former also corresponding to CORT level increases in blood. This may point to an involvement of CORT-dependent hyperarousal in learning. Furthermore, changes in CORT levels showed a limited ability to predict FS outcome in this genetically homogeneous mouse population (Fig. 4B3).

Post-FS NE and CORT levels predict long-term PTSD-like symptoms

Notably, NE and CORT levels 1 day after the FS in most cases predicted the long-term FS outcome (elevated CF34 and ASR34; Figs 4–6). Experimental day 1 levels on the other hand were less informative. It was assumed that synaptic plasticity mechanisms could underlie this phenomenon. Moreover, the strength of the acute stressor on day 1 was likely exceeding the physiological threshold. Therefore, the neurochemical responses might not predict the individual differences in response to stress (FS). However, upon priming stress, the plasticity mechanisms of the NE/CORT system in the mPFC and the HPC were well tuned to allow the prediction of long-term stress responses (hyperarousal and CF). The predictor properties of NE and CORT showed strong time- and structure-dependency.

CORT level increases initiate the differential regulation of its downstream targets, including several neurotransmitter systems and elements of HPA axis regulation. CORT-induced differences in the activity of downstream targets will determine the profile of CORT-mediated effects during the next episode of hormone release. Diverse systems regulating the biological activity of CORT were considered. Firstly, two types of receptors mediate GC actions in the brain, namely mineralocorticoid receptors (MRs) and GRs. MRs and GRs exhibit differences in binding affinity to GC and expression levels in distinct brain regions (Veldhuis et al., 1982). This dual system may work in opposite directions regulating the behavioral response (Pavlides et al., 1995). Secondly, CORT bound to corticosterone-binding globulin (CBG) is believed to be biologically inactive (Breuner & Orchnik, 2002). CBG levels, like CORT levels, are not static and were shown to decrease in response to stress (Fleschner et al., 1995). It is therefore conceivable that non-congruent changes in CORT and CBG and/or MR and GR synthesis/activity, which may be induced by the priming stress (ASR1, FS1), could result in...
structural adjustments of the CORT–CBG/MRs–GRs profile. This way, free CORT levels can serve as a reporter of the behavioral outcome, by showing a U-shape relationship with FS-potentiated ASR. The genomic effect of CORT may regulate the hippocampal-dependent fear memory (Cornelisse et al., 2014) also via changes in the NE system (Fan et al., 2014; Ha et al., 2014) and tuning its predictability 1 day after FS.

Conclusions
Acute stress-induced changes in NE and CORT signaling seem to be translated into long-term behavioral effects of FS. Upon challenges 1 day after FS, NE and CORT levels in the mPFC predicted arousal, whereas they predicted CF memory in the HPC. Both NE and CORT levels showed not a linear but a U-shape relationship with behavior. In the animal model used in this study, the ability of brain NE and CORT to predict PTSD-like symptoms showed structure- and time-specificity. Therefore, results from an assessment of NE and CORT levels for PTSD-like symptoms prognosis in the periphery should be treated with caution.

Taken together, these data provide a means of early post-physiological stress evaluation of NE signaling and CORT levels for an accurate PTSD-like symptom prognosis and give insights into time-specific pharmacological intervention.

Disclosure
The authors declare no conflict of interest.

Acknowledgements
The authors would like to thank Andreas Genevskey, MSc, and Judith Richel, MSc, for their invaluable assistance with ASR measurements.

Abbreviations

ASR, acoustic startle response; CBG, corticosterone-binding globulin; CF, contextual fear; CORT, corticosterone; FS, foot shock; GC, glucocorticoid; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; HPC, hippocampus; HPLC, high-performance liquid chromatography; LC, locus coeruleus; mPFC, medial prefrontal cortex; MR, mineralocorticoid receptor; NE, norepinephrine; PTSD, post-traumatic stress disorder.

References


Norepinephrine, corticosterone and PTSD prediction 1147


2.4. NextGen Brain Microdialysis – applying modern metabolomics technology to the analysis of extracellular fluids

Chi-Ya Kao, Elmira Anderzhanova, John M. Asara, Carsten T. Wotjak, Christoph W. Turck

Declaration of contribution: Chi-Ya Kao designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with coauthors.

- Study design and plan: in collaboration with EA, CW and CT
- Conducting the experiments: in collaboration with EA and JA
- Data analysis: in collaboration with EA, CW and CT
- Manuscript preparation: in collaboration with EA, CW and CT

Supplemental material is available on enclosed DVD and upon request.
NextGen Brain Microdialysis: Applying Modern Metabolomics Technology to the Analysis of Extracellular Fluid in the Central Nervous System

Chi-Ya Kao a, c Elmira Anderzhanova b John M. Asara d, e Carsten T. Wotjak b Christoph W. Turck a

Departments of a Translational Research in Psychiatry and b Stress Neurobiology and Neurogenetics, Max Planck Institute of Psychiatry, and c Graduate School of Systemic Neurosciences, Ludwig Maximilian University, Munich, Germany; d Division of Signal Transduction, Beth Israel Deaconess Medical Center, and e Department of Medicine, Harvard Medical School, Boston, Mass., USA

Abstract
Microdialysis is a powerful method for in vivo neurochemical analyses. It allows fluid sampling in a dynamic manner in specific brain regions over an extended period of time. A particular focus has been the neurochemical analysis of extracellular fluids to explore central nervous system functions. Brain microdialysis recovers neurotransmitters, low-molecular-weight neuromodulators and neuropeptides of special interest when studying behavior and drug effects. Other small molecules, such as central metabolites, are typically not assessed despite their potential to yield important information related to brain metabolism and activity in selected brain regions. We have implemented a liquid chromatography online mass spectrometry metabolomics platform for an expanded analysis of mouse brain microdialysates. The method is sensitive and delivers information for a far greater number of analytes than commonly used electrochemical and fluorescent detection or biochemical assays. The metabolomics platform was applied to the analysis of microdialysates in a foot shock-induced mouse model of posttraumatic stress disorder (PTSD). The rich metabolite data information was then used to delineate affected prefrontal molecular pathways that reflect individual susceptibility for developing PTSD-like symptoms. We demonstrate that hypothesis-free metabolomics can be adapted to the analysis of microdialysates for the discovery of small molecules with functional significance.

Key Words
Microdialysis · Metabolite profiling · Posttraumatic stress disorder · Molecular pathways · Biomarker · Stress susceptibility and resilience

Introduction
Microdialysis is used to dynamically monitor endogenous and exogenous substances in vivo. The procedure can be performed in any tissue [1] and body fluid [2, 3]. A major focus is the analysis of brain neurotransmission, neurochemistry and metabolism [4, 5], and microdialysis remains the method of choice for experimental neuropharmacology studies by acquiring pharmacokinetic and pharmacodynamic data from living animals [6]. For this purpose, extracellular fluids from selected brain areas are analyzed for molecular components relevant for the
physiological/pathological processes or drug response. The technique enables an assessment of the release and metabolism of neuroactive endogenous substances over a period of several days in freely moving animals. Sampling of specific brain regions, such as the medial prefrontal cortex (mPFC), paraventricular nucleus and amygdala, during behavioral experiments with rodents can be done with efficient spatial and temporal resolution of millimeters and minutes, respectively, reflecting the actual state of the animal under investigation.

Perfusion of an implanted microdialysis probe with artificial cerebrospinal fluid that resembles the brain extracellular fluid with regard to pH, ion composition and osmolarity is not accompanied by significant fluid gain or loss and tissue homeostasis disturbances, thus making microdialysis a truly physiological method for the purpose of in vivo sampling. By using a semipermeable membrane designed for microdialysis probes, macromolecules such as enzymes and proteins are excluded. This leaves collected extracellular matrices intact. Furthermore, the absence of macromolecular complexes in microdialysates does not require sample purification prior to analysis [5].

Commonly employed methods for the analysis of microdialysates include electrochemical, UV and fluorescence detection coupled with high-performance liquid chromatography (HPLC) as well as radioimmunoassay, enzyme-linked immunosorbent assay, colorimetric and spectrophotometric biochemical assays [1, 7]. The analytical methods currently used have restricted more comprehensive in-depth studies of the precious microdialysate samples. Small sample volumes limit the number of molecules, which can be simultaneously analyzed with good sensitivity using the classical detection methods. For this reason, microdialysate collection times are frequently extended, resulting in poor temporal resolution. Modern analytical methods need to be implemented in order to fully exploit microdialysis sample contents. Liquid chromatography online mass spectrometry (LC-MS) allows selective identification and sensitive quantification of a great number of molecules. Although LC-MS methods have been developed for the identification and quantification in microdialysates of neurotransmitters [8–10], neuropeptides [11] and exogenous compounds such as drugs and their metabolites [12–14], most studies have been restricted to a small number of analytes and consequently have resulted in only narrow insights in pathophysiological mechanisms.

LC-MS has become an important method in the field of metabolomics, the comprehensive analysis of metabolites. When configured in the selected reaction monitoring (SRM) mode, a great number of molecules can be identified with high confidence. This makes LC-MS the method of choice as a comprehensive screening tool for microdialysate analysis and pathway discovery pertinent to pathophysiological responses.

In the present study, we have profiled and monitored central metabolite levels in microdialysates on a large scale for the first time, using an LC-MS metabolomics platform. We have applied this approach to a posttraumatic stress disorder (PTSD) mouse model [15, 16] and have correlated metabolite levels measured prior to foot shock (FS) stress with behavioral readouts of FS resilience (early and delayed post-shock changes in arousal levels). The method is orders of magnitude more comprehensive in terms of qualitative and quantitative molecular information than traditional microdialysate analysis. This information can be exploited to gain a better understanding of pathophysiological mechanisms in mouse models.

**Materials and Methods**

**Animals**

Male C57BL/6NCrl mice, 7–8 weeks old, (Charles River GmbH, Sulzfeld, Germany) were housed in groups of four in Makrolon type II cages (23 × 16.5 × 14 cm³) under an inverse 12:12 h light-dark cycle (light off at 7:00) with food and water ad libitum. The experimental procedures were approved by the Government of Upper Bavaria. Laboratory animal care and the experiments were performed in compliance with the European Economic Community recommendations for the care and use of laboratory animals (2010/63/EU).

**Fear Conditioning and Acoustic Startle Response**

Fear conditioning for the PTSD mouse model was performed as described previously [15]. In brief, after 198 s of habituation, the mice perceived two electric FS (1.5 mA, 2 s) with a 60-second interval in between at moderate illumination (40 Lux) in a plexiglass shock chamber. The mice remained in the shock chamber for another 60 s before they were returned to their home cages. To test for arousal levels, acoustic startle response (ASR) was measured automatically as described before [16]. The level of ASR was assessed on days 1 and 2 (ASR1 and ASR2) and FS-stimulated ASR changes (ASR2/ASR1, %) were calculated. For the evaluation of PTSD-like symptoms, the levels of ASR and contextual fear response (CF) were assessed on day 34 (ASR34 and CF34).

**Brain Microdialysis**

Guide cannulas were implanted into the right mPFC (coordinates: AP: +2.20 mm, ML: 0.35 mm and DV: –1.50 mm) in accordance to Paxinos and Franklin’s Mouse Brain Atlas [17] under isoflurane (Abbott, Mumbai, India)/Metacam® (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) anesthesia/anal-
Fig. 1. Outline of the microdialysis experiment in combination with metabolomics applied to a PTSD mouse model. On experimental day 1, ASR1 was measured, followed by fear-conditioning FS1. On experimental day 2, the mice were subjected to recurrent ASR2. Microdialysates collected at Basal 1 were subjected to metabolite profiling. On experimental day 34, PTSD-like symptoms, including ASR and CF, were assessed.

Targeted Metabolomics Analysis

Four fold (v/v) ice-cold methanol was added to pooled prefrontal microdialysates (samples 1–3 for Basal 1) and vortexed for 2 min. The samples were then incubated for 2 h on dry ice and centrifuged (2,053 g, 10 min, 4°C). Supernatants were transferred to a 0.22-micrometer cellulose acetate ultrafiltration tube (Corning Life Science, Tewksbury, Mass., USA) and centrifuged (1,105 g, 2 min, 4°C) to remove undissolved particles. The filtrates were lyophilized and stored at −80°C until further analysis.

The samples were resuspended in 20 μl LC-MS grade water. Ten microliters were injected and analyzed using a 5,500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX, Framingham, Mass., USA) coupled to a Prominence UFLC HPLC system (Shimadzu, Columbia, Md., USA) via SRM of a total of 275 endogenous water soluble metabolites for steady-state analyses of the samples. The samples were delivered to the mass spectrometer via normal-phase chromatography using a 4.6 mm i.d. x 10 cm Amide Xbridge HILIC column (Waters Corp., Milford, Mass., USA) at 350 μl/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0 to 5 min; 42 to 0% B from 5 to 16 min; 0% B was held from 16 to 24 min; 0 to 85% B from 24 to 25 min; 85% B was held for 7 min to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate, pH 9.0, in 95:5 water/acetonitrile. Some metabolites were targeted in both positive and negative ion modes for a total of 292 SRM transitions using positive/negative polarity switching. ESI voltage was +4,900 V in the positive ion mode and −4,300 V in the negative ion mode. The dwell time was 4 ms per SRM transition, and the total cycle time was 1.89 s. Approximately 9–12 data points were acquired per detected metabolite. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/Sciex).

Statistics and Data Analysis

Metabolites with >20% missing value and z-score >4 were considered outliers and removed from our analyses. We normalized the intensities of each metabolite to have a mean of zero and a standard deviation of 1. Correlation analyses for the metabolite levels and behavioral readouts were performed employing GraphPad Prism 5 (GraphPad Software, La Jolla, Calif., USA). In brief, we correlated Basal 1 metabolite levels with behavioral readouts (ASR2/ASR1, ASR34 and CF34) by calculating the Pearson correlation coefficient. The significant level was set to p < 0.05. Pathway analysis was conducted using MetaboAnalyst pathway tool (http://www.metaboanalyst.ca).

Results

Metabolite Profiling of mPFC Extracellular Fluid

Microdialysate fractions were subjected to high-throughput metabolomics analyses with the help of a targeted LC-MS platform interrogating a list of close to 300 metabolites that encompass several central pathways related to energy metabolism, including citrate cycle, glycolysis or gluconeogenesis and pyruvate metabolism. In addition, the list included a number of central nervous system pathways.
**Fig. 2.** Linear correlations between Basal 1 metabolite levels and FS-induced behavioral changes. Basal 1 metabolite levels correlated with FS-induced ASR2/ASR1 (a), ASR34 (b) and CF34 (c).

(For Figure 2b and c see next page.)
system-relevant molecules that are typically analyzed in microdialysis experiments including neurotransmitters (glutamate and glycine) and endogenous ion channel agonists/antagonists (aspartate, alanine, kynurenic acid and phenylalanine) (online suppl. table S1; see www.karger.com/doi/10.1159/000381855 for all online supplementary material). In our PTSD mouse model, we were able to quantify 120 metabolites after data filtering and normalization (online suppl. table S1).

**mPFC Metabolic Fingerprints under Basal Conditions Predict FS Impact on Arousal**

Metabolic profiling of prefrontal extracellular fluid allowed us to correlate molecular data with behavioral measures in the PTSD mouse model. We identified several central metabolites, which showed linear correlations with the FS-related early changes in arousal (ASR2/ASR1; fig. 2a). We then subjected the metabolites that predicted the FS-induced arousal changes and the highly correlating expression profile metabolites (online suppl. table S2) to pathway analyses. Citrate cycle metabolism and glyoxylate and dicarboxylate metabolism were significantly enriched (table 1). These findings demonstrate the power of metabolite profiling for predicting early behavioral consequences of FS and investigating affected pathways in the PTSD mouse model.

**mPFC Metabolic Fingerprints under Basal Conditions Predict Long-Term PTSD-Like Symptoms**

In addition to the identified correlations for FS-induced early changes in arousal, a number of central metabolites showed prediction power for longer-term PTSD-symptoms, including ASR and CF 34 days after FS (fig. 2b, c). Basal 1 xanthurenic acid and glucose-1-phosphate levels positively predicted ASR34, while sarcosine levels negatively predicted ASR34. Basal 1 spermidine levels negatively predicted the CF34 outcome.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Holm-Bonferroni-adjusted p value</th>
<th>False discovery rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate cycle</td>
<td>0.0002</td>
<td>0.0002</td>
</tr>
<tr>
<td>Glyoxylate and dicarboxylate metabolism</td>
<td>0.0219</td>
<td>0.0111</td>
</tr>
</tbody>
</table>
**Discussion**

The analysis of brain microdialysate samples from individual mice with an LC-MS metabolomics platform enabled us to detect and quantify the levels of close to 120 metabolites. Our approach significantly extends the spectrum of microdialysate analytes and is able to delineate molecular pathways involved in pathological changes related to brain metabolism in a mouse model for PTSD. Metabolomics data analysis revealed numerous correlations between basal mPFC metabolite profiles and FS-associated arousal and contextual fear. Our results demonstrate that combining microdialysis with LC-MS provides a sensitive approach of a high-prediction power for deciphering the molecular signatures associated with individual susceptibility for developing PTSD-like symptoms in a given brain region. In an extension of this approach, microdialysis experiments could provide important insights into affected brain circuits underlying pathophysiological mechanisms by targeting different brain regions.

Microdialysis has been widely applied for the evaluation of extracellular dynamics of neurotransmitters and pharmacological substances in defined brain regions. Targeted quantification of up to 20 monoamines and their metabolites from microdialysis is feasible but provides rather limited insights for specific biological questions [18]. Microdialysis is also routinely used to explore changes in the extracellular levels of neuroactive amino acids including aspartate, glutamate, glutamine and alanine that are relevant not only for neurotransmission focused studies but also for neurotoxicity experiments [19, 20]. However, for sensitivity reasons, the electrochemical or fluorescent detection of amino acids in microdialysates requires pre- or post-column derivatization, which can lead to sample loss and/or measurement variability. In the present study, we were able to quantitate all 20 natural and several additional modified amino acids without derivatization by applying modern metabolomics LC-MS technology as readout for the comprehensive profiling of brain microdialysates (online suppl. table S1). This allowed us to reveal significant amino acid pathways affected by stressors and associated with PTSD-like symptom severity (table 1).

In an earlier study [21] applying the same experimental design, no significant linear correlations were found between basal prefrontal norepinephrine and corticosterone levels and behavioral responses to stress. When analyzing the here presented metabolomics data, Pearson correlation analysis showed prediction power for the metabolites with regard to behavioral changes of the PTSD mice (fig. 2). The correlations between baseline metabolite levels and FS-induced arousal and PTSD-like symptoms demonstrate the power of microdialysate metabolite profiling for the investigation of interindividual variability, in this case susceptibility or resilience towards coping with psychophysiological stress and the development of PTSD endophenotype, in an inbred mouse strain.

Several of the metabolites that show correlations to the early (day 2) and delayed (day 34) behavioral changes induced by stress have relevance for psychiatric phenotypes. Sarcosine, a glycine reuptake inhibitor, has been implicated in the regulation of synaptic plasticity, memory and cognition [22]. Our data show a positive correlation between Basal 1 sarcosine levels and FS-induced ASR2/ASR1. On the other hand, Basal 1 sarcosine levels negatively predicted the FS-induced ASR34. These data suggest that sarcosine-dependent prefrontal mechanisms of plasticity show biphasic dynamics with respect to arousal state regulation. We were also able to quantify neurokynurenines, including kynurenic acid, xanthurenic acid and nicotinate from prefrontal microdialysates. The kynurenine pathway is the major route for tryptophan metabolism and has been implicated in the pathophysiology of depression and stress-related disorders [23, 24]. Mice subjected to unpredictable chronic mild stress exhibited an elevation of the peripheral kynurenine pathway [25]. In the same study, a correlation between the peripheral kynurenine/tryptophan ratio and anxiodepressive-like behavior in mice subjected to unpredictable chronic mild stress was found. Previous studies showed that treatment with nicotinate resulted in improved human sensory register and short-term memory, possibly by facilitating neurotransmission [26]. By binding with neuronal transporters, nicotinate can be utilized as cellular precursor for nicotinamide adenine nucleotide and nicotinamide adenine dinucleotide phosphate synthesis, key events in regulating energy metabolism that has been implicated in the pathogenesis of psychiatric disorders [27–29]. In the current study, we found prefrontal nicotinate levels to positively correlate with FS-induced ASR2/ASR1 change and xanthurenic acid levels to positively predict the arousal response 34 days after shock (ASR34). These results support a possible role for neurokynurenines in mediating neuronal activity, specifically relevant for arousal mechanisms.

An additional benefit of using a great number of analytes for the interrogation of microdialysates is the ability to get information about affected molecular pathways relevant for symptom development in the mouse model under investigation. When subjecting the enriched metabo-
lites to pathway analyses, we found energy metabolism, specifically the citrate cycle pathway, to be affected in the PTSD mouse model. Previous studies have shown increased citrate cycle activity in synaptosomes of high-anxiety mice [30]. Functional synapses are enriched with mitochondria, which supply ATP and regulate calcium levels, modulating neuronal transmembrane potential [31], synaptic neurotransmission [32], receptor signaling [33] and synaptic plasticity [34]. A dysregulated citrate cycle metabolism leads to abnormal energy production that can impair neurotransmission. This may be of particular relevance in the mPFC, which exerts top-down control of emotional states. Anxiolytic medications such as monoamine oxidase inhibitors were shown to improve mitochondrial function [35]. Based on our findings, we assume that differential regulation of the citrate cycle may be involved in endophenotype-specific neurotransmission in the PTSD mouse model.

With the help of the LC-MS metabolomics platform, we were also able to quantify a number of metabolites that are directly related to neurotransmission, including glutamate, hydroxyphenylacetic acid, phenylalanine, tryptophan and the N-methyl-D-aspartate receptor agonists alamone and aspartate. Further applications of the LC-MS metabolomics platform could include studies of neurotransmitter dynamics and help delineate affected pathways involved in neuronal plasticity.

LC-MS microdialysis analysis yields high sensitivity and selectivity compared to the commonly used detection methods [36]. The method allows a reliable qualitative and quantitative determination of a wide range of metabolites. With the help of the LC-MS metabolomics platform, we were able to reveal alterations in intracellular metabolic pathways reflected as changes in metabolite profiles in the extracellular fluid. The method enables hypothesis-free physiological studies, is applicable to any rodent model and may illuminate novel targets for pharmacological intervention.

Acknowledgements

This work was supported by the Max Planck Society. C.-Y.K. was supported by the LMU Graduate School of Systemic Neurosciences.

References

Applying Modern Metabolomics to the Analysis of Extracellular Fluid

DOI: 10.1159/000381855


3. DISCUSSION AND FUTURE PERSPECTIVES

In the present study I applied transcriptomics, proteomics and metabolomics technologies in combination with *in silico* pathway analyses, as well as neurochemical microdialysis to identify molecular pathways affected in a mouse model of PTSD. For this purpose adult male C57BL/6NCrl mice were exposed to non-escapable electric FS, and tissue punches representing specific brain regions subjected to the various –omics analyses. As a second step mice were treated with either fluoxetine, an antidepressant drug used for treatment of PTSD patients, or vehicle. Affected molecular pathways were identified by interrogating transcriptomics, proteomics and metabolomics data with the help of computational biology methods. In addition, I analyzed the relationship between neuroendocrine levels, including norepinephrine and corticosterone, and PTSD-like symptoms in a structure- and time-dependent manner.

In further support of the association of the identified pathways and PTSD pathogenesis I was able to show that fluoxetine treatment rescues/prevents pathway alterations upon FS application and ameliorates PTSD-like symptoms. My results suggest new molecular mechanisms involved in PTSD pathobiology that can benefit future investigations aimed at improving PTSD diagnosis and treatment.

3.1. Energy metabolism

3.1.1. Altered energy metabolism in psychiatric diseases

My results indicate that a dysregulated citric acid cycle pathway in specific brain regions is associated with PTSD pathogenesis. Altered energy metabolism has previously been implicated in other psychiatric disorders. Filiou et al. identified decreased glycolysis and increased electron transport chain (ETC) pathways in the cingulate cortex synaptosome from
3. DISCUSSION AND FUTURE PERSPECTIVES

a high anxiety-related behavior mouse model (Filiou et al., 2011). Increased ETC activity enhances oxidative stress and results in oxidative damage and cell death. Mitochondria-targeted antioxidant treatment was shown to reduce anxiety-related behaviour in mice and rats, indicating that a dysfunctional antioxidant defense is associated with anxiety. In schizophrenia (SCZ) *post-mortem* prefrontal cortex specimens increased glucose demand was observed, with higher lactate level accumulation and cellular acidosis, again implying mitochondrial dysfunction (Rajasekaran et al., 2015). A combined transcriptomics, proteomics and metabolomics analysis also revealed altered oxidative stress in *post-mortem* SCZ prefrontal cortex (Prabakaran et al., 2004). In autism spectrum disorders (ASD) biomarkers indicative of mitochondrial dysfunction were identified, including elevated lactate, pyruvate and alanine levels and an altered lactate-to-pyruvate ratio (Correia et al., 2006; Germanò et al., 2006; László et al., 1994).

Mitochondria are involved in neurotransmission by regulating presynaptic calcium levels at central glutamatergic terminals (Billups and Forsythe, 2002). An alteration of mitochondrial function can lead to neurotransmission disequilibrium reported to be involved in anxiety disorders (Calabresi et al., 2001) and ASD (Banerjee et al., 2013). Glutamate accumulation leads to mitochondrial β-oxidation inhibition, ROS generation and GSH depletion causing secondary mitochondrial dysfunction (Pastural et al., 2009; Tirosh et al., 2000). ROS impede mitochondrial oxidative phosphorylation and result in oxidative damage of membrane lipids and proteins that have been implicated in neurodegenerative disorders (Davis et al., 2014; Halliwell, 2006). Changes in mitochondrial function may influence downstream energy metabolism, resulting in compromised ATP production and neuronal differentiation, ultimately perturbing neuronal activity that has been associated with neurological disorders (Ben-Shachar and Laifenfeld, 2004; Mattson et al., 2008). Furthermore, it was suggested that mitochondrial deficits might result in progressive disturbances in astrocyte function relevant
3. DISCUSSION AND FUTURE PERSPECTIVES

in SCZ pathogenesis (Kolomeets and Uranova, 2010). Taken together, there is accumulating evidence that mitochondrial dysfunction results in dysregulated energy metabolism and synaptic plasticity, which in turn affect neuronal circuits and brain function implicated in various psychiatric disorders. Further studies are needed in order to elucidate the specific role of mitochondrial deficits in distinct psychiatric diseases and the interactions between environmental/epigenetics factors and mitochondrial dysfunction in disease pathogenesis.

3.1.2. Interaction between energy metabolism, inflammatory response and neuroendocrine function

My results indicate an increased inflammatory pathway activity and decreased energy metabolism in the ACC of shocked mice. A cross-talk between mitochondrial function and inflammation has been recognized in various pathological conditions. ROS generated by mitochondria act as signaling molecules in inflammatory pathways and regulate the production of pro-inflammatory cytokines (Naik and Dixit, 2011). Exposure to infections during early gestation in mice activates an inflammatory response and results in a long-lasting decrease of splenocyte mitochondrial activity in the offspring (Schwartzer et al, 2013). In several recent reports inflammation has been linked to PTSD (Gola et al, 2013; Lindqvist et al, 2014; Pace and Heim, 2011). Increased levels of pro-inflammatory cytokine mRNA and ROS were identified in the brain, adrenal glands and systemic circulation in a rat model of PTSD (Wilson et al, 2013). The mitochondrial deficits and oxidative stress found in PTSD could be associated with inflammation as mitochondria are the major targets of inflammation-induced damage. Furthermore, long-lasting inflammation observed in chronic disease, such as PTSD, is sustained by reparative mechanisms fueled mainly by ATP produced by mitochondria, which in the long term can result in mitochondrial malfunction (Liu et al, 2012).
3. DISCUSSION AND FUTURE PERSPECTIVES

Adrenergic agents and GCs are also important mediators implicated in inflammatory pathway activity through their binding to receptors on immune precursor cells (Ader et al., 1995); (Elenkov et al., 2000). Lower GC production and fewer GC receptors have been observed in PTSD patients (Yehuda, 2001). A decreased GC receptor number may lead to a dysregulated inflammatory response due to a reduced capacity of immune cells to respond to anti-inflammatory signals (Miller et al., 2002b). In conclusion, the above findings suggest that exposure to psychosocial stressors induces adrenergic agent and GC production which affects the pro-inflammatory cytokine network and mitochondrial activity. The interaction of neuroendocrine, inflammation and mitochondrial function is critical for neuronal activity involved in PTSD pathogenesis.

3.1.3. Role of energy metabolism in different neurons

I found an altered energy metabolism in PrL, ACC and NAc of the shocked mice. Further interrogation of altered mitochondrial function at the neuronal level is needed for a better understanding of the association between dysregulated neuronal activity and PTSD development. Since astrocytes communicate with both GABAergic and glutamatergic neurons it is plausible that inhibitory or excitatory neurotransmitters are involved in their function. During excitatory activity oxidative metabolism is much higher in glutamatergic neurons, with more ATP produced than in other neuron types (Calvetti and Somersalo, 2012). Previous studies have indicated that alterations in hippocampal function induced by glutamatergic and GC-driven changes result in a long-term anxiety-related behavior response upon exposure to psychological stressors (Reul, 2014). GABAergic tonic inhibitory control has been suggested to regulate epigenomic responses to psychologic events in dentate gyrus granule neurons via modulation of NMDA receptor function, implying a role for hippocampal GABAergic neurons in PTSD (Papadopoulos et al., 2011). Mitochondrial
dysfunction can lead to reduced synaptic neurotransmitter release in GABAergic interneurons (Anderson and Schönborn, 2008). Without proper GABAergic neuronal function resulting from perturbed mitochondrial activity the brain may be susceptible to excitotoxicity.

Astrocytes are relevant for the preservation of neural environments, including maintenance of the extracellular milieu (Suzuki et al, 2011) and regulation of neurotransmitter synthesis (Ota et al, 2013) and ions (Lian and Stringer, 2004). Mitochondrial dysfunction in astrocytes results in neuronal glutamate excitotoxicity which is likely to be associated with their inability to covert glutamate to glutamine, a process that requires ATP (Voloboueva et al, 2007). Furthermore, co-compartmentalization of astrocytic mitochondria and glutamate transporter-1 (GLT-1) has been observed in hippocampal sections (Genda et al, 2011), and the number of astrocytic mitochondria near GLT-1 is associated with glutamate uptake (Jackson et al, 2014). Since extracellular glutamate levels regulate synaptic transmission, astrocyte mitochondrial function plays an important role in this process.

3.2. Systemic view of PTSD brain circuitry

PTSD is associated with abnormal activity in brain regions involved in fear circuitry, such as medial prefrontal cortex, amygdala, hippocampus and NAc (Chaudhury et al, 2013; Maroun, 2013; Zelikowsky et al, 2014; Zhang et al, 2014) Alterations in the functional connectivity and activities of these brain regions result in perturbed perception and interpretation of traumatic events and lead to the development of PTSD (Antoniadis and McDonald, 2006; Belzung et al, 2014).

An emerging view of prefrontal cortex (PFC) function is that multiple PFC subregions (e.g. ACC, PrL and infralimbic cortex) dynamically interact and process information in parallel,
3. DISCUSSION AND FUTURE PERSPECTIVES

rather than working as distinct modules during attention and fear expression. PrL receives sensory inputs from amygdala, hippocampus as well as other cortical regions involved in fear conditioning (Herry and Johansen, 2014) and projects back to neurons in BLA for fear expression (Anglada-Figueroa and Quirk, 2005). Previous electrophysiology studies in rats suggest that ACC neurons drive beta local field potential within PrL and induce beta rhythm in local PrL neurons during a sustained attentional task (Totah et al., 2013). In a fear conditioning rat model Almada et al. revealed that medial PFC modulates the expression of contextual fear in combination with PrL via both serotonergic and GABAergic mechanisms and ACC via GABAergic mechanism (Almada et al., 2015). These findings suggest that parallel, but distinct activities of PrL and ACC contribute to the modulation of contextual fear conditioning. Over the course of my study, FS-induced long term energy metabolism alterations were found in ACC and PrL with opposite directions. Decreased ACC activity may result in cognitive deficits in trauma-related information processing and interferes with synaptic plasticity in the PrL. Perturbed ACC activity together with PrL hyperactivation results in exaggerated fear expression critical for the development of PTSD.

Anxiety-like behaviors are regulated by various neuronal circuitries, such as dopaminergic outputs from ventral tegmental area to NAc (da Cunha et al., 2008; Doherty and Gratton, 2007). It has been shown that ventral PFC projects fibers to NAc, and PFC glutamatergic system regulates the emotional behaviors directly through the NAc (Ongür and Price, 2000; Robbins, 2000). Furthermore, previous studies suggested that dopaminergic signaling in NAc shell exerts a modulatory effect on prelimbic NMDA-induced anxiolytic-like behaviors (Ahmadi et al., 2013). In my thesis project I found a downregulation of energy metabolism in NAc and an upregulation in PrL of shocked mice. It is conceivable that upon exposure to stressors an increased PrL activity leads to perturbed neuronal signaling and cellular
metabolism in NAc, which in turn results in deficits in modulatory NAc effects on PrL function and anxiety-like behavior. In addition to PrL, results from animal studies suggest that projections from ACC to NAc regulate impulsive activity (Fineberg et al, 2010), which is associated with PTSD symptoms (Miles et al, 2015). Decreased mitochondrial activity in ACC might lead to abnormal neurotransmission and synaptic signaling to NAc, which may further disturb cellular metabolism in NAc and result in anxiety-like behavior in shocked mice. Further time course studies are needed to investigate the role of distinct affected brain regions in PTSD pathogenesis.

3.3. Fluoxetine treatment

Early intervention by fluoxetine treatment ameliorated PTSD-like symptoms in shocked mice. Results from my thesis project suggest a prominent role of the drug in the modulation of energy metabolism and the inflammatory response.

Clinical studies have resulted in mixed outcomes when PTSD patients were treated with fluoxetine (Hertzberg et al, 2000; Martenyi et al, 2007; Martenyi et al, 2002; van der Kolk et al, 1994). Hertzberg et al. found that young or female PTSD patients responded better to fluoxetine treatment than older or male combat veterans (Hertzberg et al, 2000). Fluoxetine’s mode of action includes an involvement in synaptic remodeling, neurogenesis (Guest et al, 2004).

Karpova et al. found that chronic fluoxetine treatment combined with extinction training, but neither treatment alone, induces a sustained loss of conditioned fear memory in adult animals. It was shown that fluoxetine treatment increased synaptic plasticity through local brain-derived neurotrophic factor and converted fear memory circuitry to a more vulnerable state.
3. DISCUSSION AND FUTURE PERSPECTIVES

(Karpova et al, 2011). The reactivation of juvenile plasticity by fluoxetine treatment may allow behavioral training to modify maladapted networks for better adjustment to the environment (Castrén, 2005; Maya Vetencourt et al, 2008). In conclusion, early treatment with fluoxetine upon trauma exposure seems to regulate neuronal activity and may open a window for reshaping neuronal circuitry, thereby allowing in combination with psychotherapy to improve PTSD symptoms.

3.4. Future perspectives

In my thesis project I have integrated data from state of the art transcriptomics, proteomics and metabolomics analyses to identify dysregulated molecular pathways associated with PTSD. With the help of computational biology methods I have delineated interconnected pathways in brain regions implicated in the fear response. The results reveal novel insights into the complex etiology of PTSD.

Genome-wide association studies (GWAS) are complementary to the –omics analyses carried out in my thesis project and also allow the discovery of genes and molecular pathways contributing to PTSD pathobiology. The recently established Psychiatric Genomics Consortium (PGC) group studying PTSD genetics (PGC-PTSD) has investigated over 10,000 cases and 30,000 trauma-exposed controls from several countries (Logue et al, 2015). This large-scale GWAS will without doubt lead to significant genetic associations and further insights into the pathobiology of PTSD.

Future PTSD pathway discovery should also extend to lipidomics analysis. Lipids play an important role in signal transduction events in the central nervous system (CNS). CNS lipids are reported to be involved in neurotransmitter secretion and mitochondria function (Appikatla et al, 2014; Lane-Donovan et al, 2014). Alterations at the lipidome level will shed
additional light on the connection between psychiatric disorders and lipid metabolism, and may provide new targets and strategies for therapeutic intervention. Applying modern metabolomics to the analysis of brain microdialysates, which was also carried out as part of my thesis project, allows the interrogation of altered intracellular molecular pathways during stress response in targeted brain regions of mice. The method has great potential to better understand molecular pathways affected by stressors, and can be applied to animal models for psychiatric disorders.

In addition to hypothesis-free –omics studies, further research on targeted biological parameters/candidates, such as mRNA turnover (Mitchell and Tollervey, 2001; Munchel et al, 2011), protein turnover (Price et al, 2010; Zhang et al, 2011), protein post-translational modifications (Christensen et al, 2010) in combination with alternative platforms such as single-cell analyses (Shi et al, 2012) or multimodal imaging studies (Yerys and Herrington, 2014) will lead to a more comprehensive understanding of PTSD pathobiology. The integration of data from diverse technologies and well characterized mouse models and clinical samples will bring novel insights into the biological underpinnings of PTSD and eventually lead to better diagnosis and treatment strategies.
Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


stress disorder enters the age of large-scale genomic collaboration.

*Neuropsychopharmacology.*


Bibliography


Bibliography


Bibliography


Bibliography


Bibliography


Acknowledgements

I would like to first thank Professor Chris Turck for offering me the opportunity to conduct my PhD study in his research group. I am very grateful for his continuous support, not only on my PhD work but also on helping me adapting and integrating into a foreign culture. I truly appreciate Professor Chris Turck’s generous and kind mentoring which has guided me to better scientific and personal development.

My sincere thanks to Dr. Carsten Wotjak who has guided and inspired me on my PhD project. His brilliant scientific inputs and fruitful discussions motivate me and broaden my scientific horizons. Without Dr. Carsten Wotjak’s effort in establishing the PTSD mouse model, I wouldn’t have such an interesting topic to work with.

Many thanks to Dr. Elmira Anderzhanova who patiently showed me the microdialysis technique and step-by-step guided me on scientific thinking and writing. Her enthusiasm in neuroscience inspired me and I enjoyed every moment working with her.

I would like to express my gratitude to Professor Felix Tretter who offered me nice suggestions on my PhD project and scientific career development.

I would like to extend my gratitude to Professor Philipp Khaitovich for offering the collaboration opportunity so I could conduct the transcriptomics study and for his hospitality while I was in Shanghai. Thanks to Zhisong He for his diligent effort and work on the PTSD project. It was a pleasure to collaborate with him and work together on the manuscripts.

Thanks to John Asara and his personnel at Harvard University for metabolomics measurements.

I thank Lena Bittl and her team at the Graduate School of Systemic Neurosciences for their kind support.

I would like to thank Yi-Chun Yen for being there for me at the beginning of my PhD study. Without her support, I wouldn’t fit myself in so smoothly. I sincerely appreciate her kind friendship and hours and hours of sharing/talking.

I also would like to thank Christian Webhofer, who’s always patient and friendly whenever I asked him for help; Giuseppina Maccarrone who’s always caring and willing to help me even
Acknowledgements

when she’s very busy; Maria Holzapfel for her support on my PhD study; and Carola Hetzel for her generous help on administrational affairs.

I want to thank Stefan Reckow for his patience and elaborated explanations on the problems I encountered during data processing. I also want to thank all other (former) members of the research group for all the fun and interesting discussions we had which make my PhD life so nice: Katja Weckmann, Dongik Park, Christiane Rewerts, Michaela Filiou, Daniel Martins-de-Souza, Luis Rodrigues, Cecilia Café, Jeeva Varadarajulu, Philipp Gormanns, Yi-Wen Chen, Ying He, Claudia Ditzen, Shu-Yi Su and Larysa Teplytska. I enjoyed every moment we spent together and created so much nice memories!

I am thankful for the support from Sebastian Scharf during my thesis writing. His understanding and encouragement made my writing easier and more joyful!

Last but not least, I would like to sincerely thank my family in Taiwan. My beloved parents have been always supportive on every decision I made. I can’t express how grateful I am for their love and caring. I would not have made it to Germany and the dissertation would not have been possible without their support and encouragement. Thank you for everything, Mom and Dad!
Curriculum vitae and publications

Name: Chi-Ya Kao

Date of birth: 29.10.1985

Nationality: Taiwan (R.O.C.)

Education:

2011  
Dual graduate program  
Graduate School of Systemic Neurosciences  
(University of Munich)  
Translational Psychiatry - From Bed to Bench and Back  
(Max Planck Institute of Psychiatry, Munich)

2011  
Ph.D. student  
Max Planck Institute of Psychiatry, Munich  
Department of Translational Research in Psychiatry  
Proteomics and Biomarkers  
Thesis: Pathway and biomarker discovery in a posttraumatic stress disorder (PTSD) mouse model

2010  
Master of Science  
National Cheng Kung University, Tainan, Taiwan  
Department of Medical Laboratory Science and Biotechnology  
College of Medicine  
Thesis: The role of carbon monoxide in choleresis and cholestasis.

2008  
Bachelor of Science  
National Cheng Kung University, Tainan, Taiwan  
Department of Medical Laboratory Science and Biotechnology  
College of Medicine

Laboratory internships:

2014  
Visiting Ph.D. student  
CAS-MPG Partner Institute of Computational Biology  
Department of Integrative Biology
Curriculum vitae and publications

Shanghai, China
Project: Delineation of PTSD affected brain circuits with mouse model brain punch NextGen sequencing data.

2007
Visiting undergraduate student
Department of Biology, Georgia State University
Atlanta, GA, USA

Publications:


List of author contributions

Among the manuscripts included in the dissertation, I contributed as the follows:


- Study design and plan: in collaboration with ZH, CW and CT
- Conducting the experiments: in collaboration with KH and JA
- Data analysis: in collaboration with ZH, PK and CT
- Manuscript preparation: in collaboration with ZH, CW and CT


- Study design and plan: in collaboration with ZH, CW, PK and CT
- Conducting the experiments: in collaboration with ZH and OH
- Data analysis: in collaboration with ZH, OH, PK and CT
- Manuscript preparation: in collaboration with ZH and CT


- Study design and plan: in collaboration with EA and CW
- Conducting the experiments: in collaboration with EA, GS and JS
- Data analysis: in collaboration with EA and CW
- Manuscript preparation: in collaboration with EA and CW


- Study design and plan: in collaboration with EA, CW and CT
- Conducting the experiments: in collaboration with EA and JA
- Data analysis: in collaboration with EA, CW and CT
- Manuscript preparation: in collaboration with EA, CW and CT
Affidavit

Affidavit


Muenchen, Juli 2015

Chi-Ya Kao

I hereby confirm that the dissertation ‘Pathway and biomarker discovery in a posttraumatic stress disorder mouse model’ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

Munich, July 2015

Chi-Ya Kao