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

Graduate School of
Systemic Neurosciences
LMU Munich

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 transcriptomcis 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

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 hyperaroual 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

Table of contents

Abbreviations

5-HTTLPR	serotonin transporter
ACC	anterior cingulate cortex
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
APA	American Psychiatric Association
API	atmospheric pressure ionization
BLA	basolateral amygdala
CA1	cornu ammonis 1
CAGE	cap analysis of gene expression
CeA	central nucleus of amygdala
CID	collision-induced dissociation
CNS	central nervous system
CORT	corticosterone
CRH	corticotropin-releasing hormone
CRHR1	CRH receptor 1
CR-PTSD	combat-related PTSD
CSF	cerebrospinal fluid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ESI	electrospray ionization
ETC	electron transport chain
ETD	electron transfer dissociation
FKBP5	FK506 binding protein 5
FS	foot shock
GABA	gamma-aminobutyric acid

Abbreviations

GC	glucocorticoids
GC-MS	gas chromatography mass spectrometry
GLT-1	glutamate transporter-1
GR	glucocorticoid receptors
GWAS	genome-wide association studies
HCD	high energy collision dissociation
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
ICAT	isotope-coded affinity tag
ICPL	isotope-coded protein label
IL-1	interleukin 1
IL	infralimbic cortex
iTRAQ	isobaric tag for relative and absolute quantitation
LC	locus coeruleus
LC-MS	liquid chromatography mass spectrometry
LTP	long-term potentiation
LTQ	linear ion trap
m/z	mass-to-charge
MALDI	matrix-assisted-laser-desorption ionization
mPFC	medial prefrontal cortex
MPSS	massively parallel signature sequencing
MR	mineralocorticoid receptors
MRM	multiple-reaction-monitoring
MS	mass spectrometry
NAA	N-acetylaspartate
NAc	nucleus accumbens
NCS	National Comorbidity Survey

NFκB	nuclear factor κ B
NGFI-A	nerve growth factor-induced protein A
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NPY	neuropeptide Y
PACAP	pituitary adenylate cyclase-activating peptide
PGC	Psychiatric Genomics Consortium
PrL	prelimbic cortex
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
Q	quadrupole
QqQ	triple-quadrupole
Q-TOF	quadrupole-time-of-flight
RNA-seq	RNA sequencing
ROS	reactive oxidative species
SAGE	serial analysis of gene expression
SAM	sympathetic-adrenal-medullary
SCZ	schizophrenia
SILAC	stable isotope labeling using amino acids in cell culture
SILAM	stable isotope labeling of mammals
SNP	single nucleotide polymorphisms
SPS	single prolonged stress
SRM	selected-reaction-monitoring
SSRIs	selective serotonin reuptake inhibitors
TNF-α	tumor necrosis factor-α
TOF	time-of-flight
vmPFC	ventral medial prefrontal cortex

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

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.

1.2. 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 coeruleus (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 α_2 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

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)

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

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).

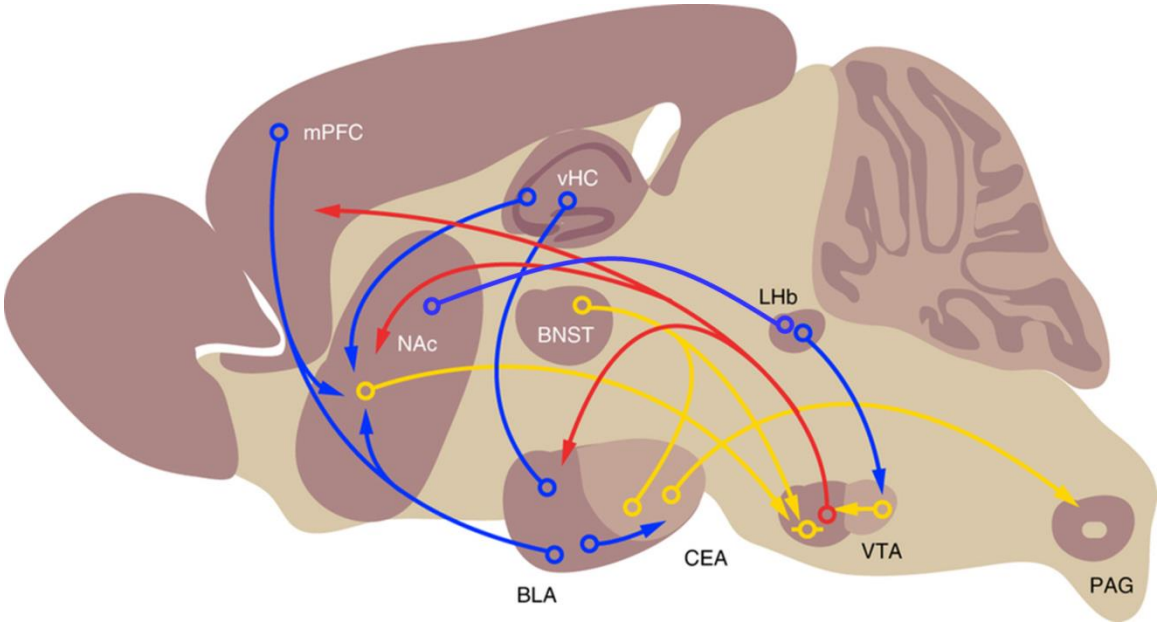


Figure 1. Brain circuits involved in fear perception. Simplified scheme depicting different projections from distinct brain regions for regulation of fear response. Red, blue and yellow arrows represent DA, glutamate, and GABA projections, respectively. Adapted from Lüthi and Lüscher (Lüthi and Lüscher,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

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

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

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 g*enes 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

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

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 fulfils the requirements for sensitive and comprehensive proteomics analysis. In addition, the LTQ-Orbitrap has a high dynamic range ($>10^3$) 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 alkylation. 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 (>10^6) 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

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

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 trasncriptomics, 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

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

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.

- 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

80804 Munich, Germany

Phone: +49-89-30622317, Fax: +49-89-30622610, E-mail: turck@psych.mpg.de

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.

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.

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 × 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 × **1**5 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}N and ^{15}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≥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 × 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 ℃ 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 ℃ 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 preciously (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 $\log_2$ 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.

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 circuity, 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

(Supplemental Table S1). 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

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 (oxolutaric 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

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.

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.

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

1. Association, A. P. (2013) *Diagnostic and statistical manual of mental health disorders: DSM-5*, American Psychiatric Publishing, Washington, DC

2. Yehuda, R., and Seckl, J. (2011) Minireview: Stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. *Endocrinology* 152, 4496-4503

3. Morris, M. C., Compas, B. E., and Garber, J. (2012) Relations among posttraumatic stress disorder, comorbid major depression, and HPA function: a systematic review and meta-analysis. *Clin Psychol Rev* 32, 301-315

4. Wu, G., Feder, A., Cohen, H., Kim, J. J., Calderon, S., Charney, D. S., and Mathé, A. A. (2013) Understanding resilience. *Front Behav Neurosci* 7, 10

5. Schmidt, U., Holsboer, F., and Rein, T. (2011) Epigenetic aspects of posttraumatic stress disorder. *Dis Markers* 30, 77-87

6. Domschke, K. (2012) Patho-genetics of posttraumatic stress disorder. *Psychiatr Danub* 24, 267-273

7. Ravindran, L. N., and Stein, M. B. (2009) Pharmacotherapy of PTSD: premises, principles, and priorities. *Brain Res* 1293, 24-39

8. Siegmund, A., and Wotjak, C. T. (2007) A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. *J Psychiatr Res* 41, 848-860

9. Kitano, H. (2002) Systems biology: a brief overview. *Science* 295, 1662-1664

10. Barabási, A. L., and Oltvai, Z. N. (2004) Network biology: understanding the cell's functional organization. *Nat Rev Genet* 5, 101-113

11. Herry, C., and Johansen, J. P. (2014) Encoding of fear learning and memory in distributed neuronal circuits. *Nat Neurosci* 17, 1644-1654

12. Robinson, O. J., Krimsky, M., Lieberman, L., Allen, P., Vytal, K., and Grillon, C. (2014) Towards a mechanistic understanding of pathological anxiety: the dorsal medial prefrontal-amygdala 'aversive amplification' circuit in unmedicated generalized and social anxiety disorders. *Lancet Psychiatry* 1, 294-302

13. Keding, T. J., and Herringa, R. J. (2015) Abnormal structure of fear circuitry in pediatric post-traumatic stress disorder. *Neuropsychopharmacology* 40, 537-545

14. Filiou, M. D., Zhang, Y., Teplytska, L., Reckow, S., Gormanns, P., Maccarrone, G., Frank, E., Kessler, M. S., Hambsch, B., Nussbaumer, M., Bunck, M., Ludwig, T., Yassouridis, A., Holsboer, F., Landgraf, R., and Turck, C. W. (2011) Proteomics and metabolomics analysis of a trait anxiety mouse model reveals divergent mitochondrial pathways. *Biol Psychiatry* 70, 1074-1082

15. Yuan, M., Breitkopf, S. B., Yang, X., and Asara, J. M. (2012) A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat Protoc* 7, 872-881

16. Golub, Y., Mauch, C. P., Dahlhoff, M., and Wotjak, C. T. (2009) Consequences of extinction training on associative and non-associative fear in a mouse model of Posttraumatic Stress Disorder (PTSD). *Behav Brain Res* 205, 544-549

17. Dahlhoff, M., Siegmund, A., Golub, Y., Wolf, E., Holsboer, F., and Wotjak, C. T. (2010) AKT/GSK-3beta/beta-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms. *Neuroscience* 169, 1216-1226

18. Paxinos G, F. K. (2001) *The mouse brain in stereotaxic coordinates*, Academic Press, San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo

19. Wiśniewski, J. R., Nagaraj, N., Zougman, A., Gnad, F., and Mann, M. (2010) Brain phosphoproteome obtained by a FASP-based method reveals plasma membrane protein topology. *J Proteome Res* 9, 3280-3289

20. Webhofer, C., Gormanns, P., Reckow, S., Lebar, M., Maccarrone, G., Ludwig, T., Pütz, B., Asara, J. M., Holsboer, F., Sillaber, I., Zieglgänsberger, W., and Turck, C. W. (2013) Proteomic and metabolomic profiling reveals time-dependent changes in hippocampal metabolism upon paroxetine treatment and biomarker candidates. *J Psychiatr Res* 47, 289-298

21. Zhang, Y., Webhofer, C., Reckow, S., Filiou, M. D., Maccarrone, G., and Turck, C. W. (2009) A MS data search method for improved 15N-labeled protein identification. *Proteomics* 9, 4265-4270

22. Keller, A., and Shteynberg, D. (2011) Software pipeline and data analysis for MS/MS proteomics: the trans-proteomic pipeline. *Methods Mol Biol* 694, 169-189

23. Pan, C., Kora, G., McDonald, W. H., Tabb, D. L., VerBerkmoes, N. C., Hurst, G. B., Pelletier, D. A., Samatova, N. F., and Hettich, R. L. (2006) ProRata: A quantitative proteomics program for accurate protein abundance ratio estimation with confidence interval evaluation. *Anal Chem* 78, 7121-7131

24. Vizcaíno, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Ríos, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P. A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H. J., Albar, J. P., Martinez-Bartolomé, S., Apweiler, R., Omenn, G. S., Martens, L., Jones, A. R., and Hermjakob, H. (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat Biotechnol* 32, 223-226

25. Xia, J., and Wishart, D. S. (2010) MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Res* 38, W71-77

26. Herrmann, L., Ionescu, I. A., Henes, K., Golub, Y., Wang, N. X., Buell, D. R., Holsboer, F., Wotjak, C. T., and Schmidt, U. (2012) Long-lasting hippocampal synaptic protein loss in a mouse model of posttraumatic stress disorder. *PLoS One* 7, e42603

27. Pamplona, F. A., Henes, K., Micale, V., Mauch, C. P., Takahashi, R. N., and Wotjak, C. T. (2011) Prolonged fear incubation leads to generalized avoidance behavior in mice. *J Psychiatr Res* 45, 354-360

28. Siegmund, A., Dahlhoff, M., Habersetzer, U., Mederer, A., Wolf, E., Holsboer, F., and Wotjak, C. T. (2009) Maternal inexperience as a risk factor of innate fear and PTSD-like symptoms in mice. *J Psychiatr Res* 43, 1156-1165

29. Billups, B., and Forsythe, I. D. (2002) Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J Neurosci* 22, 5840-5847

30. Vanden Berghe, P., Kenyon, J. L., and Smith, T. K. (2002) Mitochondrial Ca2+ uptake regulates the excitability of myenteric neurons. *J Neurosci* 22, 6962-6971

31. Kann, O., Kovács, R., and Heinemann, U. (2003) Metabotropic receptor-mediated Ca2+ signaling elevates mitochondrial Ca2+ and stimulates oxidative metabolism in hippocampal slice cultures. *J Neurophysiol* 90, 613-621

32. Naviaux, R. K. (2012) Oxidative shielding or oxidative stress? *J Pharmacol Exp Ther* 342, 608-618

33. Ehlert, U. (2013) Enduring psychobiological effects of childhood adversity. *Psychoneuroendocrinology* 38, 1850-1857

34. Rajasekaran, A., Venkatasubramanian, G., Berk, M., and Debnath, M. (2015) Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications. *Neurosci Biobehav Rev* 48, 10-21

35. Flaquer, A., Baumbach, C., Ladwig, K. H., Kriebel, J., Waldenberger, M., Grallert, H., Baumert, J., Meitinger, T., Kruse, J., Peters, A., Emeny, R., and Strauch, K. (2015) Mitochondrial genetic variants identified to be associated with posttraumatic stress disorder. *Transl Psychiatry* 5, e524

36. Lowenstein, J. (1969) *Methods in Enzymology*, Academic Press, Boston

37. Fenton, G. E., Pollard, A. K., Halliday, D. M., Mason, R., Bredy, T. W., and Stevenson, C. W. (2014) Persistent prelimbic cortex activity contributes to enhanced learned fear expression in females. *Learn Mem* 21, 55-60

38. Burgos-Robles, A., Vidal-Gonzalez, I., and Quirk, G. J. (2009) Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. *J Neurosci* 29, 8474-8482

39. Lisboa, S. F., Borges, A. A., Nejo, P., Fassini, A., Guimarães, F. S., and Resstel, L. B. (2015) Cannabinoid CB1 receptors in the dorsal hippocampus and prelimbic medial prefrontal cortex modulate anxiety-like behavior in rats: additional evidence. *Prog Neuropsychopharmacol Biol Psychiatry* 59, 76-83

40. Bénard, G., Massa, F., Puente, N., Lourenço, J., Bellocchio, L., Soria-Gómez, E., Matias, I., Delamarre, A., Metna-Laurent, M., Cannich, A., Hebert-Chatelain, E., Mulle, C., Ortega-Gutiérrez, S., Martín-Fontecha, M., Klugmann, M., Guggenhuber, S., Lutz, B., Gertsch, J., Chaouloff, F., López-Rodríguez, M. L., Grandes, P., Rossignol, R., and Marsicano, G. (2012) Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nat Neurosci* 15, 558-564

41. Totah, N. K., Jackson, M. E., and Moghaddam, B. (2013) Preparatory attention relies on dynamic interactions between prelimbic cortex and anterior cingulate cortex. *Cereb Cortex* 23, 729-738

42. Woodward, S. H., Shurick, A. A., Alvarez, J., Kuo, J., Nonyieva, Y., Blechert, J., McRae, K., and Gross, J. J. (2015) A psychophysiological investigation of emotion regulation in chronic severe posttraumatic stress disorder. *Psychophysiology* 52, 667-678

43. Wisdom, N. M., Pastorek, N. J., Miller, B. I., Booth, J. E., Romesser, J. M., Linck, J. F., and Sim, A. H. (2014) PTSD and cognitive functioning: importance of including performance validity testing. *Clin Neuropsychol* 28, 128-145

44. Kim, M. J., Chey, J., Chung, A., Bae, S., Khang, H., Ham, B., Yoon, S. J., Jeong, D. U., and Lyoo, I. K. (2008) Diminished rostral anterior cingulate activity in response to threat-related events in posttraumatic stress disorder. *J Psychiatr Res* 42, 268-277

45. Bryant, R. A., Felmingham, K., Kemp, A., Das, P., Hughes, G., Peduto, A., and Williams, L. (2008) Amygdala and ventral anterior cingulate activation predicts treatment response to cognitive behaviour therapy for post-traumatic stress disorder. *Psychol Med* 38, 555-561

46. Schuff, N., Neylan, T. C., Fox-Bosetti, S., Lenoci, M., Samuelson, K. W., Studholme, C., Kornak, J., Marmar, C. R., and Weiner, M. W. (2008) Abnormal N-acetylaspartate in hippocampus and anterior cingulate in posttraumatic stress disorder. *Psychiatry Res* 162, 147-157

47. Gasparovic, C., Arfai, N., Smid, N., and Feeney, D. M. (2001) Decrease and recovery of N-acetylaspartate/creatine in rat brain remote from focal injury. *J Neurotrauma* 18, 241-246

48. Demougeot, C., Marie, C., Giroud, M., and Beley, A. (2004) N-acetylaspartate: a literature review of animal research on brain ischaemia. *J Neurochem* 90, 776-783

49. Zell, R., Geck, P., Werdan, K., and Boekstegers, P. (1997) TNF-alpha and IL-1 alpha inhibit both pyruvate dehydrogenase activity and mitochondrial function in cardiomyocytes: evidence for primary impairment of mitochondrial function. *Mol Cell Biochem* 177, 61-67

50. Spencer, S., Brown, R. M., Quintero, G. C., Kupchik, Y. M., Thomas, C. A., Reissner, K. J., and Kalivas, P. W. (2014) α2δ-1 signaling in nucleus accumbens is necessary for cocaine-induced relapse. *J Neurosci* 34, 8605-8611

51. Wakabayashi, K. T., Myal, S. E., and Kiyatkin, E. A. (2015) Fluctuations in nucleus accumbens extracellular glutamate and glucose during motivated glucose-drinking behavior: dissecting the neurochemistry of reward. *J Neurochem* 132, 327-341

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

53. Salamone, J. D., Cousins, M. S., and Snyder, B. J. (1997) Behavioral functions of nucleus accumbens dopamine: empirical and conceptual problems with the anhedonia hypothesis. *Neurosci Biobehav Rev* 21, 341-359

54. Davidson, R. J., and Irwin, W. (1999) The functional neuroanatomy of emotion and affective style. *Trends Cogn Sci* 3, 11-21

55. Shirayama, Y., and Chaki, S. (2006) Neurochemistry of the nucleus accumbens and its relevance to depression and antidepressant action in rodents. *Curr Neuropharmacol* 4, 277-291

56. Krishnan, V., Han, M. H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., Laplant, Q., Graham, A., Lutter, M., Lagace, D. C., Ghose, S., Reister, R., Tannous, P., Green, T. A., Neve, R. L., Chakravarty, S., Kumar, A., Eisch, A. J., Self, D. W., Lee, F. S., Tamminga, C. A., Cooper, D. C., Gershenfeld, H. K., and Nestler, E. J. (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* 131, 391-404

57. Sailer, U., Robinson, S., Fischmeister, F. P., König, D., Oppenauer, C., Lueger-Schuster, B., Moser, E., Kryspin-Exner, I., and Bauer, H. (2008) Altered reward processing in

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

58. Fan, X., Li, D., Lichti, C. F., and Green, T. A. (2013) Dynamic proteomics of nucleus accumbens in response to acute psychological stress in environmentally enriched and isolated rats. *PLoS One* 8, e73689

59. Bzdok, D., Laird, A. R., Zilles, K., Fox, P. T., and Eickhoff, S. B. (2013) An investigation of the structural, connectional, and functional subspecialization in the human amygdala. *Hum Brain Mapp* 34, 3247-3266

60. Phelps, E. A., and LeDoux, J. E. (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron* 48, 175-187

61. Tan, H., Lauzon, N. M., Bishop, S. F., Chi, N., Bechard, M., and Laviolette, S. R. (2011) Cannabinoid transmission in the basolateral amygdala modulates fear memory formation via functional inputs to the prelimbic cortex. *J Neurosci* 31, 5300-5312

62. Nonaka, A., Toyoda, T., Miura, Y., Hitora-Imamura, N., Naka, M., Eguchi, M., Yamaguchi, S., Ikegaya, Y., Matsuki, N., and Nomura, H. (2014) Synaptic plasticity associated with a memory engram in the basolateral amygdala. *J Neurosci* 34, 9305-9309

63. Ventura-Silva, A. P., Melo, A., Ferreira, A. C., Carvalho, M. M., Campos, F. L., Sousa, N., and Pêgo, J. M. (2013) Excitotoxic lesions in the central nucleus of the amygdala attenuate stress-induced anxiety behavior. *Front Behav Neurosci* 7, 32

64. Grillo, C. A., Risher, M., Macht, V. A., Bumgardner, A. L., Hang, A., Gabriel, C., Mocaër, E., Piroli, G. G., Fadel, J. R., and Reagan, L. P. (2015) Repeated restraint stress-induced atrophy of glutamatergic pyramidal neurons and decreases in glutamatergic efflux in the rat amygdala are prevented by the antidepressant agomelatine. *Neuroscience* 284, 430-443

65. Vyas, A., Mitra, R., Shankaranarayana Rao, B. S., and Chattarji, S. (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* 22, 6810-6818

66. Goshen, I., Brodsky, M., Prakash, R., Wallace, J., Gradinaru, V., Ramakrishnan, C., and Deisseroth, K. (2011) Dynamics of retrieval strategies for remote memories. *Cell* 147, 678-689

67. Matrisciano, F., Busceti, C. L., Bucci, D., Orlando, R., Caruso, A., Molinaro, G., Cappuccio, I., Riozzi, B., Gradini, R., Motolese, M., Caraci, F., Copani, A., Scaccianoce, S., Melchiorri, D., Bruno, V., Battaglia, G., and Nicoletti, F. (2011) Induction of the Wnt antagonist Dickkopf-1 is involved in stress-induced hippocampal damage. *PLoS One* 6, e16447

68. Geller, D., M.B.B.S., March, J., and (CQI), T. A. C. o. Q. I. (2012) Practice parameter for the assessment and treatment of children and adolescents with obsessive-compulsive disorder. *J Am Acad Child Adolesc Psychiatry* 51, 98-113

69. Fournier, J. C., DeRubeis, R. J., Hollon, S. D., Dimidjian, S., Amsterdam, J. D., Shelton, R. C., and Fawcett, J. (2010) Antidepressant drug effects and depression severity: a patient-level meta-analysis. *JAMA* 303, 47-53

70. Guest, P. C., Knowles, M. R., Molon-Noblot, S., Salim, K., Smith, D., Murray, F., Laroque, P., Hunt, S. P., De Felipe, C., Rupniak, N. M., and McAllister, G. (2004) Mechanisms of action of the antidepressants fluoxetine and the substance P antagonist L-000760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res* 1002, 1-10

71. Wang, Y. X., Zhang, X. R., Zhang, Z. J., Li, L., Xi, G. J., Wu, D., and Wang, Y. J. (2014) Protein kinase Mζ is involved in the modulatory effect of fluoxetine on hippocampal neurogenesis in vitro. *Int J Neuropsychopharmacol* 17, 1429-1441

72. Hashioka, S., Klegeris, A., Monji, A., Kato, T., Sawada, M., McGeer, P. L., and Kanba, S. (2007) Antidepressants inhibit interferon-gamma-induced microglial production of IL-6 and nitric oxide. *Exp Neurol* 206, 33-42

73. Liu, D., Wang, Z., Liu, S., Wang, F., Zhao, S., and Hao, A. (2011) Anti-inflammatory effects of fluoxetine in lipopolysaccharide(LPS)-stimulated microglial cells. *Neuropharmacology* 61, 592-599

74. Abdel-Razaq, W., Kendall, D. A., and Bates, T. E. (2011) The effects of antidepressants on mitochondrial function in a model cell system and isolated mitochondria. *Neurochem Res* 36, 327-338

75. Scaini, G., Maggi, D., De-Nês, B., Gonçalves, C., Ferreira, G., Teodorak, B., Bez, G., Ferreira, G., Schuck, P., Quevedo, J., and Streck, E. (2011) Activity of mitochondrial respiratory chain is increased by chronic administration of antidepresants. pp. 112-118, Acta Neuropsychiatrica

76. Zhao, J., Jung, Y. H., Jang, C. G., Chun, K. H., Kwon, S. W., and Lee, J. (2015) Metabolomic identification of biochemical changes induced by fluoxetine and imipramine in a chronic mild stress mouse model of depression. *Sci Rep* 5, 8890

77. Currais, A. (2015) Ageing and inflammation - A central role for mitochondria in brain health and disease. *Ageing Res Rev* 21, 30-42

78. Taoka, Y., Okajima, K., Uchiba, M., Murakami, K., Kushimoto, S., Johno, M., Naruo, M., Okabe, H., and Takatsuki, K. (1997) Role of neutrophils in spinal cord injury in the rat. *Neuroscience* 79, 1177-1182

79. Popovich, P. G., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., and Stokes, B. T. (1999) Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. *Exp Neurol* 158, 351-365

80. Lee, J. Y., Kang, S. R., and Yune, T. Y. (2015) Fluoxetine Prevents Oligodendrocyte Cell Death by Inhibiting Microglia Activation after Spinal Cord Injury. *J Neurotrauma*

81. Raeder, M. B., Fernø, J., Glambek, M., Stansberg, C., and Steen, V. M. (2006) Antidepressant drugs activate SREBP and up-regulate cholesterol and fatty acid biosynthesis in human glial cells. *Neurosci Lett* 395, 185-190

82. Fulda, S., Galluzzi, L., and Kroemer, G. (2010) Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* 9, 447-464

83. Armstrong, L. C., Saenz, A. J., and Bornstein, P. (1999) Metaxin 1 interacts with metaxin 2, a novel related protein associated with the mammalian mitochondrial outer membrane. *J Cell Biochem* 74, 11-22

84. Ben-Shachar, D., and Laifenfeld, D. (2004) Mitochondria, synaptic plasticity, and schizophrenia. *Int Rev Neurobiol* 59, 273-296

85. Stork, C., and Renshaw, P. F. (2005) Mitochondrial dysfunction in bipolar disorder: evidence from magnetic resonance spectroscopy research. *Mol Psychiatry* 10, 900-919

Table 1. Enriched molecular pathways in shocked mice.

Brain region	Cellular fraction	Pathway (Gene Ontology/KEGG)	Fold Enrichment	Bonferroni	Benjamini	FDR	Direction
Prelimbic cortex	cytosolic	GO:0005938~cell cortex	11.470	1.1E-05	1.1E-05	1.1E-04	+
		GO:0044448~cell cortex part	12.412	5.3E-05	2.6E-05	5.5E-04	+
		GO:0015629~actin cytoskeleton	7.930	1.5E-03	4.9E-04	1.5E-02	+
		GO:0005856~cytoskeleton	3.893	1.6E-03	4.1E-04	1.7E-02	+
		GO:0030863~cortical cytoskeleton	12.594	4.0E-03	8.0E-04	4.2E-02	+
	membrane-associated	mmu00020:Citrate cycle (TCA cycle)	15.565	1.1E-03	1.1E-03	4.5E-02	+
Anterior cingulate cortex	cytosolic	GO:0044430~cytoskeletal part	2.850	7.8E-04	7.8E-04	6.1E-03	+
		GO:0007018~microtubule-based movement	5.954	1.2E-02	1.2E-02	2.7E-02	+
		GO:0005856~cytoskeleton	2.316	5.1E-03	2.5E-03	4.0E-02	+
		GO:0006461~protein complex assembly	4.253	3.8E-02	1.9E-02	8.4E-02	+
		GO:0070271~protein complex biogenesis	4.253	3.8E-02	1.9E-02	8.4E-02	+
	membrane-associated	GO:0019001~guanyl nucleotide binding	1.540	9.8E-03	9.8E-03	3.3E-02	+
		GO:0032561~guanyl ribonucleotide binding	1.540	9.8E-03	9.8E-03	3.3E-02	+
		GO:0005525~GTP binding	1.540	9.8E-03	9.8E-03	3.3E-02	+
		GO:0006091~generation of precursor metabolites and energy	1.609	5.2E-02	5.2E-02	6.4E-02	-
		GO:0003924~GTPase activity	1.850	2.8E-02	1.4E-02	9.6E-02	-
Nucleus accumbens	cytosolic	GO:0034622~cellular macromolecular complex assembly	3.160	1.0E-03	1.0E-03	1.8E-03	-
		GO:0065003~macromolecular complex assembly	3.039	1.1E-03	5.3E-04	1.9E-03	-
		GO:0034621~cellular macromolecular complex subunit organizati	3.105	1.4E-03	4.7E-04	2.5E-03	-
		GO:0043933~macromolecular complex subunit organization	2.943	2.0E-03	4.9E-04	3.5E-03	-
		GO:0051258~protein polymerization	6.020	3.4E-03	6.9E-04	6.0E-03	+
		GO:0003924~GTPase activity	3.402	6.7E-03	6.7E-03	3.8E-02	+
	membrane-associated	mmu00020:Citrate cycle (TCA cycle)	3.159	5.1E-06	5.1E-06	5.2E-05	-
		GO:0006091~generation of precursor metabolites and energy	2.102	2.7E-06	2.7E-06	2.9E-06	-
		GO:0031974~membrane-enclosed lumen	2.044	3.0E-05	3.0E-05	1.3E-04	-
		GO:0043233~organelle lumen	1.973	3.2E-04	7.9E-05	1.3E-03	-
		GO:0006084~acetyl-CoA metabolic process	3.500	2.5E-03	1.3E-03	2.7E-03	-
		GO:0070013~intracellular organelle lumen	1.949	6.7E-04	1.3E-04	2.8E-03	-
		GO:0043232~intracellular non-membrane-bounded organelle	1.519	7.8E-04	1.3E-04	3.2E-03	-
		GO:0043228~non-membrane-bounded organelle	1.519	7.8E-04	1.3E-04	3.2E-03	-
		GO:0006099~tricarboxylic acid cycle	3.482	8.4E-03	2.8E-03	9.2E-03	-
		GO:0046356~acetyl-CoA catabolic process	3.482	8.4E-03	2.8E-03	9.2E-03	-
		GO:0006007~glucose catabolic process	2.813	8.9E-03	2.2E-03	9.7E-03	-
		GO:0046365~monosaccharide catabolic process	2.813	8.9E-03	2.2E-03	9.7E-03	-
		GO:0019320~hexose catabolic process	2.813	8.9E-03	2.2E-03	9.7E-03	-
		GO:0006006~glucose metabolic process	2.396	1.5E-02	3.0E-03	1.6E-02	-
		GO:0019318~hexose metabolic process	2.396	1.5E-02	3.0E-03	1.6E-02	-
		GO:0016052~carbohydrate catabolic process	2.639	1.8E-02	3.0E-03	1.9E-02	-
		GO:0044275~cellular carbohydrate catabolic process	2.639	1.8E-02	3.0E-03	1.9E-02	-
		GO:0005996~monosaccharide metabolic process	2.331	2.7E-02	4.0E-03	3.0E-02	-
		GO:0006096~glycolysis	2.857	2.8E-02	3.6E-03	3.1E-02	-
		GO:0005759~mitochondrial matrix	2.270	6.8E-03	9.7E-04	2.8E-02	-
		GO:0031980~mitochondrial lumen	2.270	6.8E-03	9.7E-04	2.8E-02	-
		GO:0009109~coenzyme catabolic process	3.250	3.2E-02	3.6E-03	3.5E-02	-
		GO:0009060~aerobic respiration	3.250	3.2E-02	3.6E-03	3.5E-02	-
		GO:0051187~cofactor catabolic process	3.088	3.3E-02	3.3E-03	3.6E-02	-
		GO:0015980~energy derivation by oxidation of organic compounds	2.386	4.5E-02	4.2E-03	5.0E-02	-
		GO:0050662~coenzyme binding	2.257	1.5E-02	1.5E-02	4.5E-02	-
		GO:0015672~monovalent inorganic cation transport	6.658	1.8E-03	1.8E-03	4.5E-03	-
		GO:0006096~glycolysis	6.053	4.8E-03	2.4E-03	1.2E-02	-
		GO:0051258~protein polymerization	8.194	6.0E-03	2.0E-03	1.5E-02	-
Basolateral amygdala	cytosolic	GO:0042623~ATPase activity, coupled	5.644	9.9E-03	9.9E-03	6.9E-02	-
		GO:0005874~microtubule	4.276	1.3E-02	1.3E-02	8.6E-02	-
Central nucleus of amygdala	cytosolic	mmu04540:Gap junction	9.882	6.1E-05	6.1E-05	1.5E-03	+
		GO:0051258~protein polymerization	17.762	5.1E-06	5.1E-06	2.4E-05	+
		GO:0043623~cellular protein complex assembly	12.212	1.5E-04	7.7E-05	7.2E-04	+
		GO:0007018~microtubule-based movement	11.493	2.6E-04	8.6E-05	1.2E-03	+
		GO:0003924~GTPase activity	7.914	3.2E-04	3.2E-04	3.5E-03	+
		GO:0007017~microtubule-based process	7.850	9.3E-04	2.3E-04	4.3E-03	+
		GO:0006461~protein complex assembly	9.769	9.5E-04	1.9E-04	4.4E-03	+
		GO:0070271~protein complex biogenesis	9.769	9.5E-04	1.9E-04	4.4E-03	+
		GO:0005874~microtubule	7.663	3.1E-04	3.1E-04	4.0E-03	+
		GO:0005198~structural molecule activity	5.500	4.0E-04	2.0E-04	4.5E-03	+
		GO:0034622~cellular macromolecular complex assembly	6.737	1.5E-02	2.5E-03	7.1E-02	+
		GO:0034621~cellular macromolecular complex subunit organizati	6.737	1.5E-02	2.5E-03	7.1E-02	+
		GO:0044430~cytoskeletal part	3.621	5.7E-03	2.9E-03	7.2E-02	+
CA1	cytosolic	GO:0005198~structural molecule activity	6.424	4.2E-09	4.2E-09	6.3E-08	-
		GO:0007017~microtubule-based process	8.439	1.0E-05	1.0E-05	4.8E-05	-
		GO:0051258~protein polymerization	11.763	1.4E-04	6.8E-05	6.3E-04	-
		GO:0007018~microtubule-based movement	10.859	2.8E-04	9.4E-05	1.3E-03	-
		GO:0043623~cellular protein complex assembly	8.304	2.8E-03	6.9E-04	1.3E-02	-
		GO:0044430~cytoskeletal part	3.206	1.9E-03	1.9E-03	2.6E-02	-
		GO:0005882~intermediate filament	8.172	4.1E-03	2.0E-03	5.7E-02	-
		GO:0045111~intermediate filament cytoskeleton	8.172	4.1E-03	2.0E-03	5.7E-02	-
		GO:0006461~protein complex assembly	6.416	2.0E-02	4.0E-03	9.3E-02	-
		GO:0070271~protein complex biogenesis	6.416	2.0E-02	4.0E-03	9.3E-02	-
	membrane-associated	GO:0043209~myelin sheath	43.667	2.9E-03	2.9E-03	3.8E-02	-

Table 2. Rescued pathways upon chronic fluoxetine treatment in shocked mice.

Brain region	DAVID terms	P(rescued)[a] < 0.05
ACC	transit peptide	1.14E-13
	acetylation	4.93E-13
	GO:0005525~GTP binding	6.67E-11
	gtp-binding	1.90E-10
	GO:0006091~generation of precursor metabolites and energy	1.36E-08
	iron	2.86E-06
	GO:0003924~GTPase activity	2.10E-05
	mmu04540:Gap junction	0.001281604
CA1	transmembrane protein	1.53E-05
	GO:0007017~microtubule-based process	0.001953125
	GO:0043209~myelin sheath	0.015625
	microtubule	0.015625
	myelin	0.015625
	PIRSF002306:tubulin	0.03125
NAc	mmu00020:Citrate cycle (TCA cycle)	0.009605408

[a]P(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.

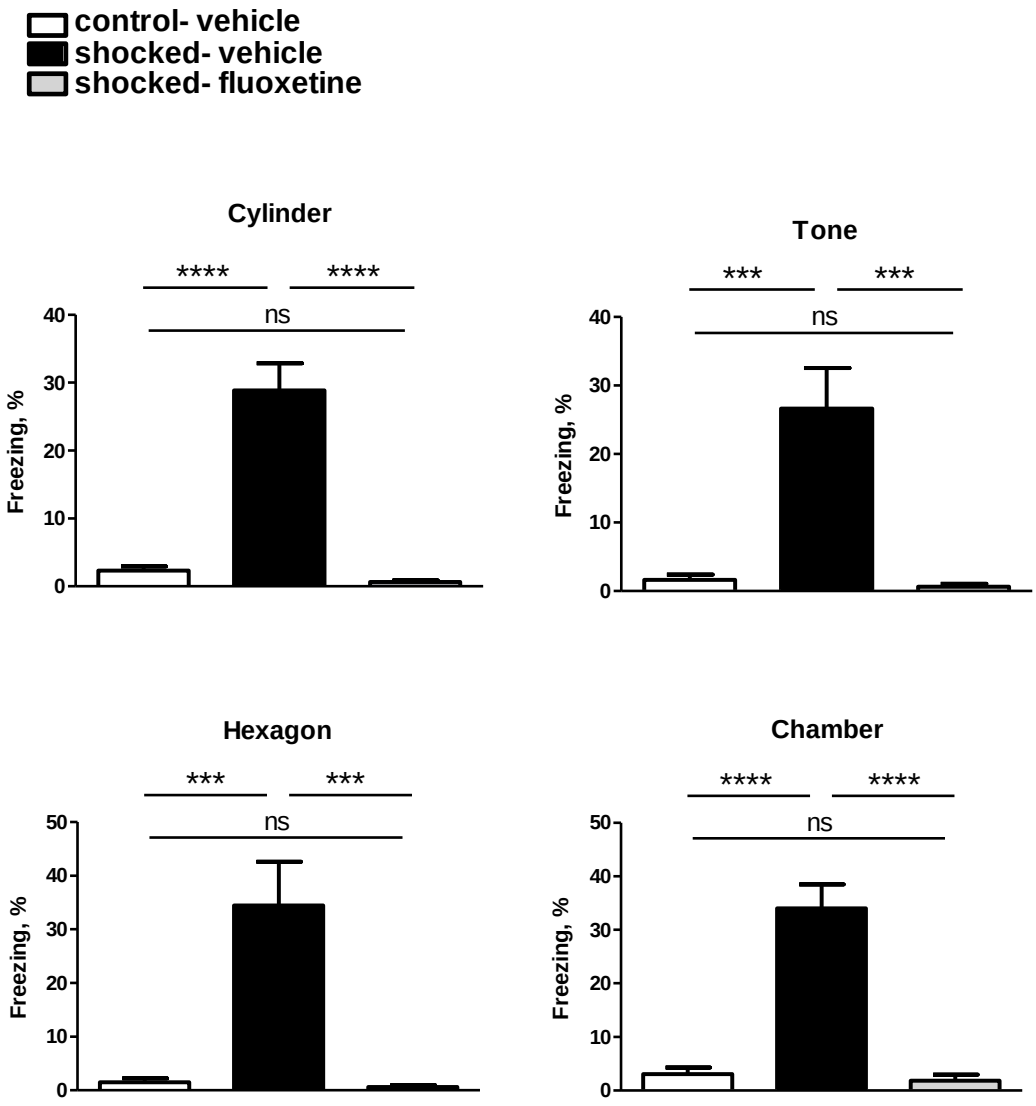

control- vehicle
shocked- vehicle
shocked- fluoxetine
Cylinder
****
****
ns
Freezing, %
40
30
20
10
0
Tone
***
***
ns
Freezing, %
40
30
20
10
0
Hexagon
***
***
ns
Freezing, %
50
40
30
20
10
0
Chamber
****
****
ns
Freezing, %
50
40
30
20
10
0

Figure 1

A

Pyruvic acid (HMDB00243)
NAD (HMDB00902)
L-Malic acid (HMDB00156)
Pyruvate dehydrogenase complex (P35486, Q9D051, Q8BMF4, O08749)
Malate dehydrogenase, mitochondrial (P08249)
ATP (HMDB00538)
Fumarate hydratase, mitochondrial (P97807)
NADH (HMDB01487)
Pyruvate carboxylase, mitochondrial (Q05920)
Acetyl-CoA (HMDB01206)
Citrate synthase, mitochondrial (Q9CZU6)
Citric acid (HMDB00094)
Fumaric acid (HMDB00134)
Oxalacetic acid (HMDB00223)
ADP (HMDB01341)
Aconitate hydratase, mitochondrial (Q99KI0)
FADH2 (HMDB01197)
Cis-Aconitic acid (HMDB00072)
Succinate dehydrogenase (Q8K2B3, Q9CQA3, Q9CZB0, Q9CXV1)
Aconitate hydratase, mitochondrial (Q99KI0)
GDP (HMDB01201)
FAD (HMDB01248)
Isocitric acid (HMDB00193)
NAD (HMDB00902)
NAc (SV-NSV)
NAD (HMDB00902)
Succinic acid (HMDB00254)
Oxoglutarate dehydrogenase complex (Q60597, Q9D2G2, O08749)
Isocitrate dehydrogenase, mitochondrial (Q9D6R2, Q91VA7, P70404)
NADH (HMDB01487)
Succinyl-CoA ligase (Q9WUM5, Q9Z2I8)
Oxoglutaric acid (HMDB00208)
NADH (HMDB01487)
GTP (HMDB01273)
Coenzyme A (HMDB01423)
Succinyl-CoA (HMDB01022)
NADH (HMDB01487)

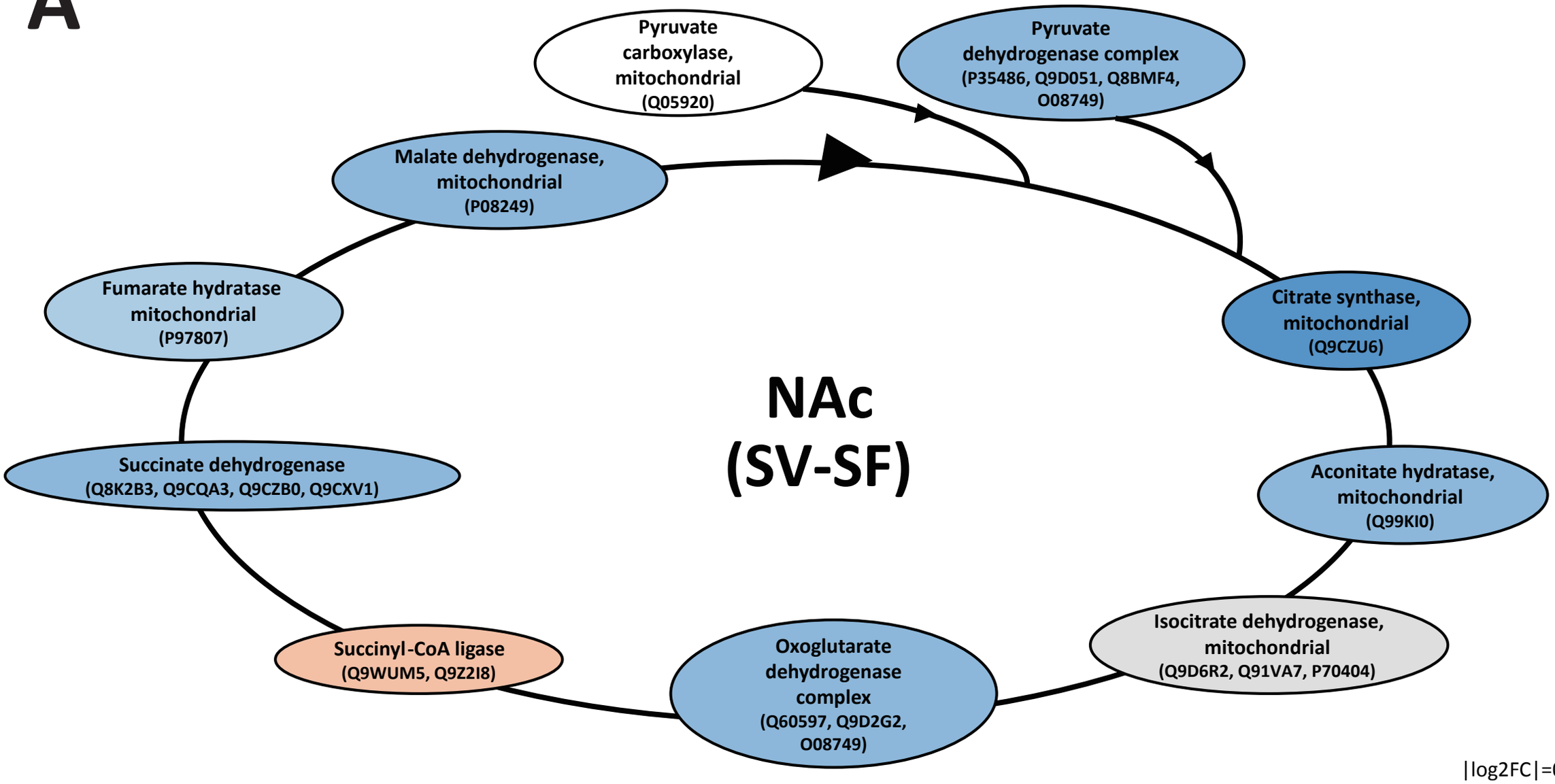
Metabolites
Enzymes
|log2FC|=0
|log2FC|=0
0<|log2FC|≤0.1
0.1<|log2FC|≤0.2
0.2<|log2FC|≤0.3
0.3<|log2FC|≤0.4
0.4<|log2FC|≤0.5
|log2FC|>0.5
0<|log2FC|≤0.5
0.5<|log2FC|≤1
1<|log2FC|≤1.5
1.5<|log2FC|≤2
|log2FC|>2
Mixture of increase and decrease
Not measured

B

Pyruvic acid (HMDB00243)
NAD (HMDB00902)
L-Malic acid (HMDB00156)
Pyruvate dehydrogenase complex (P35486, Q9D051, Q8BMF4, O08749)
Malate dehydrogenase, mitochondrial (P08249)
ATP (HMDB00538)
Fumarate hydratase, mitochondrial (P97807)
NADH (HMDB01487)
Pyruvate carboxylase, mitochondrial (Q05920)
Acetyl-CoA (HMDB01206)
Citrate synthase, mitochondrial (Q9CZU6)
Citric acid (HMDB00094)
Fumaric acid (HMDB00134)
Oxalacetic acid (HMDB00223)
ADP (HMDB01341)
Aconitate hydratase, mitochondrial (Q99KI0)
FADH2 (HMDB01197)
Cis-Aconitic acid (HMDB00072)
Succinate dehydrogenase (Q8K2B3, Q9CQA3, Q9CZB0, Q9CXV1)
Aconitate hydratase, mitochondrial (Q99KI0)
GDP (HMDB01201)
FAD (HMDB01248)
Isocitric acid (HMDB00193)
NAD (HMDB00902)
ACC (SV-NSV)
NAD (HMDB00902)
Succinic acid (HMDB00254)
Oxoglutarate dehydrogenase complex (Q60597, Q9D2G2, O08749)
Isocitrate dehydrogenase, mitochondrial (Q9D6R2, Q91VA7, P70404)
NADH (HMDB01487)
Succinyl-CoA ligase (Q9WUM5, Q9Z2I8)
Oxoglutaric acid (HMDB00208)
NADH (HMDB01487)
GTP (HMDB01273)
Coenzyme A (HMDB01423)
Succinyl-CoA (HMDB01022)
NADH (HMDB01487)

Figure 2

A


Pyruvate carboxylase, mitochondrial (Q05920)
Pyruvate dehydrogenase complex (P35486, Q9D051, Q8BMF4, O08749)
Malate dehydrogenase, mitochondrial (P08249)
Fumarate hydratase mitochondrial (P97807)
Citrate synthase, mitochondrial (Q9CZU6)
NAc (SV-SF)
Succinate dehydrogenase (Q8K2B3, Q9CQA3, Q9CZB0, Q9CXV1)
Aconitate hydratase, mitochondrial (Q99KI0)
Succinyl-CoA ligase (Q9WUM5, Q9Z2I8)
Oxoglutarate dehydrogenase complex (Q60597, Q9D2G2, O08749)
Isocitrate dehydrogenase, mitochondrial (Q9D6R2, Q91VA7, P70404)
Enzymes
|log2FC|=0
0<|log2FC|≤0.5
0.5<|log2FC|≤1
1<|log2FC|≤1.5
1.5<|log2FC|≤2
|log2FC|>2
Increase and decrease
Not measured

B

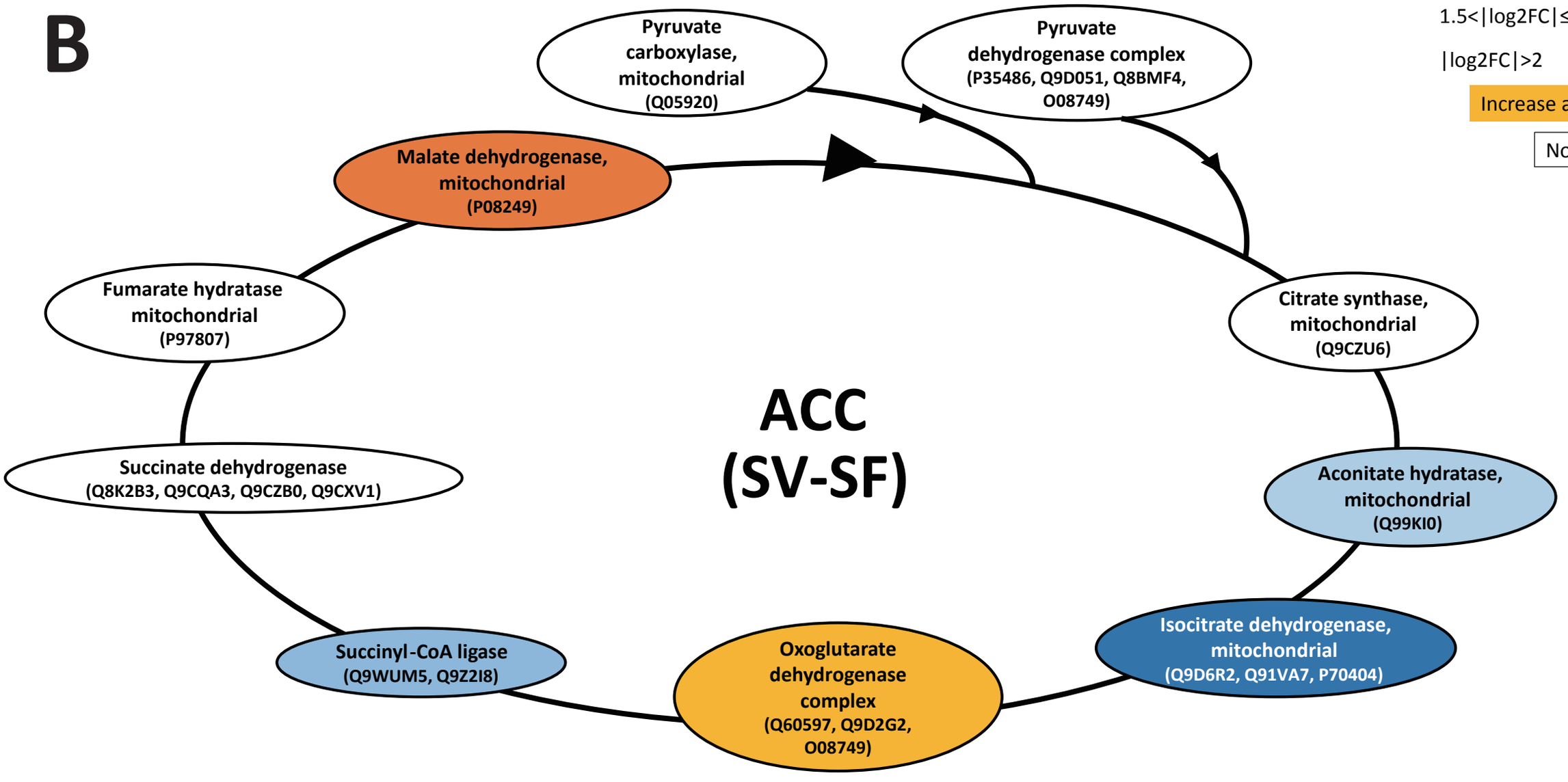

Pyruvate carboxylase, mitochondrial (Q05920)
Pyruvate dehydrogenase complex (P35486, Q9D051, Q8BMF4, O08749)
Malate dehydrogenase, mitochondrial (P08249)
Fumarate hydratase mitochondrial (P97807)
Citrate synthase, mitochondrial (Q9CZU6)
ACC (SV-SF)
Succinate dehydrogenase (Q8K2B3, Q9CQA3, Q9CZB0, Q9CXV1)
Aconitate hydratase, mitochondrial (Q99KI0)
Succinyl-CoA ligase (Q9WUM5, Q9Z2I8)
Oxoglutarate dehydrogenase complex (Q60597, Q9D2G2, O08749)
Isocitrate dehydrogenase, mitochondrial (Q9D6R2, Q91VA7, P70404)

Figure 3

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 Kao[1,2,†], Zhisong He[3,†], Oliver Hahn[3], Carsten T. Wotjak[4], Philipp Khaitovich[3,†], Christoph W. Turck[1,†*]

[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

[†] 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.

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 cm^3) 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 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 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,

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℃ 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℃ 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

read count of the resulting 14591 protein-coding genes for normalization (Supplementary Methods and Materials).

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

$|log2FC_{rescued}| < |log2FC_{PTSD}|$, 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-1ß (52, 53), IL-8 (54) and interferon-α and ß (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 *NFκB* work synergistically to transactivate downstream targets associated with inflammation (68). *NFκB* is the master transcriptional regulator of the immune response and is associated with neurogenesis (69). *NFκB* function can be manipulated through the activities of Toll-like-receptors (TLRs) (70,

71). 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 NFκB pathway in posttraumatic anxiety. In accordance with previous studies, our findings support an important role for *NFκB, 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-α, IL-1ß 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.

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.

Reference

1. Association AP (2013): *Diagnostic and statistical manual of mental health disorders: DSM-5*. Washington, DC: American Psychiatric Publishing.

2. Eckart C, Engler H, Riether C, Kolassa S, Elbert T, Kolassa IT (2009): No PTSD-related differences in diurnal cortisol profiles of genocide survivors. *Psychoneuroendocrinology*. 34:523-531.

3. Pitman RK, Orr SP (1990): Twenty-four hour urinary cortisol and catecholamine excretion in combat-related posttraumatic stress disorder. *Biol Psychiatry*. 27:245-247.

4. Cohen H, Kaplan Z, Koresh O, Matar MA, Geva AB, Zohar J (2011): Early post-stressor intervention with propranolol is ineffective in preventing posttraumatic stress responses in an animal model for PTSD. *Eur Neuropsychopharmacol*. 21:230-240.

5. Dębiec J, Bush DE, LeDoux JE (2011): Noradrenergic enhancement of reconsolidation in the amygdala impairs extinction of conditioned fear in rats--a possible mechanism for the persistence of traumatic memories in PTSD. *Depress Anxiety*. 28:186-193.

6. Murrough JW, Huang Y, Hu J, Henry S, Williams W, Gallezot JD, et al. (2011): Reduced amygdala serotonin transporter binding in posttraumatic stress disorder. *Biol Psychiatry*. 70:1033-1038.

7. Gressier F, Calati R, Balestri M, Marsano A, Alberti S, Antypa N, et al. (2013): The 5-HTTLPR polymorphism and posttraumatic stress disorder: a meta-analysis. *J Trauma Stress*. 26:645-653.

8. Segman RH, Cooper-Kazaz R, Macciardi F, Goltser T, Halfon Y, Dobroborski T, et al. (2002): Association between the dopamine transporter gene and posttraumatic stress disorder. *Mol Psychiatry*. 7:903-907.

9. Geracioti TD, Jefferson-Wilson L, Strawn JR, Baker DG, Dashevsky BA, Horn PS, et al. (2013): Effect of traumatic imagery on cerebrospinal fluid dopamine and serotonin metabolites in posttraumatic stress disorder. *J Psychiatr Res*. 47:995-998.

10. Amstadter AB, Nugent NR, Yang BZ, Miller A, Siburian R, Moorjani P, et al. (2011): Corticotrophin-releasing hormone type 1 receptor gene (CRHR1) variants predict posttraumatic stress disorder onset and course in pediatric injury patients. *Dis Markers*. 30:89-99.

11. Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, et al. (2008): Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *JAMA*. 299:1291-1305.

12. Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, et al. (2011): Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature*. 470:492-497.

13. Siegmund A, Wotjak CT (2006): Toward an animal model of posttraumatic stress disorder. *Ann N Y Acad Sci*. 1071:324-334.

14. Bremner JD (2002): Neuroimaging studies in post-traumatic stress disorder. *Curr Psychiatry Rep*. 4:254-263.

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

extinction paradigm in women with childhood sexual-abuse-related post-traumatic stress disorder. *Psychol Med*. 35:791-806.

16. Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, et al. (2002): Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. *Nat Neurosci*. 5:1242-1247.

17. Siegmund A, Wotjak CT (2007): A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. *J Psychiatr Res*. 41:848-860.

18. Golub Y, Mauch CP, Dahlhoff M, Wotjak CT (2009): Consequences of extinction training on associative and non-associative fear in a mouse model of Posttraumatic Stress Disorder (PTSD). *Behav Brain Res*. 205:544-549.

19. Pamplona FA, Henes K, Micale V, Mauch CP, Takahashi RN, Wotjak CT (2011): Prolonged fear incubation leads to generalized avoidance behavior in mice. *J Psychiatr Res*. 45:354-360.

20. Golub Y, Kaltwasser SF, Mauch CP, Herrmann L, Schmidt U, Holsboer F, et al. (2011): Reduced hippocampus volume in the mouse model of Posttraumatic Stress Disorder. *J Psychiatr Res*. 45:650-659.

21. Dahlhoff M, Siegmund A, Golub Y, Wolf E, Holsboer F, Wotjak CT (2010): AKT/GSK-3beta/beta-catenin signalling within hippocampus and amygdala reflects genetically determined differences in posttraumatic stress disorder like symptoms. *Neuroscience*. 169:1216-1226.

22. Paxinos G FK (2001): *The mouse brain in stereotaxic coordinates*. San Diego, San Francisco, New York, Boston, London, Sydney, Tokyo: Academic Press.

23. Huang da W, Sherman BT, Lempicki RA (2009): Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*. 4:44-57.

24. Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, et al. (2007): DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic acids research*. 35:W169-175.

25. Martenyi F, Soldatenkova V (2006): Fluoxetine in the acute treatment and relapse prevention of combat-related post-traumatic stress disorder: Analysis of the veteran group of a placebo-controlled, randomized clinical trial. *Eur Neuropsychopharmacol*. 16:340-349.

26. Antoniadis EA, McDonald RJ (2006): Fornix, medial prefrontal cortex, nucleus accumbens, and mediodorsal thalamic nucleus: roles in a fear-based context discrimination task. *Neurobiol Learn Mem*. 85:71-85.

27. Belzung C, Turiault M, Griebel G (2014): Optogenetics to study the circuits of fear- and depression-like behaviors: a critical analysis. *Pharmacol Biochem Behav*. 122:144-157.

28. Maroun M (2013): Medial prefrontal cortex: multiple roles in fear and extinction. *Neuroscientist*. 19:370-383.

29. Zelikowsky M, Hersman S, Chawla MK, Barnes CA, Fanselow MS (2014): Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. *J Neurosci*. 34:8462-8466.

30. Zhang CL, Houbaert X, Lepleux M, Deshors M, Normand E, Gambino F, et al. (2014): The hippocampo-amygdala control of contextual fear expression is affected in a model of intellectual disability. *Brain Struct Funct*.

31. Chaudhury D, Walsh JJ, Friedman AK, Juarez B, Ku SM, Koo JW, et al. (2013): Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature*. 493:532-536.

32. Brown VM, Morey RA (2012): Neural systems for cognitive and emotional processing in posttraumatic stress disorder. *Front Psychol*. 3:449.

33. Khani A, Kermani M, Hesam S, Haghparast A, Argandoña EG, Rainer G (2014): Activation of cannabinoid system in anterior cingulate cortex and orbitofrontal cortex modulates cost-benefit decision making. *Psychopharmacology (Berl)*.

34. Economides M, Guitart-Masip M, Kurth-Nelson Z, Dolan RJ (2014): Anterior cingulate cortex instigates adaptive switches in choice by integrating immediate and delayed components of value in ventromedial prefrontal cortex. *J Neurosci*. 34:3340-3349.

35. Decety J, Jackson PL (2004): The functional architecture of human empathy. *Behav Cogn Neurosci Rev*. 3:71-100.

36. Bush G, Luu P, Posner MI (2000): Cognitive and emotional influences in anterior cingulate cortex. *Trends Cogn Sci*. 4:215-222.

37. Hoerst M, Weber-Fahr W, Tunc-Skarka N, Ruf M, Bohus M, Schmahl C, et al. (2010): Correlation of glutamate levels in the anterior cingulate cortex with self-reported impulsivity in patients with borderline personality disorder and healthy controls. *Arch Gen Psychiatry*. 67:946-954.

38. O'Doherty DC, Chitty KM, Saddiqui S, Bennett MR, Lagopoulos J (2015): A systematic review and meta-analysis of magnetic resonance imaging measurement of structural volumes in posttraumatic stress disorder. *Psychiatry Res*.

39. Felmingham K, Kemp A, Williams L, Das P, Hughes G, Peduto A, et al. (2007): Changes in anterior cingulate and amygdala after cognitive behavior therapy of posttraumatic stress disorder. *Psychol Sci*. 18:127-129.

40. Bryant RA, Felmingham K, Kemp A, Das P, Hughes G, Peduto A, et al. (2008): Amygdala and ventral anterior cingulate activation predicts treatment response to cognitive behaviour therapy for post-traumatic stress disorder. *Psychol Med*. 38:555-561.

41. Schuff N, Neylan TC, Fox-Bosetti S, Lenoci M, Samuelson KW, Studholme C, et al. (2008): Abnormal N-acetylaspartate in hippocampus and anterior cingulate in posttraumatic stress disorder. *Psychiatry Res*. 162:147-157.

42. Gola H, Engler H, Sommershof A, Adenauer H, Kolassa S, Schedlowski M, et al. (2013): Posttraumatic stress disorder is associated with an enhanced spontaneous production of pro-inflammatory cytokines by peripheral blood mononuclear cells. *BMC Psychiatry*. 13:40.

43. Pace TW, Heim CM (2011): A short review on the psychoneuroimmunology of posttraumatic stress disorder: from risk factors to medical comorbidities. *Brain Behav Immun*. 25:6-13.

44. Lindqvist D, Wolkowitz OM, Mellon S, Yehuda R, Flory JD, Henn-Haase C, et al. (2014): Proinflammatory milieu in combat-related PTSD is independent of depression and early life stress. *Brain Behav Immun*. 42:81-88.

45. Goshen I, Kreisel T, Ben-Menachem-Zidon O, Licht T, Weidenfeld J, Ben-Hur T, et al. (2008): Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. *Mol Psychiatry*. 13:717-728.

46. Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, et al. (2010): Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. *Brain Behav Immun*. 24:1058-1068.

47. Marques RE, Guabiraba R, Russo RC, Teixeira MM (2013): Targeting CCL5 in inflammation. *Expert Opin Ther Targets*. 17:1439-1460.

48. Maghazachi AA, Al-Aoukaty A, Schall TJ (1996): CC chemokines induce the generation of killer cells from CD56+ cells. *Eur J Immunol*. 26:315-319.

49. Stellwagen D, Malenka RC (2006): Synaptic scaling mediated by glial TNF-alpha. *Nature*. 440:1054-1059.

50. Li AJ, Katafuchi T, Oda S, Hori T, Oomura Y (1997): Interleukin-6 inhibits long-term potentiation in rat hippocampal slices. *Brain Res*. 748:30-38.

51. Tancredi V, D'Antuono M, Cafè C, Giovedì S, Buè MC, D'Arcangelo G, et al. (2000): The inhibitory effects of interleukin-6 on synaptic plasticity in the rat hippocampus are associated with an inhibition of mitogen-activated protein kinase ERK. *J Neurochem*. 75:634-643.

52. Cunningham AJ, Murray CA, O'Neill LA, Lynch MA, O'Connor JJ (1996): Interleukin-1 beta (IL-1 beta) and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. *Neurosci Lett*. 203:17-20.

53. Katsuki H, Nakai S, Hirai Y, Akaji K, Kiso Y, Satoh M (1990): Interleukin-1 beta inhibits long-term potentiation in the CA3 region of mouse hippocampal slices. *Eur J Pharmacol*. 181:323-326.

54. Xiong H, Boyle J, Winkelbauer M, Gorantla S, Zheng J, Ghorpade A, et al. (2003): Inhibition of long-term potentiation by interleukin-8: implications for human immunodeficiency virus-1-associated dementia. *J Neurosci Res*. 71:600-607.

55. D'Arcangelo G, Grassi F, Ragozzino D, Santoni A, Tancredi V, Eusebi F (1991): Interferon inhibits synaptic potentiation in rat hippocampus. *Brain Res*. 564:245-248.

56. Mendoza-Fernández V, Andrew RD, Barajas-López C (2000): Interferon-alpha inhibits long-term potentiation and unmasks a long-term depression in the rat hippocampus. *Brain Res*. 885:14-24.

57. Balschun D, Wetzel W, Del Rey A, Pitossi F, Schneider H, Zuschratter W, et al. (2004): Interleukin-6: a cytokine to forget. *FASEB J*. 18:1788-1790.

58. Bie B, Brown DL, Naguib M (2011): Increased synaptic GluR1 subunits in the anterior cingulate cortex of rats with peripheral inflammation. *Eur J Pharmacol*. 653:26-31.

59. Bryant RA, Felmingham KL, Kemp AH, Barton M, Peduto AS, Rennie C, et al. (2005): Neural networks of information processing in posttraumatic stress disorder: a functional magnetic resonance imaging study. *Biol Psychiatry*. 58:111-118.

60. Shin LM, Bush G, Milad MR, Lasko NB, Brohawn KH, Hughes KC, et al. (2011): Exaggerated activation of dorsal anterior cingulate cortex during cognitive interference: a monozygotic twin study of posttraumatic stress disorder. *Am J Psychiatry*. 168:979-985.

61. Torres-Platas SG, Cruceanu C, Chen GG, Turecki G, Mechawar N (2014): Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. *Brain Behav Immun*. 42:50-59.

62. Setiawan E, Wilson AA, Mizrahi R, Rusjan PM, Miler L, Rajkowska G, et al. (2015): Role of translocator protein density, a marker of neuroinflammation, in the brain during major depressive episodes. *JAMA Psychiatry*. 72:268-275.

63. Carotta S, Wu L, Nutt SL (2010): Surprising new roles for PU.1 in the adaptive immune response. *Immunol Rev*. 238:63-75.

64. Gjoneska E, Pfenning AR, Mathys H, Quon G, Kundaje A, Tsai LH, et al. (2015): Conserved epigenomic signals in mice and humans reveal immune basis of Alzheimer's disease. *Nature*. 518:365-369.

65. Daskalakis NP, Cohen H, Cai G, Buxbaum JD, Yehuda R (2014): Expression profiling associates blood and brain glucocorticoid receptor signaling with trauma-related individual differences in both sexes. *Proc Natl Acad Sci U S A*. 111:13529-13534.

66. Kumar P, Garg R, Bolden G, Pandey KN (2010): Interactive roles of Ets-1, Sp1, and acetylated histones in the retinoic acid-dependent activation of guanylyl cyclase/atrial natriuretic peptide receptor-A gene transcription. *J Biol Chem*. 285:37521-37530.

67. Garrett-Sinha LA (2013): Review of Ets1 structure, function, and roles in immunity. *Cell Mol Life Sci*. 70:3375-3390.

68. Katoh M (2009): Integrative genomic analyses of ZEB2: Transcriptional regulation of ZEB2 based on SMADs, ETS1, HIF1alpha, POU/OCT, and NF-kappaB. *Int J Oncol*. 34:1737-1742.

69. Zhang Y, Shen GL, Shangguan LJ, Yu Y, He ML (2015): Involvement of NFκB signaling in mediating the effects of GRK5 on neural stem cells. *Brain Res*. 1608:31-39.

70. Krieg AM (2006): Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov*. 5:471-484.

71. Krieg AM, Vollmer J (2007): Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. *Immunol Rev*. 220:251-269.

72. Zimmerman G, Shaltiel G, Barbash S, Cohen J, Gasho CJ, Shenhar-Tsarfaty S, et al. (2012): Post-traumatic anxiety associates with failure of the innate immune receptor TLR9 to evade the pro-inflammatory NFκB pathway. *Transl Psychiatry*. 2:e78.

73. Terrando N, Monaco C, Ma D, Foxwell BM, Feldmann M, Maze M (2010): Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline. *Proc Natl Acad Sci U S A*. 107:20518-20522.

74. O'Donovan A, Chao LL, Paulson J, Samuelson KW, Shigenaga JK, Grunfeld C, et al. (2015): Altered inflammatory activity associated with reduced hippocampal volume and more severe posttraumatic stress symptoms in Gulf War veterans. *Psychoneuroendocrinology*. 51:557-566.

75. Pezze MA, Feldon J (2004): Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol*. 74:301-320.

76. Michels L, Schulte-Vels T, Schick M, O'Gorman RL, Zeffiro T, Hasler G, et al. (2014): Prefrontal GABA and glutathione imbalance in posttraumatic stress disorder: preliminary findings. *Psychiatry Res*. 224:288-295.

77. Gao J, Wang H, Liu Y, Li YY, Chen C, Liu LM, et al. (2014): Glutamate and GABA imbalance promotes neuronal apoptosis in hippocampus after stress. *Med Sci Monit*. 20:499-512.

78. Pennington DL, Abé C, Batki SL, Meyerhoff DJ (2014): A preliminary examination of cortical neurotransmitter levels associated with heavy drinking in posttraumatic stress disorder. *Psychiatry Res*. 224:281-287.

79. Voineagu I, Wang X, Johnston P, Lowe JK, Tian Y, Horvath S, et al. (2011): Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature*. 474:380-384.

80. Ying W, Chen Y, Alano CC, Swanson RA (2002): Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *J Cereb Blood Flow Metab*. 22:774-779.

81. Szabó C, Dawson VL (1998): Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci*. 19:287-298.

82. Fineberg NA, Potenza MN, Chamberlain SR, Berlin HA, Menzies L, Bechara A, et al. (2010): Probing compulsive and impulsive behaviors, from animal models to endophenotypes: a narrative review. *Neuropsychopharmacology*. 35:591-604.

83. Miles SR, Menefee DS, Wanner J, Teten Tharp A, Kent TA (2015): The Relationship Between Emotion Dysregulation and Impulsive Aggression in Veterans With Posttraumatic Stress Disorder Symptoms. *J Interpers Violence*.

84. Ravindran LN, Stein MB (2009): Pharmacotherapy of PTSD: premises, principles, and priorities. *Brain Res*. 1293:24-39.

85. Guest PC, Knowles MR, Molon-Noblot S, Salim K, Smith D, Murray F, et al. (2004): Mechanisms of action of the antidepressants fluoxetine and the substance P antagonist L-

000760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res*. 1002:1-10.

86. Wang YX, Zhang XR, Zhang ZJ, Li L, Xi GJ, Wu D, et al. (2014): Protein kinase Mζ is involved in the modulatory effect of fluoxetine on hippocampal neurogenesis in vitro. *Int J Neuropsychopharmacol*. 17:1429-1441.

87. Kobayashi K, Haneda E, Higuchi M, Suhara T, Suzuki H (2012): Chronic fluoxetine selectively upregulates dopamine D_1-like receptors in the hippocampus. *Neuropsychopharmacology*. 37:1500-1508.

88. Herrmann L, Ionescu IA, Henes K, Golub Y, Wang NX, Buell DR, et al. (2012): Long-lasting hippocampal synaptic protein loss in a mouse model of posttraumatic stress disorder. *PLoS One*. 7:e42603.

89. Ha E, Jung KH, Choe BK, Bae JH, Shin DH, Yim SV, et al. (2006): Fluoxetine increases the nitric oxide production via nuclear factor kappa B-mediated pathway in BV2 murine microglial cells. *Neurosci Lett*. 397:185-189.

90. Hashioka S, Klegeris A, Monji A, Kato T, Sawada M, McGeer PL, et al. (2007): Antidepressants inhibit interferon-gamma-induced microglial production of IL-6 and nitric oxide. *Exp Neurol*. 206:33-42.

91. Liu D, Wang Z, Liu S, Wang F, Zhao S, Hao A (2011): Anti-inflammatory effects of fluoxetine in lipopolysaccharide(LPS)-stimulated microglial cells. *Neuropharmacology*. 61:592-599.

92. Obuchowicz E, Bielecka AM, Paul-Samojedny M, Pudełko A, Kowalski J (2014): Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. *Pharmacol Rep*. 66:34-43.

93. Levkovitz Y, Fenchel D, Kaplan Z, Zohar J, Cohen H (2015): Early post-stressor intervention with minocycline, a second-generation tetracycline, attenuates post-traumatic stress response in an animal model of PTSD. *Eur Neuropsychopharmacol*. 25:124-132.

94. Muhie S, Gautam A, Meyerhoff J, Chakraborty N, Hammamieh R, Jett M (2015): Brain transcriptome profiles in mouse model simulating features of post-traumatic stress disorder. Molecular Brain.

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.

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.

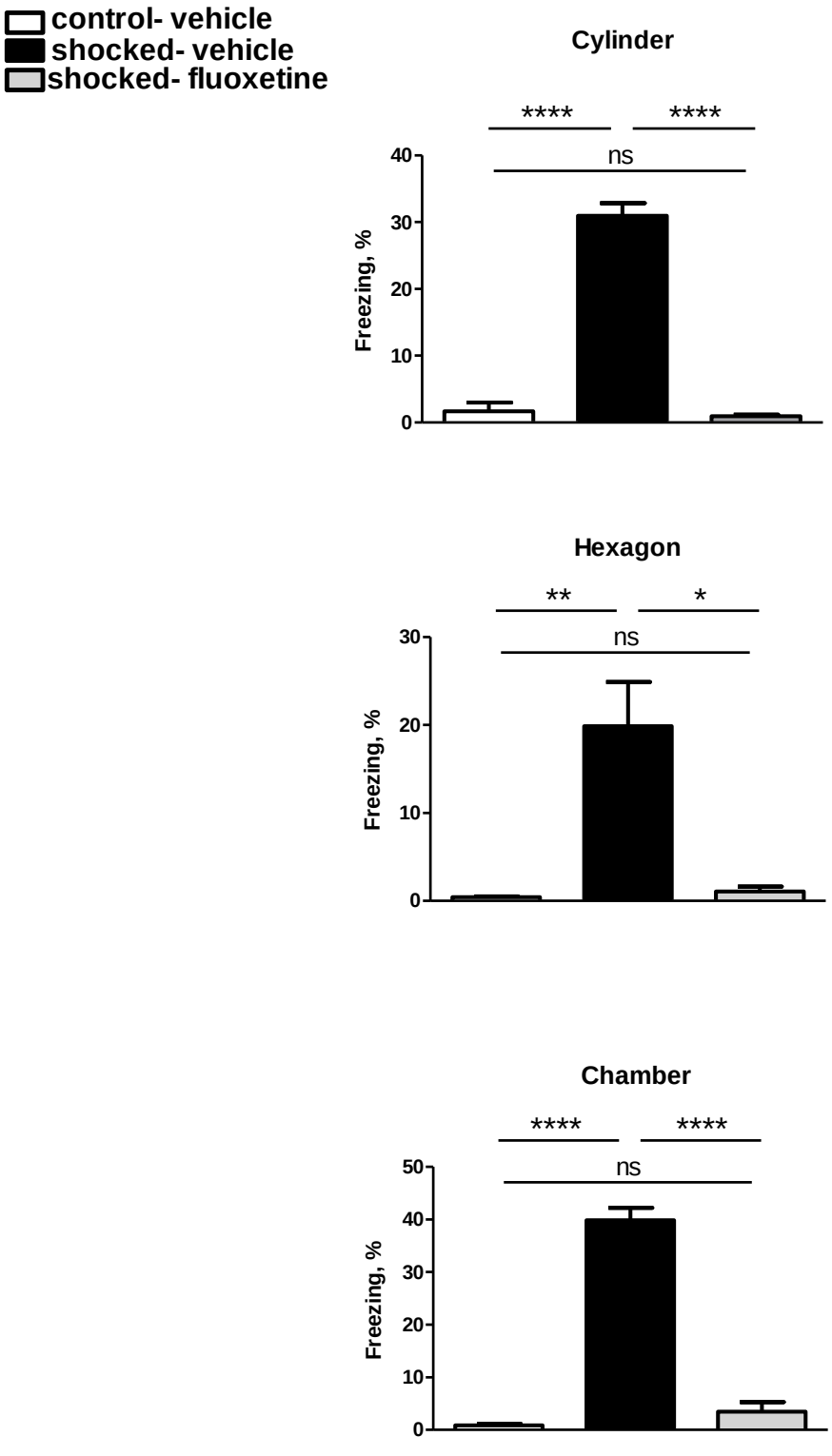

control- vehicle
shocked- vehicle
shocked- fluoxetine
Cylinder
****
****
ns
Freezing, %
40
30
20
10
0
Hexagon
**
*
ns
Freezing, %
30
20
10
0
Chamber
****
****
ns
Freezing, %
50
40
30
20
10
0

Figure 1

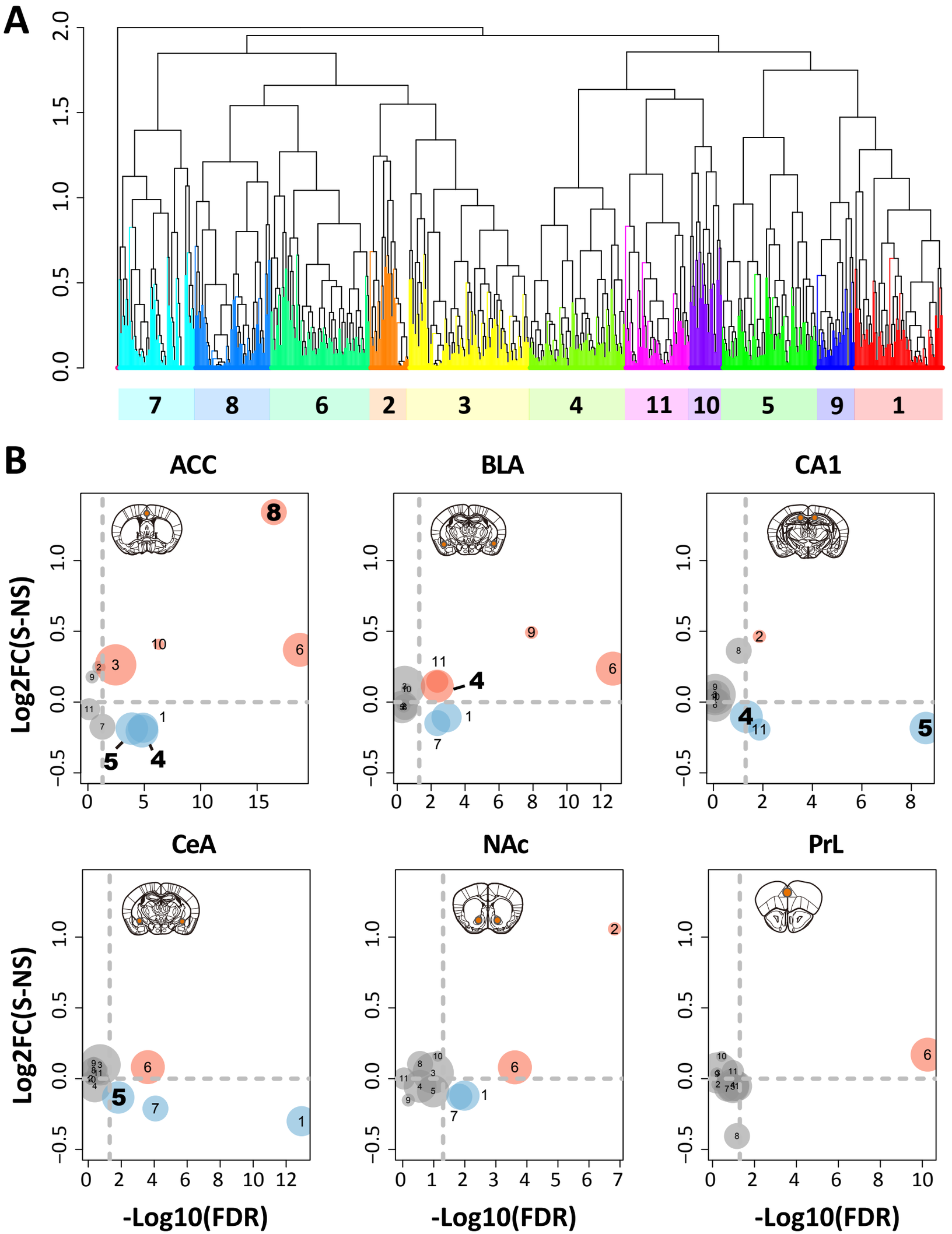

A
2.0
1.5
1.0
0.5
0.0
7
8
6
2
3
4
11
10
5
9
1
B
ACC
BLA
CA1
CeA
NAc
PrL
Log2FC(S-NS)
-Log10(FDR)

Figure 2

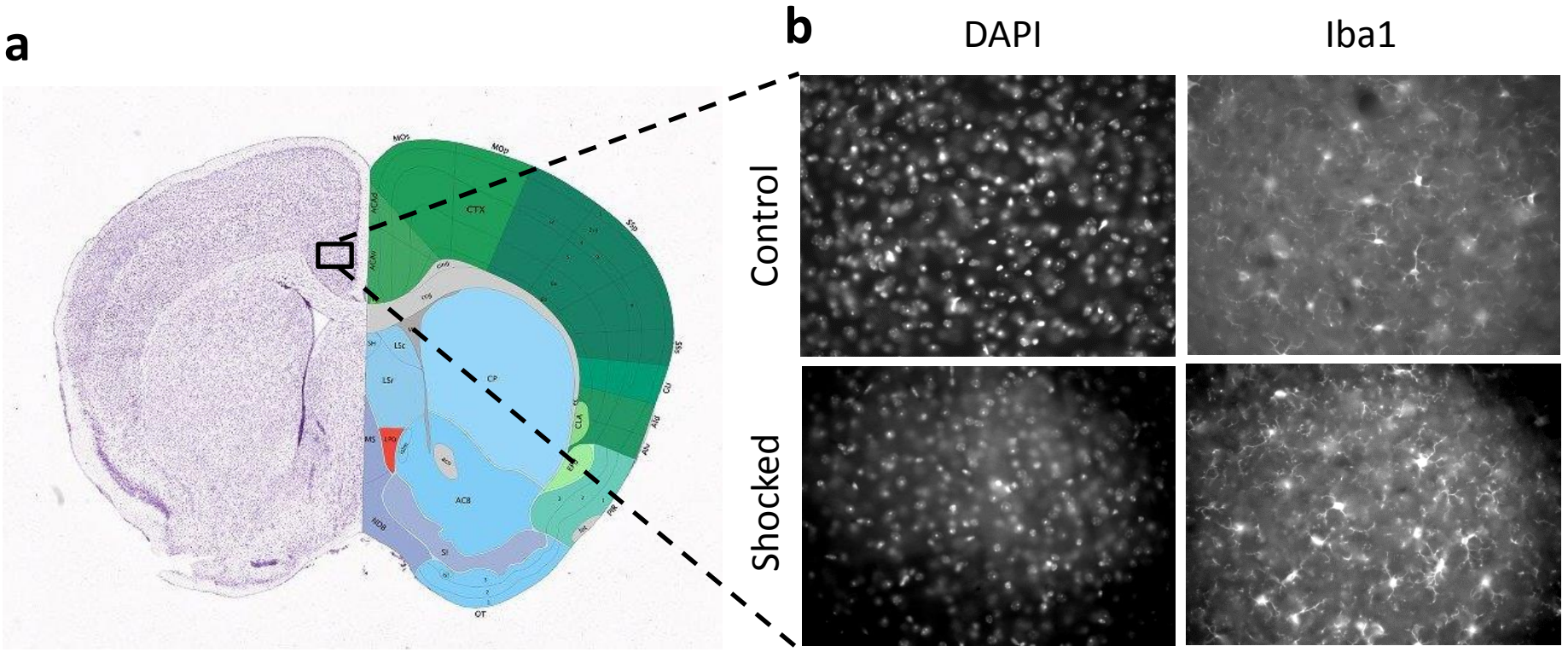

a
b
DAPI
Iba1
Control
Shocked
MOs
MOp
CTX
SSp
ACAd
ACAv
LSc
LSr
CP
CLA
AId
AIv
MS
ACB
RDB
SI
PIR
OT
c
Mean integrated intensity
50000
40000
30000
20000
10000
0
Control
Shocked
Mean microglia numbers
10
8
6
4
2
0
Control
Shocked

Figure 3

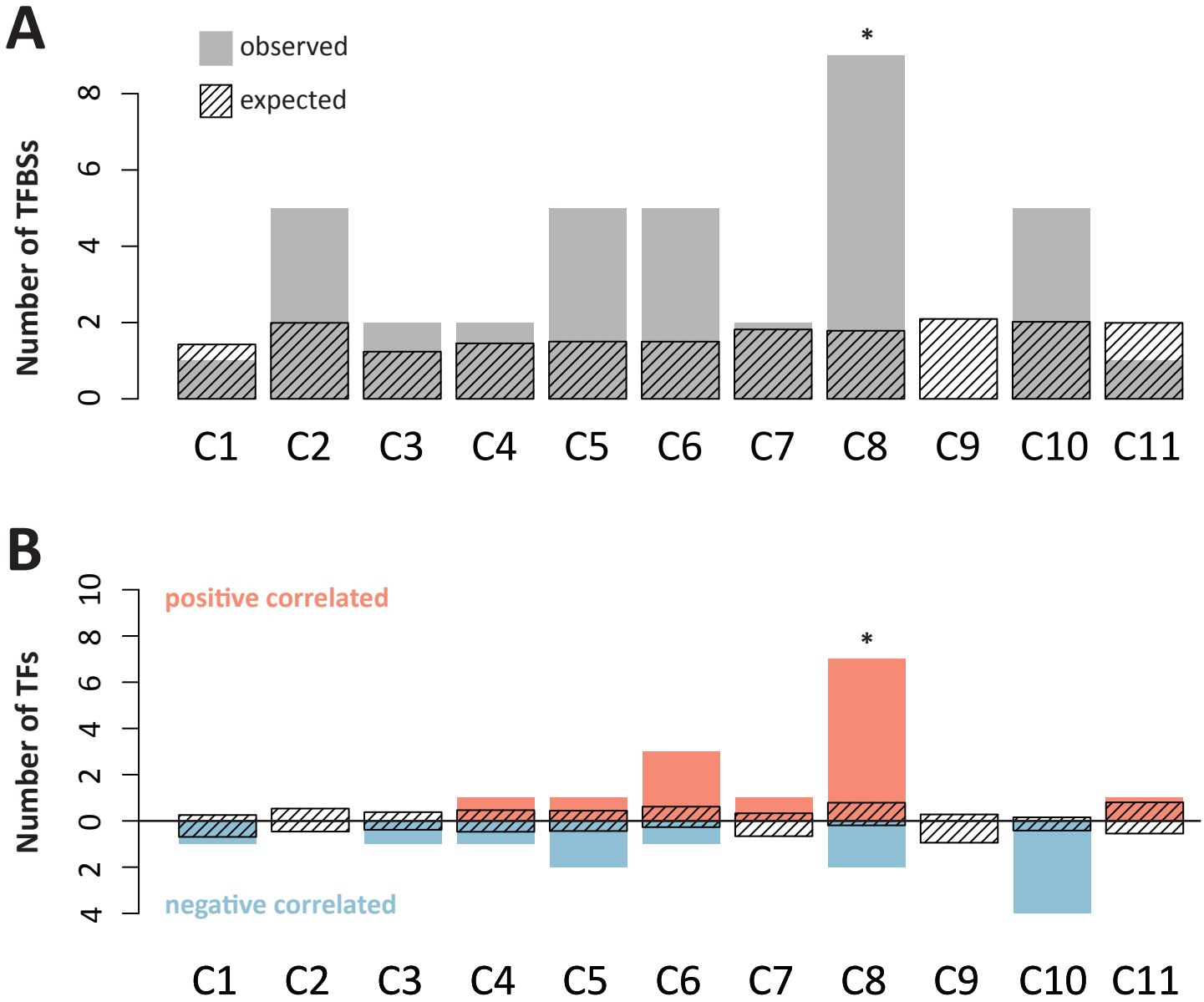

A
observed
expected
Number of TFBSs
0
2
4
6
8
*
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
B
positive correlated
negative correlated
Number of TFs
4
2
0
2
4
6
8
10
*
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11

Figure 4

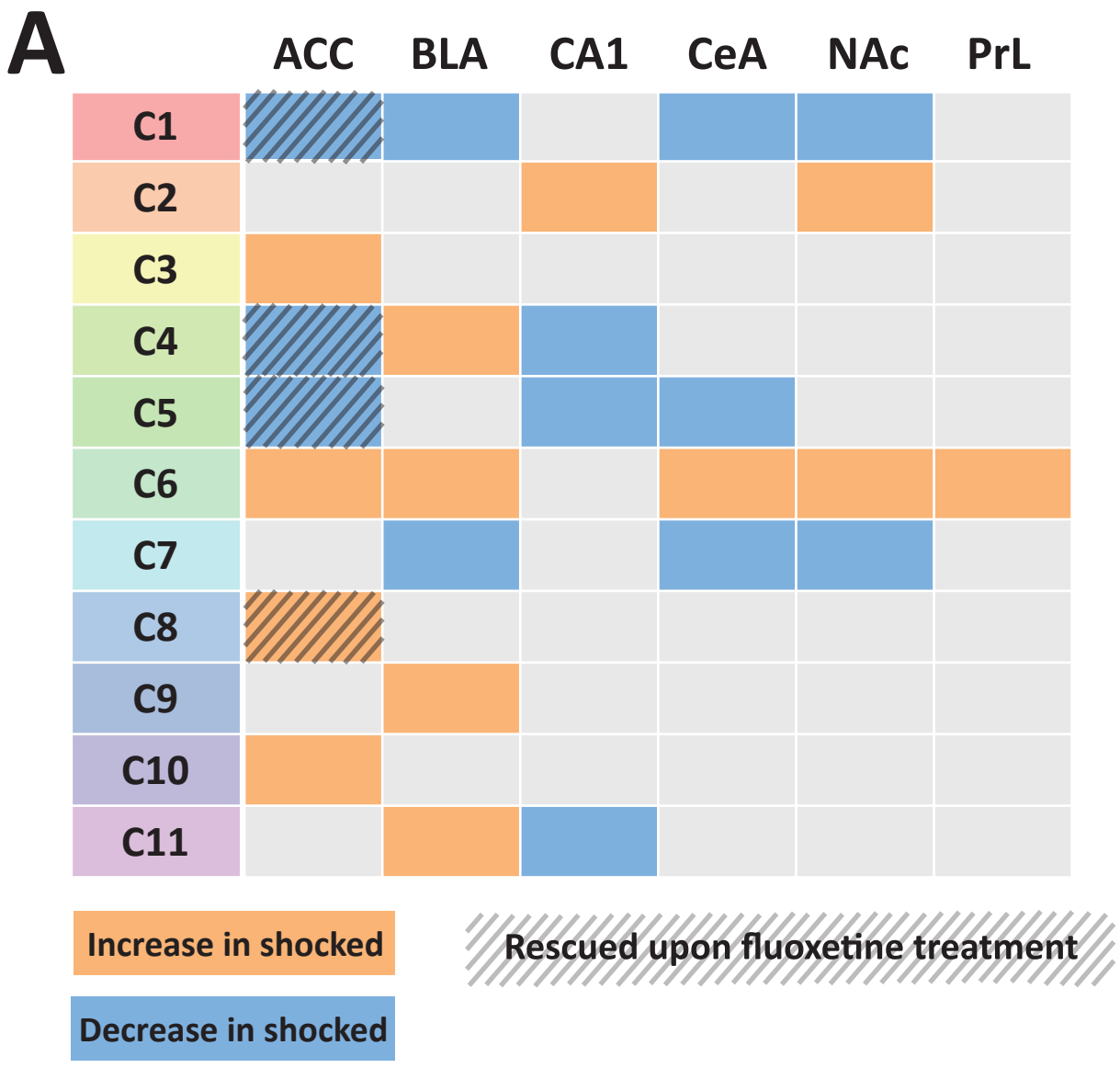

A
ACC
BLA
CA1
CeA
NAc
PrL
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
Increase in shocked
Decrease in shocked
Rescued upon fluoxetine treatment
B
ACC
NS
S
SF
Expression Level
(z-transformed)
C1
C4
C5
C8

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

EJN European Journal of Neuroscience

FENS Federation of European Neuroscience Societies

European Journal of Neuroscience, Vol. 41, pp. 1139–1148, 2015 doi:10.1111/ejn.12860

BEHAVIORAL NEUROSCIENCE

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. Wotjak[4] and E. Anderzhanova[4]

[1]Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany
[2]Graduate School of Systemic Neurosciences, Ludwig-Maximilians-University Munich, Planegg-Martinsried, Germany
[3]Department of Clinical Research, Max Planck Institute of Psychiatry, Munich, Germany
[4]Department 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-direct 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 α_2-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; Debiec *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

Correspondence: Dr E. Anderzhanova, as above.
E-mail: anderzhanova@psych.mpg.de

Received 30 September 2014, accepted 26 January 2015

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 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 ($23 \times 16.5 \times 14$ cm^3) 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).

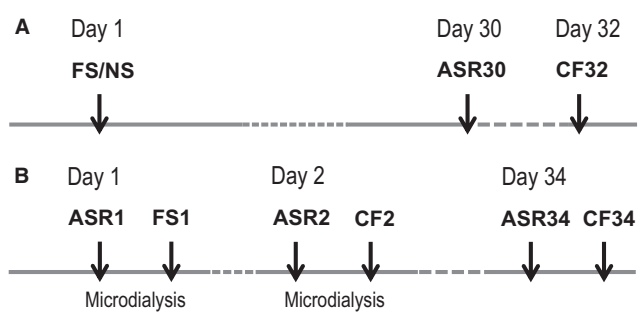


FIG. 1. (A) Experimental outline for the PTSD model validation. PTSD-like endophenotype was evaluated by acoustic startle response (ASR) on day 30 (ASR30) and contextual fear (CF) response on day 32 (CF32) after foot shock (FS). Control mice remained non-shocked (NS). (B) Outline for microdialysis experiment with PTSD model. On experimental day 1, mice were subjected to acoustic startle stimulation (ASR1), followed by fear conditioning protocol (FS1). On experimental day 2, mice were subjected to recurrent ASR2 and exposed to FS chamber for CF recall (CF2). The strength of fear conditioning was further examined 34 days after the FS (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 ($16 \times 16 \times 32$ 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 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 pseudorandom 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 −3.0 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 ($16 \times 16 \times 32$ cm^3) 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; $CaCl_2$, 1.2; $MgCl_2$, 1.0; Na_2HPO_4, 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/Coulochem 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_3), applying the following equation: $x_i(\%) = 200 \cdot (x_i^2)/(x_i^2 + x_3^2)$. 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) $F_{1,3} = 16.81$, $P = 0.0003$; 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 NE 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

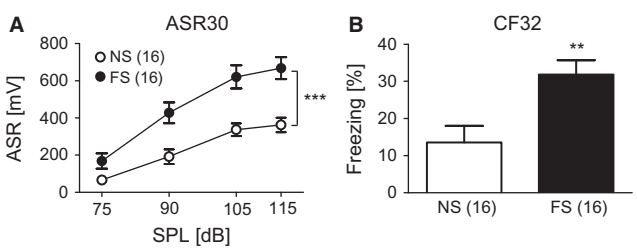


FIG. 2. PTSD mouse model validation. Foot shock (FS) resulted in facilitation of the acoustic startle response (ASR) (A) and retention of contextual fear (CF) memory (B) measured on days 30 and 32 in shocked (FS, closed circles) and non-shocked (NS, open circles) mice. $^{**}P < 0.01$; $^{***}P < 0.001$. 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.

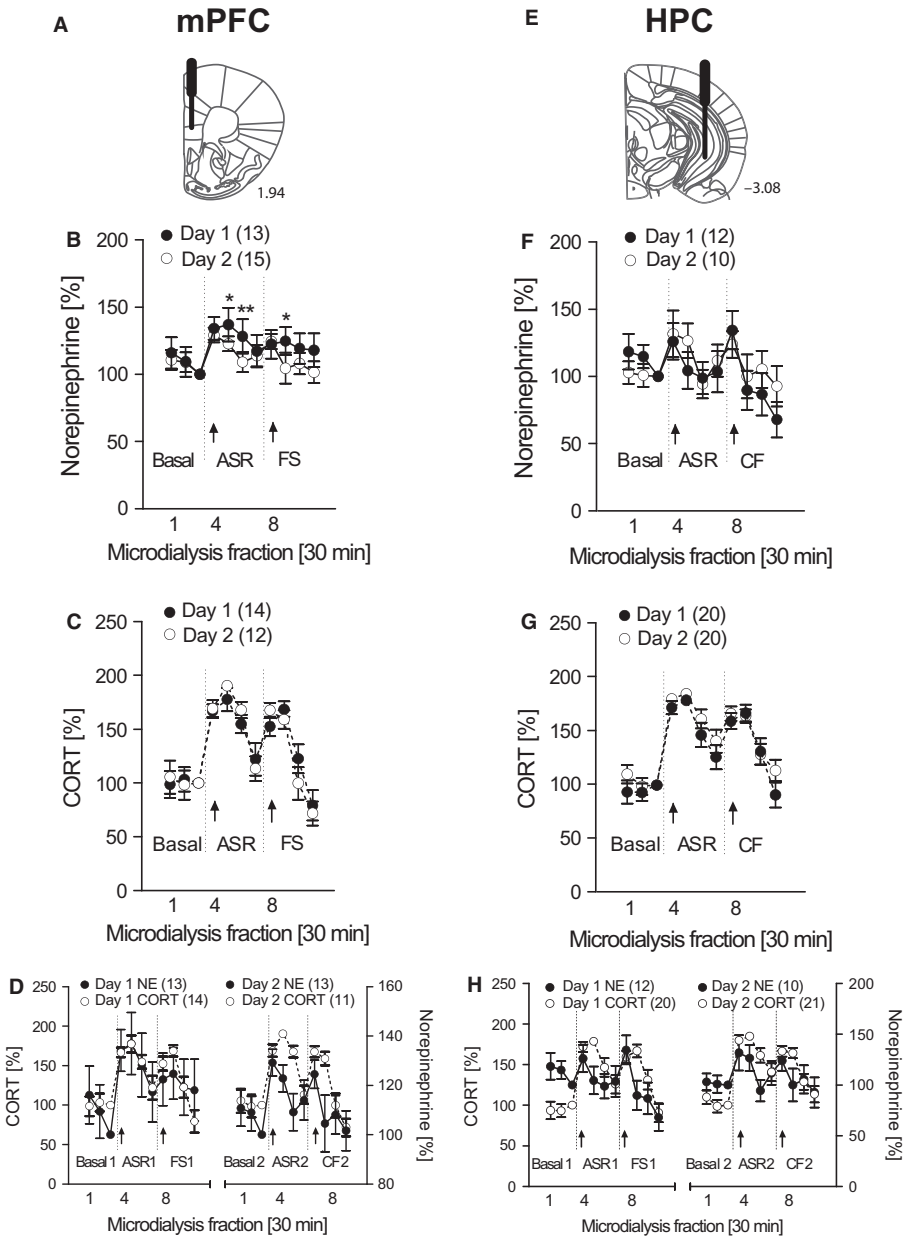


FIG. 3. Microdialysis data. The schematic diagrams show the targeted areas for microdialysis probe implantation in the medial prefrontal cortex (mPFC) (A) and the hippocampus (HPC) (E). The computer-based atlas by Paxinos & Franklin (2001) was used to mark probe locations; numbers refer to distances 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 (%) and CORT (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, $t_8 = 0.6761$, $P = 0.5180$; 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: $t_8 = 2.562$, $P = 0.0335$; Fig. 5A3) and on day 34 (Student's *t*-test: $t_8 = 2.366$, $P = 0.0455$; 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: $t_8 = 2.566$, $P = 0.0333$; Fig. 5B3) and a tendency to express higher CF on day 34 (Student's *t*-test: $t_8 = 1.997$, $P = 0.081$) 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-Korcsok *et al.*, 2003; Jedema *et al.*, 2008). Stress could induce a selective increase in α2-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 α2-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.

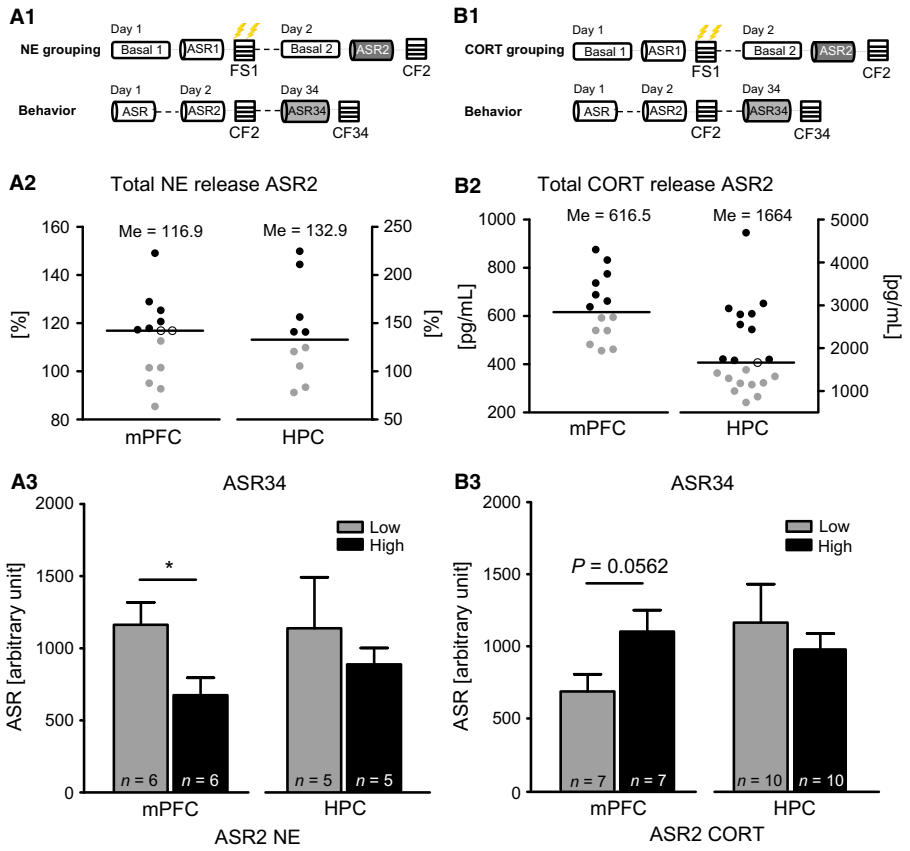


FIG. 4. Predictability of low and high norepinephrine (NE) and corticosterone (CORT) levels for arousal. (A) NE release in the medial prefrontal cortex (mPFC), but not in the hippocampus (HPC), coinciding with acoustic startle response (ASR)2 (A1) predicts ASR34 response (A3). Gray and black bars correspond 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;

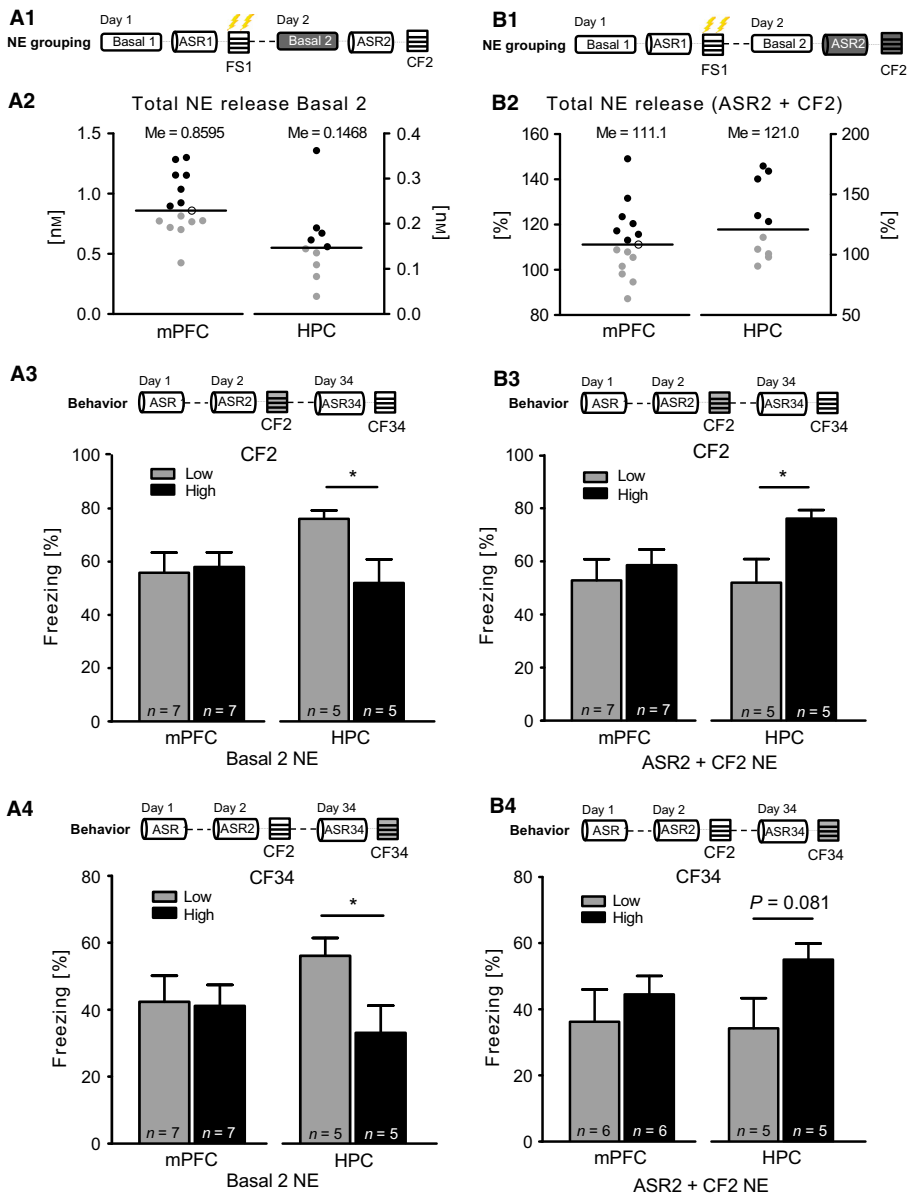


FIG. 5. Predictability of low and high norepinephrine (NE) levels for contextual fear (CF). (A) NE release in the hippocampus (HPC) during basal condition on day 2 (A1) corresponds to CF2 (A3) and CF34 (A4). (B) NE release in the HPC, but not in the medial prefrontal cortex (mPFC), coinciding with ASR2 + CF2 (B1) corresponding to CF2 (B3). The number of mice was reduced in the CF34 test due to an occasional mouse death. For details of grouping, see Fig. 4. Numbers inside the bars correspond to the sample size. $^*P < 0.05$. ASR, acoustic startle response; CF, contextual fear.

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 γ-aminobutyric acid (GABA)-mediated], both increasing and decreasing its efficiency that depends on the receptors involved (Pralong *et al.*, 2002). The innate difference in β-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. 6B), suggesting that intermediate NE levels promoted fear memory consolidation. Without considering the specifics of the memorised 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.

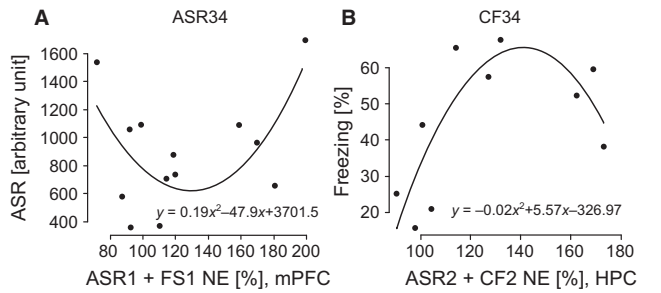


FIG. 6. U-shape relationship between norepinephrine (NE) and behavior. (A) NE release in the medial prefrontal cortex (mPFC) upon stressors' presentation on day 1 [acoustic startle response (ASR)1 + foot shock (FS)1 NE] showed a tendency to correlation with ASR on day 34 (ASR34) in a U-shape manner (adjusted $R^2 = 0.3133$, $P = 0.061$). (B) NE release in the hippocampus (HPC) upon stress presentations on day 2 [ASR2 + conditioned fear (CF)2 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.

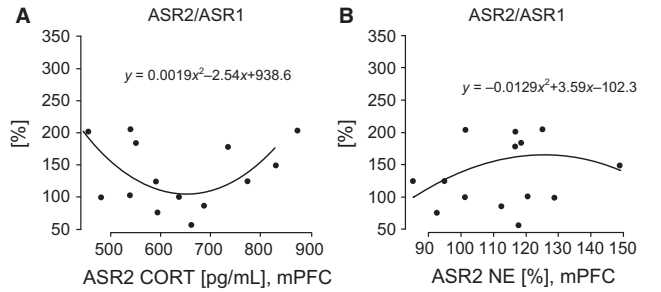


FIG. 7. U-shape relationship between corticosterone (CORT) and behavior. (A) In the medial prefrontal cortex (mPFC), free CORT accumulation during acoustic startle response (ASR)2 showed a U-shape relationship with FS-potentiated ASR (ASR2/ASR1, %; adjusted $R^2 = 0.344$, $P = 0.09$). (B) Notably, no 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 naïve (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 (Karst *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 (Croft *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 & Orchinik, 2002). CBG levels, like CORT levels, are not static and were shown to decrease in response to stress (Fleshner *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; Hu *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-psychophysiological 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 Genewsky, MSc, and Judith Reichel, MSc, for their invaluable assistance with ASR experiments.

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

American Psychiatric Association. (2000) *American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR*, 4th Edn. text rev., Author, Washington, DC.

Anderzhanova, E.A., Bächli, H., Buneeva, O.A., Narkevich, V.B., Medvedev, A.E., Thoeringer, C.K., Wotjak, C.T. & Kudrin, V.S. (2013) Strain differences in profiles of dopaminergic neurotransmission in the prefrontal cortex of the BALB/C vs. C57Bl/6 mice: consequences of stress and afobazole. *Eur. J. Pharmacol.*, **708**, 95–104.

Arnsten, A.F., Raskind, M.A., Taylor, F.B. & Connor, D.F. (2015) The effects of stress exposure on prefrontal cortex: translating basic research into successful treatments for post-traumatic stress disorder. *Ann. NY Acad. Sci.*, **1**, 89–99.

Aston-Jones, G. & Cohen, J.D. (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annu. Rev. Neurosci.*, **28**, 403–450.

Baker, D.G., West, S.A., Nicholson, W.E., Ekhator, N.N., Kasckow, J.W., Hill, K.K., Bruce, A.B., Orth, D.N. & Geracioti, T.D. Jr. (1999) Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am. J. Psychiat.*, **156**, 585–588.

Barsegyan, A., McGaugh, J.L. & Roozendaal, B. (2014) Noradrenergic activation of the basolateral amygdala modulates the consolidation of object-in-context recognition memory. *Front. Behav. Neurosci.*, **8**, 160.

Bondi, C.O., Jett, J.D. & Morilak, D.A. (2010) Beneficial effects of desipramine on cognitive function of chronically stressed rats are mediated by alpha1-adrenergic receptors in medial prefrontal cortex. *Prog. Neuropsychoph.*, **34**, 913–923.

Bremner, J.D., Licinio, J., Darnell, A., Krystal, J.H., Owens, M.J., Southwick, S.M., Nemeroff, C.B. & Charney, D.S. (1997a) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am. J. Psychiat.*, **54**, 624–629.

Bremner, J.D., Innis, R.B., Ng, C.K., Staib, L.H., Salomon, R.M., Bronen, R.A., Duncan, J., Southwick, S.M., Krystal, J.H., Rich, D., Zubal, G., Dey, H., Soufer, R. & Charney, D.S. (1997b) Positron emission tomography measurement of cerebral metabolic correlates of yohimbine administration in combat-related posttraumatic stress disorder. *Arch. Gen. Psychiat.*, **54**, 246–254.

Breuner, C.W. & Orchinik, M. (2002) Beyond carrier proteins plasma binding proteins as mediators of corticosteroid action in vertebrates. *J. Endocrinol.*, **175**, 99–112.

Buss, C., Wolf, O.T., Witt, J. & Hellhammer, D.H. (2004) Autobiographic memory impairment following acute cortisol administration. *Psychoneuroendocrinology*, **29**, 1093–1096.

Cerqueira, J.J., Mailliet, F., Almeida, O.F., Jay, T.M. & Sousa, N. (2007) The prefrontal cortex as a key target of the maladaptive response to stress. *J. Neurosci.*, **27**, 2781–2787.

Cohen, H., Kaplan, Z., Koresh, O., Matar, M.A., Geva, A.B. & Zohar, J. (2011) Early post-stressor intervention with propranolol is ineffective in preventing posttraumatic stress responses in an animal model for PTSD. *Eur. Neuropsychopharm.*, **21**, 230–240.

Cornelisse, S., van Ast, V.A., Joëls, M. & Kindt, M. (2014) Delayed effects of cortisol enhance fear memory of trace conditioning. *Psychoneuroendocrinology*, **40**, 257–268.

Croft, A.P., O'Callaghan, M.J., Shaw, S.G., Connolly, G., Jacquot, C. & Little, H.J. (2008) Effects of minor laboratory procedures, adrenalectomy, social defeat or acute alcohol on regional brain concentrations of corticosterone. *Brain Res.*, **1238**, 12–22.

Daskalakis, N.P., Diamantopoulou, A., Claessens, S.E., Remmers, E., Tjälve, M., Oitzl, M.S., Champagne, D.L. & de Kloet, E.R. (2014) Early experience of a novel-environment in isolation primes a fearful phenotype characterized by persistent amygdala activation. *Psychoneuroendocrinology*, **39**, 39–57.

Debiec, J., Bush, D.E. & LeDoux, J.E. (2011) Noradrenergic enhancement of reconsolidation in the amygdala impairs extinction of conditioned fear in rats: a possible mechanism for the persistence of traumatic memories in PTSD. *Depress. Anxiety*, **28**, 186–193.

Eckart, C., Engler, H., Riether, C., Kolassa, S., Elbert, T. & Kolassa, I.T. (2009) No PTSD-related differences in diurnal cortisol profiles of genocide survivors. *Psychoneuroendocrinology*, **34**, 523–531.

Fan, Y., Chen, P., Li, Y., Cui, K., Noel, D.M., Cummins, E.D., Peterson, D.J., Brown, R.W. & Zhu, M.Y. (2014) Corticosterone administration upregulated expression of norepinephrine transporter and dopamine β-hydroxylase in rat locus coeruleus and its terminal regions. *J. Neurochem.*, **128**, 445–458.

Fiorenza, N.G., Rosa, J., Izquierdo, I. & Myskiw, J.C. (2012) Modulation of the extinction of two different fear-motivated tasks in three distinct brain areas. *Behav. Brain Res.*, **232**, 210–216.

Fleshner, M., Deak, T., Spencer, R.L., Laudenslager, M.L., Watkins, L.R. & Maier, S.F. (1995) A long term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. *Endocrinology*, **136**, 5336–5342.

Geracioti, T.D. Jr., Baker, D.G., Ekhator, N.N., West, S.A., Hill, K.K., Bruce, A.B., Schmidt, D., Rounds-Kugler, B., Yehuda, R., Keck, P.E. Jr. & Kasckow, J.W. (2001) CSF norepinephrine concentrations in posttraumatic stress disorder. *Am. J. Psychiat.*, **158**, 1227–1230.

Golub, Y., Mauch, C.P., Dahlhoff, M. & Wotjak, C.T. (2009) Consequences of extinction training on associative and non-associative fear in a mouse model of Posttraumatic Stress Disorder (PTSD). *Behav. Brain Res.*, **205**, 544–549.

Hajós-Korcsok, E., Robinson, D.D., Yu, J.H., Fitch, C.S., Walker, E. & Merchant, K.M. (2003) Rapid habituation of hippocampal serotonin and norepinephrine release and anxiety-related behaviors, but not plasma corticosterone levels, to repeated footshock stress in rats. *Pharmacol. Biochem. Be.*, **74**, 609–616.

Herrmann, L., Ionescu, I.A., Henes, K., Golub, Y., Wang, N.X., Buell, D.R., Holsboer, F., Wotjak, C.T. & Schmidt, U. (2012) Long-lasting hippocampal synaptic protein loss in a mouse model of posttraumatic stress disorder. *PLoS One*, **7**, e42603.

Hillard, C.J. (2014) Stress regulates endocannabinoid-CB1 receptor signaling. *Semin. Immunol.*, **26**, 380–388.
Hinkelmann, K., Yassouridis, A., Mass, R., Tenge, H., Kellner, M., Jahn, H., Wiedemann, K. & Wolf, K. (2010) CCK-4: psychophysiological conditioning elicits features of spontaneous panic attacks. *J. Psychiat. Res.*, **44**, 1148–1153.
Hu, L., Yang, J., Song, T., Hou, N., Liu, Y., Zhao, X., Zhang, D., Wang, L., Wang, T. & Huang, C. (2014) A new stress model, a scream sound, alters learning and monoamine levels in rat brain. *Physiol. Behav.*, **123**, 105–113.
Introini-Collison, I.B., Castellano, C. & McGaugh, J.L. (1994) Interaction of GABAergic and beta noradrenergic drugs in the regulation of memory storage. *Behav. Neural Biol.*, **61**, 150–155.
Izquierdo, I. & Medina, J.H. (1997) Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol. Learn. Mem.*, **68**, 285–316.
Jedema, H.P., Gold, S.J., Gonzalez-Burgos, G., Sved, A.F., Tobe, B.J., Wensel, T. & Grace, A.A. (2008) Chronic cold exposure increases RGS7 expression and decreases alpha(2)-autoreceptor-mediated inhibition of noradrenergic locus coeruleus neurons. *Eur. J. Neurosci.*, **9**, 2433–2443.
Jett, J.D. & Morilak, D.A. (2013) Too much of a good thing: blocking noradrenergic facilitation in medial prefrontal cortex prevents the detrimental effects of chronic stress on cognition. *Neuropsychopharmacology*, **38**, 585–595.
Joëls, M., Pu, Z. & Krugers, H.J. (2006) Learning under stress: how does it work? *Trends Cogn. Sci.*, **10**, 152–158.
Karst, H., Berger, S., Turiault, M., Tronche, F., Schütz, G. & Joëls, M. (2005) Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proc. Natl. Acad. Sci. USA*, **102**, 19204–19207.
Lapiz, M.D. & Morilak, D.A. (2006) Noradrenergic modulation of cognitive function in rat medial prefrontal cortex as measured by attentional set shifting capability. *Neuroscience*, **137**, 1039–1049.
Little, H.J., Croft, A.P., O'Callaghan, M.J., Brooks, S.P., Wang, G. & Shaw, S.G. (2008) Selective increases in regional brain glucocorticoid: a novel effect of chronic alcohol. *Neuroscience*, **156**, 1017–1027.
Ma, S. & Morilak, D.A. (2005) Chronic intermittent cold stress sensitises the hypothalamic-pituitary-adrenal response to a novel acute stress by enhancing noradrenergic influence in the rat paraventricular nucleus. *J. Neuroendocrinol.*, **17**, 761–769.
Morilak, D.A., Barrera, G., Echevarria, D.J., Garcia, A.S., Hernandez, A., Ma, S. & Petre, C.O. (2005) Role of brain norepinephrine in the behavioral response to stress. *Prog. Neuro-psychoph.*, **29**, 1214–1224.
Murchison, C.F., Zhang, X.Y., Zhang, W.P., Ouyang, M., Lee, A. & Thomas, S.A. (2004) A distinctrole for norepinephrine in memoryretrieval. *Cell*, **117**, 131–143.
Nicholson, E.L., Bryant, R.A. & Felmingham, K.L. (2014) Interaction of noradrenaline and cortisol predicts negative intrusive memories in posttraumatic stress disorder. *Neurobiol. Learn. Mem.*, **112**, 204–211.
Norrholm, S.D., Glover, E.M., Stevens, J.S., Fani, N., Galatzer-Levy, I.R., Bradley, B., Ressler, K.J. & Jovanovic, T. (2014) Fear load: the psychophysiological over-expression of fear as an intermediate phenotype associated with trauma reactions. *Int. J. Psychophysiol.*, doi: 10.1016/j.ijpsycho.2014.11.005. [Epub ahead of print].
Pamplona, F.A., Henes, K., Micale, V., Mauch, C.P., Takahashi, R.N. & Wotjak, C.T. (2011) Prolonged fear incubation leads to generalized avoidance behavior in mice. *J. Psychiat. Res.*, **45**, 354–360.
Pavlides, C., Watanabe, Y., Magariños, A.M. & McEwen, B.S. (1995) Opposing roles of type I and type II adrenal steroid receptors in hippocampal long-term potentiation. *Neuroscience*, **68**, 387–394.
Paxinos, G. & Franklin, K.B.J. (2001) *A Stereotaxic Atlas of the Mouse Brain*. Academic Press, San Diego, CA.
Pitman, R.K. & Orr, S.P. (1990) Twenty-four hour urinary cortisol and cathecholamine excretion in combat-related PTSD. *Biol. Psychiat.*, **27**, 245–249.
Pitman, R.K., Sanders, K.M., Zusman, R.M., Healy, A.R., Cheema, F., Lasko, N.B., Cahill, L. & Orr, S.P. (2002) Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biol. Psychiat.*, **51**, 189–192.
Pralong, E., Magistretti, P. & Stoop, R. (2002) Cellular perspectives on the glutamate-monoamine interactions in limbic lobe structures and their relevance for some psychiatric disorders. *Prog. Neurobiol.*, **67**, 173–202.
Reyes, B.A., Szot, P., Sikkema, C., Cathel, A.M., Kirby, L.G. & Van Bockstaele, E.J. (2012) Stress-induced sensitization of cortical adrenergic receptors following a history of cannabinoid exposure. *Exp. Neurol.*, **236**, 327–335.
Robertson, S.D., Plummer, N.W., de Marchena, J. & Jensen, P. (2013) Developmental origins of central norepinephrine neuron diversity. *Nat. Neurosci.*, **16**, 1016–1023.
Salehi, B., Cordero, M.I. & Sandi, C. (2010) Learning under stress: the inverted-U-shape function revisited. *Learn. Memory*, **17**, 522–530.
Schroeter, S., Apparsundaram, S., Wiley, R.G., Miner, L.H., Sesack, S.R. & Blakely, R.D. (2000) Immunolocalization of the cocaine and antidepressant-sensitive l-norepinephrine transporter. *J. Comp. Neurol.*, **420**, 211–232.
Siegmund, A. & Wotjak, C.T. (2007) A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitized fear. *J. Psychiat. Res.*, **41**, 848–860.
Soeter, M. & Kindt, M. (2011) Noradrenergic enhancement of associative fear memory in humans. *Neurobiol. Learn. Mem.*, **96**, 263–271.
Southwick, S.M., Krystal, J.H., Bremner, J.D., Morgan, C.A. 3rd, Nicolaou, A.L., Nagy, L.M., Johnson, D.R., Heninger, G.R. & Charney, D.S. (1997) Noradrenergic and serotonergic function in posttraumatic stress disorder. *Arch. Gen. Psychiat.*, **54**, 749–758.
Stein, M.B., Kerridge, C., Dimsdale, J.E. & Hoyt, D.B. (2007) Pharmacotherapy to prevent PTSD: results from a randomized controlled proof-of-concept trial in physically injured patients. *J. Trauma. Stress*, **20**, 923–932.
Thomas, S.A. & Palmiter, R.D. (1997) Disruption of the dopamine beta-hydroxylase gene in mice suggests roles for norepinephrine in motor function, learning, and memory. *Behav. Neurosci.*, **111**, 579–589.
Urry, H.L., van Reekum, C.M., Johnstone, T., Kalin, N.H., Thurow, M.E., Schaefer, H.S., Jackson, C.A., Frye, C.J., Greischar, L.L., Alexander, A.L. & Davidson, R.J. (2006) Amygdala and ventromedial prefrontal cortex are inversely coupled during regulation of negative affect and predict the diurnal pattern of cortisol secretion among older adults. *J. Neurosci.*, **26**, 4415–4425.
Veldhuis, H.D., Van Koppen, C., Van Ittersum, M. & de Kloet, E.R. (1982) Specificity of the adrenal steroid receptor system in rat hippocampus. *Endocrinology*, **110**, 2044–2051.
Wolf, O.T., Convit, A., McHugh, P.F., Kandil, E., Thorn, E.L., De Santi, S., McEwen, B.S. & de Leon, M.J. (2001) Cortisol differentially affects memory in young and elderly men. *Behav. Neurosci.*, **115**, 1002–1011.
Yan, W.W., Wang, C.Y., Zeng, J., Liu, Q.Y., Xu, S.T., Liu, W.X., Xiao, P. & Li, C.H. (2014) Low-frequency stimulation of dorsal norephinephrine bundle reverses behavioral long-term potentiation and learning performance in rats. *Neuroscience*, **265**, 238–244.
Yehuda, R., Southwick, S.M., Nussbaum, G., Wahby, V., Giller, E.L. Jr. & Mason, J.W. (1990) Low urinary cortisol excretion in patients with posttraumatic stress disorder. *J. Nerv. Ment. Dis.*, **178**, 366–369.
Yen, Y.C., Mauch, C.P., Dahlhoff, M., Micale, V., Bunck, M., Sartori, S.B., Singewald, N., Landgraf, R. & Wotjak, C.T. (2012) Increased levels of conditioned fear and avoidance behaviour coincide with changes in phosphorylation of the protein kinase B (AKT) within the amygdala in a mouse morel of extremes in trait anxiety. *Neurobiol. Learn. Mem.*, **98**, 56–65.
Zhang, L., Ouyang, M., Ganellin, C.R. & Thomas, S.A. (2013) The slow after hyperpolarization: a target of β1-adrenergic signaling in hippocampus-dependent memory retrieval. *J. Neurosci.*, **33**, 5006–5016.

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.

Original Paper

Molecular Neuropsychiatry

Mol Neuropsychiatry 2015;1:60–67
DOI: 10.1159/000381855

Received: February 26, 2015
Accepted: March 24, 2015
Published online: April 29, 2015

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

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

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.

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

2296–9209/15/0011–0060$39.50/0

E-Mail karger@karger.com
www.karger.com/mnp

Christoph W. Turck
Department of Translational Research in Psychiatry
Max Planck Institute of Psychiatry, Kraepelinstrasse 2–10
DE–80804 Munich (Germany)
E-Mail turck@psych.mpg.de

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 ($23 \times 16.5 \times 14$ cm^3) 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 (Abbot, Mumbai, India)/Metacam® (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany) anesthesia/anal-

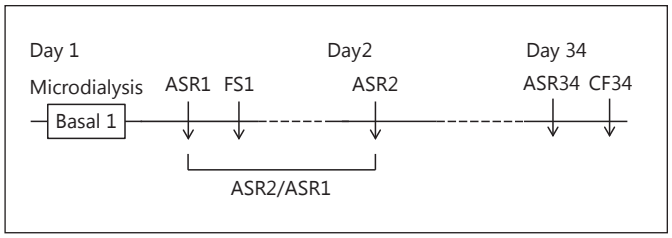


Fig. 1. Outline of the microdialysis experiment in combination with metabolomics applied to a PTSD mouse model. On experimental day 1, ASR1 was measured, followed by fear-conditioning FS1. On experimental day 2, the mice were subjected to recurrent ASR2. Microdialysates collected at Basal 1 were subjected to metabolite profiling. On experimental day 34, PTSD-like symptoms, including ASR and CF, were assessed.

gesia. Recovery in experimental plexiglass home cages (16 × 16 × 32 cm^3) lasted for 1 week and included Metacam supplementation (0.25 mg/100 ml) with drinking water for 3 days after surgery, with daily inspection for food intake. One day before the experiment, the mice were shortly anesthetized with isoflurane. Microdialysis probes with a molecular cutoff of 6 kDa (o.d. 0.2 mm, cuprophane membrane 2 mm of length, MAB 4.15.2. Cu; Microbiotech/se AB, Stockholm, Sweden) were inserted and connected to the perfusion lines, which consist of FEP tubing and low-volume liquid swivel TCS2–23 (Eicom, Kyoto, Japan). From the moment of insertion, the probes were continuously perfused with sterile artificial cerebrospinal fluid (NaCl 145 mM, KCl 2.7 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1.0 mM and Na_2HPO_4 2.0 mM; pH 7.4) at a flow rate of 0.3 μl/min.

Microdialysis fractions (30 min) were constantly collected at a perfusion rate of 1.5 μl/min into 300-microliter microtubes (Microbiotech/se AB) following a 1.5 h equilibration period. During the collection time, the tubes were kept in a refrigerated Univentor 820 Microsampler (Univentor, Zejtun, Malta) at 4°C. The dead volume of the outlet line was compensated with a delay in fraction sampling (7 min). The perfusion flow rate was reduced to 0.3 μl/min for overnight perfusion.

The microdialysis experiment is outlined in figure 1. On experimental day 1, the mice were kept in the experimental plexiglass home cages (16 × 16 × 32 cm^3) for baseline microdialysis sample collection for 1.5 h. The mice were then subjected to a 20-minute ASR test in a sound and protected environment. To permit a continuous dialysis in microdialysis experiments, we used a custom-made startle chamber equipped with a commercially available ASR apparatus (San Diego Instruments, San Diego, Calif., USA). Response was expressed in arbitrary units. At the end of each test, the mice were transferred back to their home cages, and the chamber was cleaned thoroughly with soap water. Two hours later, the mice were subjected to a FS for fear conditioning. Microdialysis was done as described previously [18]. The microdialysate fractions corresponding to the periods of Basal 1 (samples 1–3), ASR1 (samples 4–7) and FS1 (samples 8–11) on experimental day 1 were collected. Only baseline samples were analyzed for metabolite content.

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 × 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 –4,500 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

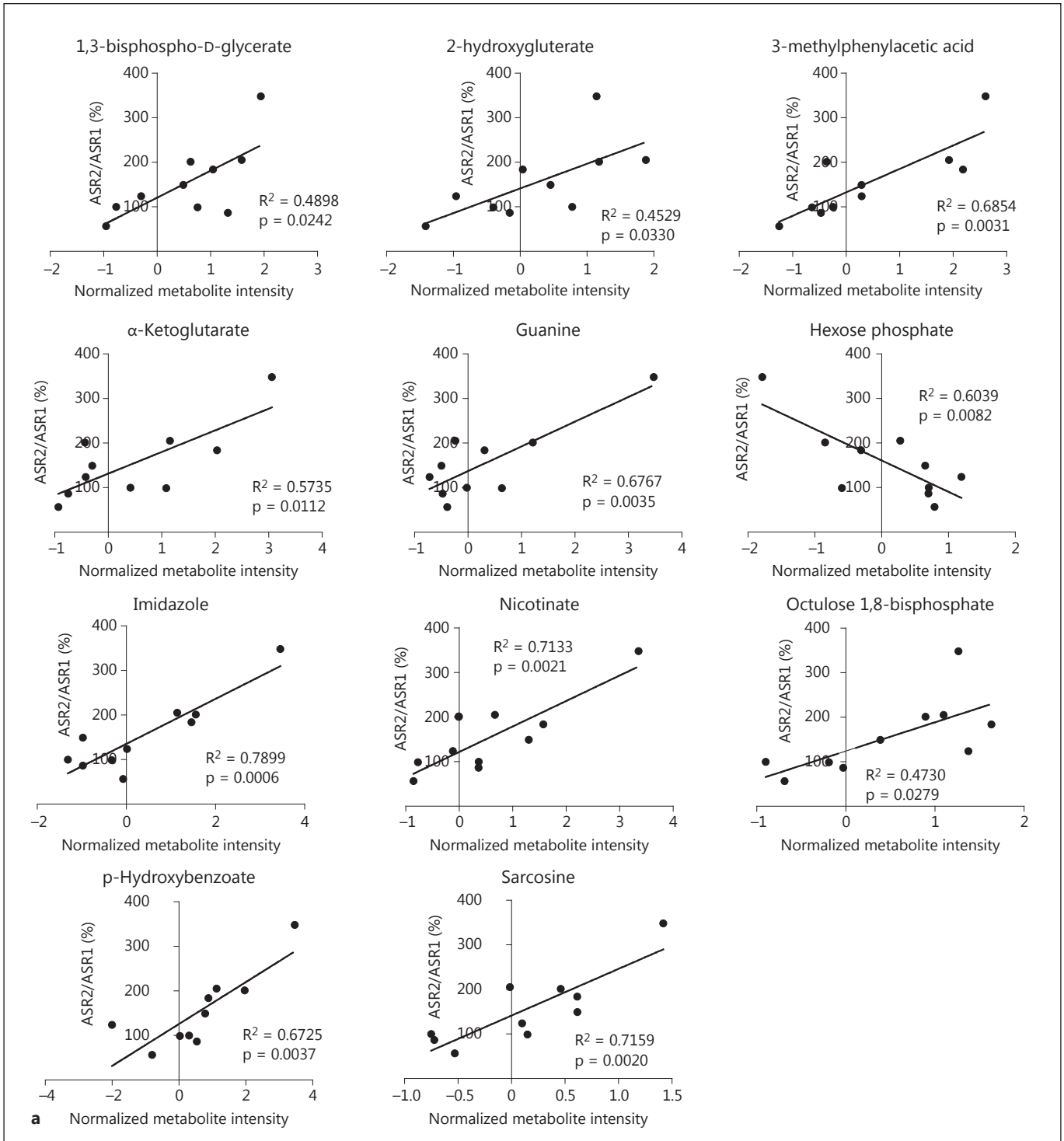
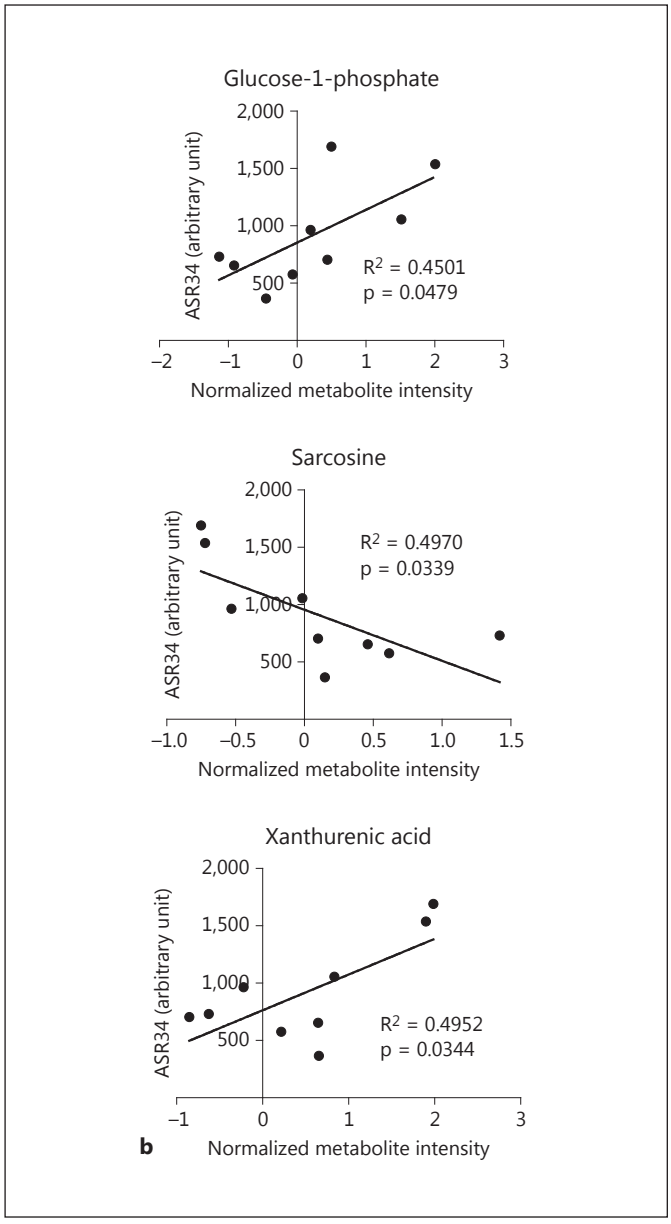


Fig. 2. Linear correlations between Basal 1 metabolite levels and FS-induced behavioral changes. Basal 1 metabolite levels correlated with FS-induced ASR2/ASR1 (**a**), ASR34 (**b**) and CF34 (**c**).

(For Figure 2b and c see next page.)



2

Table 1. Pathways enriched by metabolites correlating with FS-induced ASR2/ASR1 and metabolites with a correlating expression profile

Pathway	Holm-Bonferroni-adjusted p value	False discovery rate
Citrate cycle	0.0002	0.0002
Glyoxylate and dicarboxylate metabolism	0.0219	0.0111

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 wwww.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.

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 alanine 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 microdialysate 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

1 Rittenhouse KD, Pollack GM: Microdialysis and drug delivery to the eye. Adv Drug Deliv Rev 2008;45:229–241.
2 Tsai TH, Cheng FC, Chen YF, Chen CF: Online microdialysis coupled with microbore liquid chromatography with ultraviolet detection for continuous monitoring of free cefsulodin in rat blood. J Chromatogr A 2001;914:83–87.
3 Chang YL, Chou MH, Lin MF, Chen CF, Tsai TH: Determination and pharmacokinetic study of meropenem in rat bile using on-line microdialysis and liquid chromatography. J Chromatogr A 2002;961:119–124.
4 Ungerstedt U, Pycock C: Functional correlates of dopamine neurotransmission. Bull Schweiz Akad Med Wiss 1974;30:44–55.
5 Anderzhanova E, Wotjak CT: Brain microdialysis and its applications in experimental neurochemistry. Cell Tissue Res 2013;1:27–39.
6 Westerhout J, Ploeger B, Smeets J, Danhof M, de Lange EC: Physiologically based pharmacokinetic modeling to investigate regional brain distribution kinetics in rats. AAPS J 2012;14:543–553.
7 Guihen E, O'Connor WT: Current separation and detection methods in microdialysis the drive towards sensitivity and speed. Electrophoresis 2009;30:2062–2075.
8 Zhang MY, Hughes ZA, Kerns EH, Lin Q, Beyer CE: Development of a liquid chromatography/tandem mass spectrometry method for the quantitation of acetylcholine and related neurotransmitters in brain microdialysis samples. J Pharm Biomed Anal 2007;44:586–593.
9 Hows ME, Lacroix L, Heidbreder C, Organ AJ, Shah AJ: High-performance liquid chromatography/tandem mass spectrometric assay for the simultaneous measurement of dopamine, norepinephrine, 5-hydroxytryptamine and cocaine in biological samples. J Neurosci Methods 2004;138:123–132.
10 Berna MJ, Ackermann BL: Quantification of serine enantiomers in rat brain microdialysate using Marfey's reagent and LC/MS/MS. J Chromatogr B 2007;846:359–363.
11 Haskins WE, Wang Z, Watson CJ, Rostand RR, Witowski SR, Powell DH, Kennedy RT: Capillary LC-MS2 at the attomole level for monitoring and discovering endogenous peptides in microdialysis samples collected in vivo. Anal Chem 2001;73:5005–5014.
12 Dias C, Nashed Y, Atluri H, Mitra A: Ocular penetration of acyclovir and its peptide prodrugs valacyclovir and val-valacyclovir following systemic administration in rabbits: an evaluation using ocular microdialysis and LC-MS. Curr Eye Res 2002;25:243–252.
13 Chen YF, Chang CH, Wang SC, Tsai TH: Measurement of unbound cocaine in blood, brain and bile of anesthetized rats using microdialysis coupled with liquid chromatography and verified by tandem mass spectrometry. Biomed Chromatogr 2005;19:402–408.
14 Bengtsson J, Jansson B, Hammarlund-Udenaes M: On-line desalting and determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in microdialysis and plasma samples using column switching and liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2005;19:2116–2122.
15 Siegmund A, Wotjak CT: A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitized fear. J Psychiatr Res 2007;41:848–860.
16 Golub Y, Mauch CP, Dahlhoff M, Wotjak CT: Consequences of extinction training on associative and non-associative fear in a mouse model of posttraumatic stress disorder (PTSD). Behav Brain Res 2009;205:544–549.
17 Paxinos G, Franklin KBJ: A Stereotaxic Atlas of the Mouse Brain. San Diego, Academic Press, 2002.
18 Anderzhanova E, Bächli H, Buneeva OA, Narkevich VB, Medvedev AE, Thoeringer CK, Wotjak CT, Kudrin VS: Strain differences in profiles of dopaminergic neurotransmission in the prefrontal cortex of the BALB/C vs. C57Bl/6 mice: consequences of stress and afobazole. Eur J Pharmacol 2013;708:95–104.

19 Wölfer J, Gorji A, Speckmann EJ, Wassmann H: Interstitial amino acid concentrations in rodent brain tissue during chemical ischemia. J Neurosci Res 2014;92:955–963.
20 Anderzhanova E, Rayevsky KS, Saransaari P, Riitamaa E, Oja SS: Effects of acute toxic doses of psychostimulants on extracellular levels of excitatory amino acids and taurine in rats: comparison of d-amphetamine and sydnocarb. Ann NY Acad Sci 2002;965:193–203.
21 Kao C-Y, Stalla G, Stalla J, Wotjak CT, Anderzhanova E: Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice. Eur J Neurosci 2015, E-pub ahead of print.
22 McDonald JW, Johnston MV: Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Rev 1990;15:41–70.
23 Curzon G, Bridges PK: Tryptophan metabolism in depression. J Neurol Neurosurg Psychiatry 1970;33:698–704.
24 Lapin IP: Neurokynurenines (NEKY) as common neurochemical links of stress and anxiety. Adv Exp Med Biol 2003;527:121–125.
25 Laugeray A, Launay JM, Callebert J, et al: Evidence for a key role of the peripheral kynurenine pathway in the modulation of anxiety- and depression-like behaviours in mice: focus on individual differences. Pharmacol Biochem Behav 2011;98:161–168.
26 Loriaux SM, Deijen JB, Orlebeke JF, De Swart JH: The effects of nicotinic acid and xanthinol nicotinate on human memory in different categories of age. A double blind study. Psychopharmacology (Berl) 1985;87:390–395.
27 Mallei A, Failler M, Corna S, Racagni G, Mathé AA, Popoli M: Synaptoproteomic analysis of a rat gene-environment model of depression reveals involvement of energy metabolism and cellular remodeling pathways. Int J Neuropsychopharmacol 2014; 18:pyu067.
28 Einat H, Yuan P, Manji HK: Increased anxiety-like behaviors and mitochondrial dysfunction in mice with targeted mutation of the Bcl-2 gene: further support for the involvement of mitochondrial function in anxiety disorders. Behav Brain Res 2005;165:172–180.
29 Mancuso C, Scapagini G, Currò D, Giuffrida Stella AM, De Marco C, Butterfield DA, Calabrese V: Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. Front Biosci 2007;12:1107–1123.
30 Filiou MD, Zhang Y, Teplytska L, Reckow S, Gormanns P, Maccarrone G, Frank E, Kessler MS, Hambsch B, Nussbaumer M, Bunck M, Ludwig T, Yassouridis A, Holsboer F, Landgraf R, Turck CW: Proteomics and metabolomics analysis of a trait anxiety mouse model reveals divergent mitochondrial pathways. Biol Psychiatry 2011;70:1074–1082.
31 Mattson MP, Partin J: Evidence for mitochondrial control of neuronal polarity. J Neurosci Res 1999;56:8–20.
32 Billups B, Forsythe ID: Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. J Neurosci 2002;22:5840–5847.
33 Kann O, Kovacs R, Heinemann U: Metabotropic receptor-mediated Ca2+ signaling elevates mitochondrial Ca2+ and stimulates oxidative metabolism in hippocampal slice cultures. J Neurophysiol 2003;90:613–621.
34 Vanden Berghe P, Kenyon JL, Smith TK: Mitochondrial Ca2+ uptake regulates the excitability of myenteric neurons. J Neurosci 2002; 22:6962–6971.
35 Thiffault C, Quirion R, Poirier J: The effect of L-deprenyl, D-deprenyl and MDL72974 on mitochondrial respiration: a possible mechanism leading to an adaptive increase in superoxide dismutase activity. Brain Res Mol Brain Res 1997;49:127–136.
36 Hows ME, Organ AJ, Murray S, Dawson LA, Foxton R, Heidbreder C, Hughes ZA, Lacroix L, Shah AJ: High-performance liquid chromatography/tandem mass spectrometry assay for the rapid high sensitivity measurement of basal acetylcholine from microdialysates. J Neurosci Methods 2002;121:33–39.

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

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

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).

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,

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

(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

Abelson JL, Curtis GC (1996). Hypothalamic-pituitary-adrenal axis activity in panic disorder. 24-hour secretion of corticotropin and cortisol. *Arch Gen Psychiatry* **53**(4): 323-331.

Abram HS (1970). *Psychological aspects of stress.* Thomas: Springfield, IL.

Adam GC, Sorensen EJ, Cravatt BF (2002). Chemical strategies for functional proteomics. *Mol Cell Proteomics* **1**(10): 781-790.

Adamec R, Hebert M, Blundell J, Mervis RF (2012). Dendritic morphology of amygdala and hippocampal neurons in more and less predator stress responsive rats and more and less spontaneously anxious handled controls. *Behav Brain Res* **226**(1): 133-146.

Adamec RE, Blundell J, Burton P (2005). Neural circuit changes mediating lasting brain and behavioral response to predator stress. *Neurosci Biobehav Rev* **29**(8): 1225-1241.

Adamec RE, Blundell J, Collins A (2001). Neural plasticity and stress induced changes in defense in the rat. *Neurosci Biobehav Rev* **25**(7-8): 721-744.

Ader R, Cohen N, Felten D (1995). Psychoneuroimmunology: interactions between the nervous system and the immune system. *Lancet* **345**(8942): 99-103.

Afifi TO, Asmundson GJ, Taylor S, Jang KL (2010). The role of genes and environment on trauma exposure and posttraumatic stress disorder symptoms: a review of twin studies. *Clin Psychol Rev* **30**(1): 101-112.

Aga-Mizrachi S, Cymerblit-Sabba A, Gurman O, Balan A, Shwam G, Deshe R, *et al* (2014). Methylphenidate and desipramine combined treatment improves PTSD symptomatology in a rat model. *Transl Psychiatry* **4**: e447.

Ahmadi H, Nasehi M, Rostami P, Zarrindast MR (2013). Involvement of the nucleus accumbens shell dopaminergic system in prelimbic NMDA-induced anxiolytic-like behaviors. *Neuropharmacology* **71**: 112-123.

Albucher RC, Liberzon I (2002). Psychopharmacological treatment in PTSD: a critical review. *J Psychiatr Res* **36**(6): 355-367.

Almada RC, Coimbra NC, Brandão ML (2015). Medial prefrontal cortex serotonergic and GABAergic mechanisms modulate the expression of contextual fear: intratelencephalic pathways and differential involvement of cortical subregions. *Neuroscience* **284**: 988-997.

Altemus M, Cloitre M, Dhabhar FS (2003). Enhanced cellular immune response in women with PTSD related to childhood abuse. *Am J Psychiatry* **160**(9): 1705-1707.

Amstadter AB, Nugent NR, Yang BZ, Miller A, Siburian R, Moorjani P, *et al* (2011). Corticotrophin-releasing hormone type 1 receptor gene (CRHR1) variants predict posttraumatic stress disorder onset and course in pediatric injury patients. *Dis Markers* **30**(2-3): 89-99.

Anderson TR, Schönborn KJ (2008). Bridging the educational research-teaching practice gap: Conceptual understanding, part 1: The multifaceted nature of expert knowledge. *Biochem Mol Biol Educ* **36**(4): 309-315.

Anderzhanova E, Wotjak CT (2013). Brain microdialysis and its applications in experimental neurochemistry. *Cell Tissue Res* **354**(1): 27-39.

Anglada-Figueroa D, Quirk GJ (2005). Lesions of the basal amygdala block expression of conditioned fear but not extinction. *J Neurosci* **25**(42): 9680-9685.

Antoniadis EA, McDonald RJ (2006). Fornix, medial prefrontal cortex, nucleus accumbens, and mediodorsal thalamic nucleus: roles in a fear-based context discrimination task. *Neurobiol Learn Mem* **85**(1): 71-85.

Anwyl R (2009). Metabotropic glutamate receptor-dependent long-term potentiation. *Neuropharmacology* **56**(4): 735-740.

Appikatla S, Bessert D, Lee I, Hüttemann M, Mullins C, Somayajulu-Nitu M, *et al* (2014). Insertion of proteolipid protein into oligodendrocyte mitochondria regulates extracellular pH and adenosine triphosphate. *Glia* **62**(3): 356-373.

Association AP (1952). *Diagnostic and statistical manual of mental disorders (1st ed.)* American Psychiatric Association: Washington, DC.

Association AP (1980). *Diagnostic and statistical manual of mental disorders (3rd ed.)* American Psychiatric Association: Washington, DC.

Association AP (1987). *Diagnostic and statistical manual of mental disorders (3rd ed. revised)* American Psychiatric Association: Washington, DC.

Association AP (2013). *Diagnostic and statistical manual of mental health disorders: DSM-5* American Psychiatric Publishing: Washington, DC.

Atmaca M, Kuloglu M, Tezcan E, Onal S, Ustundag B (2002). Neopterin levels and dexamethasone suppression test in posttraumatic stress disorder. *Eur Arch Psychiatry Clin Neurosci* **252**(4): 161-165.

Baker DG, Ekhator NN, Kasckow JW, Hill KK, Zoumakis E, Dashevsky BA, *et al* (2001). Plasma and cerebrospinal fluid interleukin-6 concentrations in posttraumatic stress disorder. *Neuroimmunomodulation* **9**(4): 209-217.

Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, Hill KK, *et al* (1999). Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am J Psychiatry* **156**(4): 585-588.

Ballenger JC, Davidson JR, Lecrubier Y, Nutt DJ, Foa EB, Kessler RC, *et al* (2000). Consensus statement on posttraumatic stress disorder from the International Consensus Group on Depression and Anxiety. *J Clin Psychiatry* **61 Suppl 5**: 60-66.

Balogh SA, Radcliffe RA, Logue SF, Wehner JM (2002). Contextual and cued fear conditioning in C57BL/6J and DBA/2J mice: context discrimination and the effects of retention interval. *Behav Neurosci* **116**(6): 947-957.

Banerjee A, García-Oscos F, Roychowdhury S, Galindo LC, Hall S, Kilgard MP, *et al* (2013). Impairment of cortical GABAergic synaptic transmission in an environmental rat model of autism. *Int J Neuropsychopharmacol* **16**(6): 1309-1318.

Bantscheff M, Lemeer S, Savitski MM, Kuster B (2012). Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present. *Anal Bioanal Chem* **404**(4): 939-965.

Barabási AL, Oltvai ZN (2004). Network biology: understanding the cell's functional organization. *Nat Rev Genet* **5**(2): 101-113.

Barnes PJ, Adcock IM (2009). Glucocorticoid resistance in inflammatory diseases. *Lancet* **373**(9678): 1905-1917.

Barsegyan A, McGaugh JL, Roozendaal B (2014). Noradrenergic activation of the basolateral amygdala modulates the consolidation of object-in-context recognition memory. *Front Behav Neurosci* **8**: 160.

Belzung C, Turiault M, Griebel G (2014). Optogenetics to study the circuits of fear- and depression-like behaviors: a critical analysis. *Pharmacol Biochem Behav* **122**: 144-157.

Ben-Shachar D, Laifenfeld D (2004). Mitochondria, synaptic plasticity, and schizophrenia. *Int Rev Neurobiol* **59**: 273-296.

Berger W, Mendlowicz MV, Marques-Portella C, Kinrys G, Fontenelle LF, Marmar CR, *et al* (2009). Pharmacologic alternatives to antidepressants in posttraumatic stress disorder: a systematic review. *Prog Neuropsychopharmacol Biol Psychiatry* **33**(2): 169-180.

Bielczyk NZ, Buitelaar JK, Glennon JC, Tiesinga PH (2015). Circuit to construct mapping: a mathematical tool for assisting the diagnosis and treatment in major depressive disorder. *Front Psychiatry* **6**: 29.

Billups B, Forsythe ID (2002). Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J Neurosci* **22**(14): 5840-5847.

Binder EB, Bradley RG, Liu W, Epstein MP, Deveau TC, Mercer KB, *et al* (2008). Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults. *JAMA* **299**(11): 1291-1305.

Black PH, Garbutt LD (2002). Stress, inflammation and cardiovascular disease. *J Psychosom Res* **52**(1): 1-23.

Blundell J, Adamec R, Burton P (2005). Role of NMDA receptors in the syndrome of behavioral changes produced by predator stress. *Physiol Behav* **86**(1-2): 233-243.

Bossini L, Tavanti M, Calossi S, Lombardelli A, Polizzotto NR, Galli R, *et al* (2008). Magnetic resonance imaging volumes of the hippocampus in drug-naïve patients with post-traumatic stress disorder without comorbidity conditions. *J Psychiatr Res* **42**(9): 752-762.

Bremner JD (2002). Neuroimaging studies in post-traumatic stress disorder. *Curr Psychiatry Rep* **4**(4): 254-263.

Bremner JD, Innis RB, Ng CK, Staib LH, Salomon RM, Bronen RA, *et al* (1997a). Positron emission tomography measurement of cerebral metabolic correlates of yohimbine administration in combat-related posttraumatic stress disorder. *Arch Gen Psychiatry* **54**(3): 246-254.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, *et al* (1997b). Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. *Am J Psychiatry* **154**(5): 624-629.

Bremner JD, Mletzko T, Welter S, Quinn S, Williams C, Brummer M, *et al* (2005a). Effects of phenytoin on memory, cognition and brain structure in post-traumatic stress disorder: a pilot study. *J Psychopharmacol* **19**(2): 159-165.

Bremner JD, Southwick S, Brett E, Fontana A, Rosenheck R, Charney DS (1992). Dissociation and posttraumatic stress disorder in Vietnam combat veterans. *Am J Psychiatry* **149**(3): 328-332.

Bremner JD, Southwick SM, Darnell A, Charney DS (1996). Chronic PTSD in Vietnam combat veterans: course of illness and substance abuse. *Am J Psychiatry* **153**(3): 369-375.

Bremner JD, Southwick SM, Johnson DR, Yehuda R, Charney DS (1993). Childhood physical abuse and combat-related posttraumatic stress disorder in Vietnam veterans. *Am J Psychiatry* **150**(2): 235-239.

Bremner JD, Vermetten E, Schmahl C, Vaccarino V, Vythilingam M, Afzal N, *et al* (2005b). Positron emission tomographic imaging of neural correlates of a fear acquisition and extinction paradigm in women with childhood sexual-abuse-related post-traumatic stress disorder. *Psychol Med* **35**(6): 791-806.

Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, *et al* (2000). Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotechnol* **18**(6): 630-634.

Breslau N, Chilcoat HD, Kessler RC, Davis GC (1999). Previous exposure to trauma and PTSD effects of subsequent trauma: results from the Detroit Area Survey of Trauma. *Am J Psychiatry* **156**(6): 902-907.

Breslau N, Kessler RC, Chilcoat HD, Schultz LR, Davis GC, Andreski P (1998). Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma. *Arch Gen Psychiatry* **55**(7): 626-632.

Brunello N, Davidson JR, Deahl M, Kessler RC, Mendlewicz J, Racagni G, *et al* (2001). Posttraumatic stress disorder: diagnosis and epidemiology, comorbidity and social consequences, biology and treatment. *Neuropsychobiology* **43**(3): 150-162.

Brunet A, Orr SP, Tremblay J, Robertson K, Nader K, Pitman RK (2008). Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. *J Psychiatr Res* **42**(6): 503-506.

Bu Z, Callaway DJ (2011). Proteins move! Protein dynamics and long-range allostery in cell signaling. *Adv Protein Chem Struct Biol* **83**: 163-221.

Calabresi P, Gubellini P, Picconi B, Centonze D, Pisani A, Bonsi P, *et al* (2001). Inhibition of mitochondrial complex II induces a long-term potentiation of NMDA-mediated synaptic excitation in the striatum requiring endogenous dopamine. *J Neurosci* **21**(14): 5110-5120.

Calvetti D, Somersalo E (2012). Ménage à trois: the role of neurotransmitters in the energy metabolism of astrocytes, glutamatergic, and GABAergic neurons. *J Cereb Blood Flow Metab* **32**(8): 1472-1483.

Carrasco GA, Van de Kar LD (2003). Neuroendocrine pharmacology of stress. *Eur J Pharmacol* **463**(1-3): 235-272.

Caruth C (1996). *Unclaimed experience: Trauma, narrative, and history*. Hopkins University Press: Baltimore, MA.

Castrén E (2005). Is mood chemistry? *Nat Rev Neurosci* **6**(3): 241-246.

Chadman KK, Yang M, Crawley JN (2009). Criteria for validating mouse models of psychiatric diseases. *Am J Med Genet B Neuropsychiatr Genet* **150B**(1): 1-11.

Chan YL, Chou MH, Lin MF, Chen CF, Tsai TH (2002). Determination and pharmacokinetic study of meropenem in rat bile using on-line microdialysis and liquid chromatography. *J Chromatogr A* **961**(1): 119-124.

Charney DS, Deutch AY, Krystal JH, Southwick SM, Davis M (1993). Psychobiologic mechanisms of posttraumatic stress disorder. *Arch Gen Psychiatry* **50**(4): 295-305.

Chaudhury D, Walsh JJ, Friedman AK, Juarez B, Ku SM, Koo JW, *et al* (2013). Rapid regulation of depression-related behaviours by control of midbrain dopamine neurons. *Nature* **493**(7433): 532-536.

Christensen GL, Kelstrup CD, Lyngsø C, Sarwar U, Bøgebo R, Sheikh SP, *et al* (2010). Quantitative phosphoproteomics dissection of seven-transmembrane receptor signaling using full and biased agonists. *Mol Cell Proteomics* **9**(7): 1540-1553.

Chrousos GP, Castro M, Leung DY, Webster E, Kino T, Bamberger C, *et al* (1996). Molecular mechanisms of glucocorticoid resistance/hypersensitivity. Potential clinical implications. *Am J Respir Crit Care Med* **154**(2 Pt 2): S39-43; discussion S43-34.

Cloonan N, Forrest AR, Kolle G, Gardiner BB, Faulkner GJ, Brown MK, *et al* (2008). Stem cell transcriptome profiling via massive-scale mRNA sequencing. *Nat Methods* **5**(7): 613-619.

Cohen H, Kaplan Z, Koresh O, Matar MA, Geva AB, Zohar J (2011). Early post-stressor intervention with propranolol is ineffective in preventing posttraumatic stress responses in an animal model for PTSD. *Eur Neuropsychopharmacol* **21**(3): 230-240.

Cohen S, Janicki-Deverts D, Doyle WJ, Miller GE, Frank E, Rabin BS, *et al* (2012). Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. *Proc Natl Acad Sci U S A* **109**(16): 5995-5999.

Collins F, Galas D (1993). A new five-year plan for the U.S. Human Genome Project. *Science* **262**(5130): 43-46.

Corbo V, Clément MH, Armony JL, Pruessner JC, Brunet A (2005). Size versus shape differences: contrasting voxel-based and volumetric analyses of the anterior cingulate cortex in individuals with acute posttraumatic stress disorder. *Biol Psychiatry* **58**(2): 119-124.

Corcoran KA, Quirk GJ (2007). Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. *J Neurosci* **27**(4): 840-844.

Cornelis MC, Nugent NR, Amstadter AB, Koenen KC (2010). Genetics of post-traumatic stress disorder: review and recommendations for genome-wide association studies. *Curr Psychiatry Rep* **12**(4): 313-326.

Correia C, Coutinho AM, Diogo L, Grazina M, Marques C, Miguel T, *et al* (2006). Brief report: High frequency of biochemical markers for mitochondrial dysfunction in autism: no association with the mitochondrial aspartate/glutamate carrier SLC25A12 gene. *J Autism Dev Disord* **36**(8): 1137-1140.

Cross D, Crow T, Powers A, Bradley B (2015). Childhood trauma, PTSD, and problematic alcohol and substance use in low-income, African-American men and women. *Child Abuse Negl*.

Czell D, Efe T, Preuss M, Schofer MD, Becker R (2012). Influence of intraventricular application of baclofen on arterial blood pressure and neurotransmitter concentrations in the hypothalamic paraventricular nucleus of rats. *Neurochem Res* **37**(2): 381-386.

da Cunha IC, Lopes AP, Steffens SM, Ferraz A, Vargas JC, de Lima TC, *et al* (2008). The microinjection of AMPA receptor antagonist into the accumbens shell, but not into the accumbens core, induces anxiolysis in an animal model of anxiety. *Behav Brain Res* **188**(1): 91-99.

Dai Y, Li Z, Xue L, Dou C, Zhou Y, Zhang L, *et al* (2010). Metabolomics study on the anti-depression effect of xiaoyaosan on rat model of chronic unpredictable mild stress. *J Ethnopharmacol* **128**(2): 482-489.

Dansky BS, Roitzsch JC, Brady KT, Saladin ME (1997). Posttraumatic stress disorder and substance abuse: use of research in a clinical setting. *J Trauma Stress* **10**(1): 141-148.

Davidson JR, Hughes D, Blazer DG, George LK (1991). Post-traumatic stress disorder in the community: an epidemiological study. *Psychol Med* **21**(3): 713-721.

Davis J, Moylan S, Harvey BH, Maes M, Berk M (2014). Neuroprogression in schizophrenia: Pathways underpinning clinical staging and therapeutic corollaries. *Aust N Z J Psychiatry* **48**(6): 512-529.

de Baumont A, Maschietto M, Lima L, Carraro DM, Olivieri EH, Fiorini A, *et al* (2015). Innate immune response is differentially dysregulated between bipolar disease and schizophrenia. *Schizophr Res* **161**(2-3): 215-221.

de Kloet ER, Sibug RM, Helmerhorst FM, Schmidt MV, Schmidt M (2005). Stress, genes and the mechanism of programming the brain for later life. *Neurosci Biobehav Rev* **29**(2): 271-281.

de Leenheer AP, Thienpont LM (1992). Applications of isotope dilution-mass spectrometry in clinical chemistry, pharmacokinetics, and toxicology. *Mass Spectrometry Reviews* **11**(4): 259-307.

de Quervain DJ (2008). Glucocorticoid-induced reduction of traumatic memories: implications for the treatment of PTSD. *Prog Brain Res* **167**: 239-247.

de Quervain DJ, Aerni A, Schelling G, Roozendaal B (2009). Glucocorticoids and the regulation of memory in health and disease. *Front Neuroendocrinol* **30**(3): 358-370.

Delahanty DL, Nugent NR (2006). Predicting PTSD prospectively based on prior trauma history and immediate biological responses. *Ann N Y Acad Sci* **1071**: 27-40.

Delahanty DL, Nugent NR, Christopher NC, Walsh M (2005). Initial urinary epinephrine and cortisol levels predict acute PTSD symptoms in child trauma victims. *Psychoneuroendocrinology* **30**(2): 121-128.

Dickie EW, Brunet A, Akerib V, Armony JL (2008). An fMRI investigation of memory encoding in PTSD: influence of symptom severity. *Neuropsychologia* **46**(5): 1522-1531.

Diehl LA, Alvares LO, Noschang C, Engelke D, Andreazza AC, Gonçalves CA, *et al* (2012). Long-lasting effects of maternal separation on an animal model of post-traumatic stress disorder: effects on memory and hippocampal oxidative stress. *Neurochem Res* **37**(4): 700-707.

Dielenberg RA, McGregor IS (2001). Defensive behavior in rats towards predatory odors: a review. *Neurosci Biobehav Rev* **25**(7-8): 597-609.

Dierks MR, Jordan JK, Sheehan AH (2007). Prazosin treatment of nightmares related to posttraumatic stress disorder. *Ann Pharmacother* **41**(6): 1013-1017.

Dillman AA, Cookson MR (2014). Transcriptomic changes in brain development. *Int Rev Neurobiol* **116**: 233-250.

Ding J, Han F, Shi Y (2010). Single-prolonged stress induces apoptosis in the amygdala in a rat model of post-traumatic stress disorder. *J Psychiatr Res* **44**(1): 48-55.

Doherty M, Gratton A (2007). Differential involvement of ventral tegmental GABA(A) and GABA(B) receptors in the regulation of the nucleus accumbens dopamine response to stress. *Brain Res* **1150**: 62-68.

Duman RS, Strada SJ, Enna SJ (1989). Glucocorticoid administration increases receptor-mediated and forskolin-stimulated cyclic AMP accumulation in rat brain cerebral cortical slices. *Brain Res* **477**(1-2): 166-171.

Durstewitz D, Seamans JK, Sejnowski TJ (2000). Neurocomputational models of working memory. *Nat Neurosci* **3 Suppl**: 1184-1191.

Dębiec J, Bush DE, LeDoux JE (2011). Noradrenergic enhancement of reconsolidation in the amygdala impairs extinction of conditioned fear in rats--a possible mechanism for the persistence of traumatic memories in PTSD. *Depress Anxiety* **28**(3): 186-193.

Ehring T, Ehlers A, Cleare AJ, Glucksman E (2008). Do acute psychological and psychobiological responses to trauma predict subsequent symptom severities of PTSD and depression? *Psychiatry Res* **161**(1): 67-75.

Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES (2000). The sympathetic nerve--an integrative interface between two supersystems: the brain and the immune system. *Pharmacol Rev* **52**(4): 595-638.

Elzinga BM, Bremner JD (2002). Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? *J Affect Disord* **70**(1): 1-17.

Eng JK, McCormack AL, Yates JR (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**(11): 976-989.

Epstein JN, Saunders BE, Kilpatrick DG, Resnick HS (1998). PTSD as a mediator between childhood rape and alcohol use in adult women. *Child Abuse Negl* **22**(3): 223-234.

Felmingham K, Kemp A, Williams L, Das P, Hughes G, Peduto A, *et al* (2007). Changes in anterior cingulate and amygdala after cognitive behavior therapy of posttraumatic stress disorder. *Psychol Sci* **18**(2): 127-129.

Fendt M, Bürki H, Imobersteg S, Lingenhöhl K, McAllister KH, Orain D, *et al* (2009). Fear-reducing effects of intra-amygdala neuropeptide Y infusion in animal models of conditioned fear: an NPY Y1 receptor independent effect. *Psychopharmacology (Berl)* **206**(2): 291-301.

Fenton GE, Pollard AK, Halliday DM, Mason R, Bredy TW, Stevenson CW (2014). Persistent prelimbic cortex activity contributes to enhanced learned fear expression in females. *Learn Mem* **21**(2): 55-60.

Fields HL, Hjelmstad GO, Margolis EB, Nicola SM (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu Rev Neurosci* **30**: 289-316.

Filiou MD, Zhang Y, Teplytska L, Reckow S, Gormanns P, Maccarrone G, *et al* (2011). Proteomics and metabolomics analysis of a trait anxiety mouse model reveals divergent mitochondrial pathways. *Biol Psychiatry* **70**(11): 1074-1082.

Fineberg NA, Potenza MN, Chamberlain SR, Berlin HA, Menzies L, Bechara A, *et al* (2010). Probing compulsive and impulsive behaviors, from animal models to endophenotypes: a narrative review. *Neuropsychopharmacology* **35**(3): 591-604.

Fink KB, Göthert M (2007). 5-HT receptor regulation of neurotransmitter release. *Pharmacol Rev* **59**(4): 360-417.

Fiori LM, Turecki G (2008). Implication of the polyamine system in mental disorders. *J Psychiatry Neurosci* **33**(2): 102-110.

Foa EB, Stein DJ, McFarlane AC (2006). Symptomatology and psychopathology of mental health problems after disaster. *J Clin Psychiatry* **67 Suppl 2**: 15-25.

Funder JW (2010). Minireview: Aldosterone and mineralocorticoid receptors: past, present, and future. *Endocrinology* **151**(11): 5098-5102.

Gao J, Wang H, Liu Y, Li YY, Chen C, Liu LM, *et al* (2014). Glutamate and GABA imbalance promotes neuronal apoptosis in hippocampus after stress. *Med Sci Monit* **20**: 499-512.

Genda EN, Jackson JG, Sheldon AL, Locke SF, Greco TM, O'Donnell JC, *et al* (2011). Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. *J Neurosci* **31**(50): 18275-18288.

Geracioti TD, Baker DG, Ekhator NN, West SA, Hill KK, Bruce AB, *et al* (2001). CSF norepinephrine concentrations in posttraumatic stress disorder. *Am J Psychiatry* **158**(8): 1227-1230.

Geracioti TD, Baker DG, Kasckow JW, Strawn JR, Jeffrey Mulchahey J, Dashevsky BA, *et al* (2008). Effects of trauma-related audiovisual stimulation on cerebrospinal fluid norepinephrine and corticotropin-releasing hormone concentrations in post-traumatic stress disorder. *Psychoneuroendocrinology* **33**(4): 416-424.

Geracioti TD, Jefferson-Wilson L, Strawn JR, Baker DG, Dashevsky BA, Horn PS, *et al* (2013). Effect of traumatic imagery on cerebrospinal fluid dopamine and serotonin metabolites in posttraumatic stress disorder. *J Psychiatr Res* **47**(7): 995-998.

Germanò E, Gagliano A, Magazù A, Calarese T, Calabrò ME, Bonsignore M, *et al* (2006). [Neurobiology of autism: Study of a sample of autistic children]. *Minerva Pediatr* **58**(2): 109-120.

Geschwind DH, Konopka G (2009). Neuroscience in the era of functional genomics and systems biology. *Nature* **461**(7266): 908-915.

Geuze E, Westenberg HG, Jochims A, de Kloet CS, Bohus M, Vermetten E, *et al* (2007). Altered pain processing in veterans with posttraumatic stress disorder. *Arch Gen Psychiatry* **64**(1): 76-85.

Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, *et al* (2002). Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. *Nat Neurosci* **5**(11): 1242-1247.

Glaser R, Kiecolt-Glaser JK (2005). Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol* **5**(3): 243-251.

Glatt SJ, Tylee DS, Chandler SD, Pazol J, Nievergelt CM, Woelk CH, *et al* (2013). Blood-based gene-expression predictors of PTSD risk and resilience among deployed marines: a pilot study. *Am J Med Genet B Neuropsychiatr Genet* **162B**(4): 313-326.

Gola H, Engler H, Sommershof A, Adenauer H, Kolassa S, Schedlowski M, *et al* (2013). Posttraumatic stress disorder is associated with an enhanced spontaneous production of pro-inflammatory cytokines by peripheral blood mononuclear cells. *BMC Psychiatry* **13**: 40.

Golier JA, Yehuda R, De Santi S, Segal S, Dolan S, de Leon MJ (2005). Absence of hippocampal volume differences in survivors of the Nazi Holocaust with and without posttraumatic stress disorder. *Psychiatry Res* **139**(1): 53-64.

Golub Y, Kaltwasser SF, Mauch CP, Herrmann L, Schmidt U, Holsboer F, *et al* (2011). Reduced hippocampus volume in the mouse model of Posttraumatic Stress Disorder. *J Psychiatr Res* **45**(5): 650-659.

Gray JR, Braver TS, Raichle ME (2002). Integration of emotion and cognition in the lateral prefrontal cortex. *Proc Natl Acad Sci U S A* **99**(6): 4115-4120.

Gressier F, Calati R, Balestri M, Marsano A, Alberti S, Antypa N, *et al* (2013). The 5-HTTLPR polymorphism and posttraumatic stress disorder: a meta-analysis. *J Trauma Stress* **26**(6): 645-653.

Grillon C, Pine DS, Baas JM, Lawley M, Ellis V, Charney DS (2006). Cortisol and DHEA-S are associated with startle potentiation during aversive conditioning in humans. *Psychopharmacology (Berl)* **186**(3): 434-441.

Grinevich V, Fournier A, Pelletier G (1997). Effects of pituitary adenylate cyclase-activating polypeptide (PACAP) on corticotropin-releasing hormone (CRH) gene expression in the rat hypothalamic paraventricular nucleus. *Brain Res* **773**(1-2): 190-196.

Group. BDW (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* **69**(3): 89-95.

Guest PC, Knowles MR, Molon-Noblot S, Salim K, Smith D, Murray F, *et al* (2004). Mechanisms of action of the antidepressants fluoxetine and the substance P antagonist L-000760735 are associated with altered neurofilaments and synaptic remodeling. *Brain Res* **1002**(1-2): 1-10.

Guihen E, O'Connor WT (2009). Current separation and detection methods in microdialysis the drive towards sensitivity and speed. *Electrophoresis* **30**(12): 2062-2075.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* **17**(10): 994-999.

Halliwell B (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol* **141**(2): 312-322.

Hambrecht-Wiedbusch VS, Mitchell MF, Firn KA, Baghdoyan HA, Lydic R (2014). Benzodiazepine site agonists differentially alter acetylcholine release in rat amygdala. *Anesth Analg* **118**(6): 1293-1300.

Hampel H, Frank R, Broich K, Teipel SJ, Katz RG, Hardy J, *et al* (2010). Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. *Nat Rev Drug Discov* **9**(7): 560-574.

Harbers M, Carninci P (2005). Tag-based approaches for transcriptome research and genome annotation. *Nat Methods* **2**(7): 495-502.

Hebb AL, Zacharko RM, Dominguez H, Laforest S, Gauthier M, Levac C, *et al* (2003). Changes in brain cholecystokinin and anxiety-like behavior following exposure of mice to predator odor. *Neuroscience* **116**(2): 539-551.

Heim C, Newport DJ, Heit S, Graham YP, Wilcox M, Bonsall R, *et al* (2000). Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *JAMA* **284**(5): 592-597.

Henningsen K, Palmfeldt J, Christiansen S, Baiges I, Bak S, Jensen ON, *et al* (2012). Candidate hippocampal biomarkers of susceptibility and resilience to stress in a rat model of depression. *Mol Cell Proteomics* **11**(7): M111.016428.

Herman JP, Ostrander MM, Mueller NK, Figueiredo H (2005). Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry* **29**(8): 1201-1213.

Herry C, Johansen JP (2014). Encoding of fear learning and memory in distributed neuronal circuits. *Nat Neurosci* **17**(12): 1644-1654.

.

Hertzberg MA, Feldman ME, Beckham JC, Kudler HS, Davidson JR (2000). Lack of efficacy for fluoxetine in PTSD: a placebo controlled trial in combat veterans. *Ann Clin Psychiatry* **12**(2): 101-105.

Hou C, Liu J, Wang K, Li L, Liang M, He Z, *et al* (2007). Brain responses to symptom provocation and trauma-related short-term memory recall in coal mining accident survivors with acute severe PTSD. *Brain Res* **1144**: 165-174.

Imam S, Noguera DR, Donohue TJ (2015). An integrated approach to reconstructing genome-scale transcriptional regulatory networks. *PLoS Comput Biol* **11**(2): e1004103.

Initiative BISaT (2000). NIH working definition of bioinformatics and computational biology.

Jackson JG, O'Donnell JC, Takano H, Coulter DA, Robinson MB (2014). Neuronal activity and glutamate uptake decrease mitochondrial mobility in astrocytes and position mitochondria near glutamate transporters. *J Neurosci* **34**(5): 1613-1624.

Jalkanen AJ, Leikas JV, Forsberg MM (2014). Prolyl oligopeptidase inhibition decreases extracellular acetylcholine levels in rat hippocampus and prefrontal cortex. *Neurosci Lett* **579**: 110-113.

Jett JD, Morilak DA (2013). Too much of a good thing: blocking noradrenergic facilitation in medial prefrontal cortex prevents the detrimental effects of chronic stress on cognition. *Neuropsychopharmacology* **38**(4): 585-595.

Joseph MH, Kennett GA (1983). Corticosteroid response to stress depends upon increased tryptophan availability. *Psychopharmacology (Berl)* **79**(1): 79-81.

Jovanovic T, Phifer JE, Sicking K, Weiss T, Norrholm SD, Bradley B, *et al* (2011). Cortisol suppression by dexamethasone reduces exaggerated fear responses in posttraumatic stress disorder. *Psychoneuroendocrinology* **36**(10): 1540-1552.

Joëls M, Pu Z, Wiegert O, Oitzl MS, Krugers HJ (2006). Learning under stress: how does it work? *Trends Cogn Sci* **10**(4): 152-158.

Kaddurah-Daouk R, Krishnan KR (2009). Metabolomics: a global biochemical approach to the study of central nervous system diseases. *Neuropsychopharmacology* **34**(1): 173-186.

Karas M, Hillenkamp F (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* **60**(20): 2299-2301.

Karl A, Schaefer M, Malta LS, Dörfel D, Rohleder N, Werner A (2006). A meta-analysis of structural brain abnormalities in PTSD. *Neurosci Biobehav Rev* **30**(7): 1004-1031.

Karl A, Werner A (2010a). The use of proton magnetic resonance spectroscopy in PTSD research--meta-analyses of findings and methodological review. *Neurosci Biobehav Rev* **34**(1): 7-22.

Karl T, Chesworth R, Duffy L, Herzog H (2010b). Acoustic startle response and sensorimotor gating in a genetic mouse model for the Y1 receptor. *Neuropeptides* **44**(3): 233-239.

Karpova NN, Pickenhagen A, Lindholm J, Tiraboschi E, Kulesskaya N, Agústsdóttir A, *et al* (2011). Fear erasure in mice requires synergy between antidepressant drugs and extinction training. *Science* **334**(6063): 1731-1734.

Karst H, Berger S, Turiault M, Tronche F, Schütz G, Joëls M (2005). Mineralocorticoid receptors are indispensable for nongenomic modulation of hippocampal glutamate transmission by corticosterone. *Proc Natl Acad Sci U S A* **102**(52): 19204-19207.

Keen-Rhinehart E, Michopoulos V, Toufexis DJ, Martin EI, Nair H, Ressler KJ, *et al* (2009). Continuous expression of corticotropin-releasing factor in the central nucleus of the amygdala emulates the dysregulation of the stress and reproductive axes. *Mol Psychiatry* **14**(1): 37-50.

Kessler RC (2000). Posttraumatic stress disorder: the burden to the individual and to society. *J Clin Psychiatry* **61 Suppl 5**: 4-12; discussion 13-14.

Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry* **52**(12): 1048-1060.

Kim MJ, Chey J, Chung A, Bae S, Khang H, Ham B, *et al* (2008). Diminished rostral anterior cingulate activity in response to threat-related events in posttraumatic stress disorder. *J Psychiatr Res* **42**(4): 268-277.

King DW, King LA, Foy DW, Gudanowski DM (1996). Prewar factors in combat-related posttraumatic stress disorder: structural equation modeling with a national sample of female and male Vietnam veterans. *J Consult Clin Psychol* **64**(3): 520-531.

Kitano H (2002). Systems biology: a brief overview. *Science* **295**(5560): 1662-1664.

Kodzius R, Kojima M, Nishiyori H, Nakamura M, Fukuda S, Tagami M, *et al* (2006). CAGE: cap analysis of gene expression. *Nat Methods* **3**(3): 211-222.

Koenigs M, Huey ED, Raymont V, Cheon B, Solomon J, Wassermann EM, *et al* (2008). Focal brain damage protects against post-traumatic stress disorder in combat veterans. *Nat Neurosci* **11**(2): 232-237.

Kohda K, Harada K, Kato K, Hoshino A, Motohashi J, Yamaji T, *et al* (2007). Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: a putative post-traumatic stress disorder model. *Neuroscience* **148**(1): 22-33.

Kolomeets NS, Uranova N (2010). Ultrastructural abnormalities of astrocytes in the hippocampus in schizophrenia and duration of illness: a postortem morphometric study. *World J Biol Psychiatry* **11**(2 Pt 2): 282-292.

Kosten TR, Mason JW, Giller EL, Ostroff RB, Harkness L (1987). Sustained urinary norepinephrine and epinephrine elevation in post-traumatic stress disorder. *Psychoneuroendocrinology* **12**(1): 13-20.

Krettek JE, Price JL (1978). A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. *J Comp Neurol* **178**(2): 255-280.

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, *et al* (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* **131**(2): 391-404.

Lane-Donovan C, Philips GT, Herz J (2014). More than cholesterol transporters: lipoprotein receptors in CNS function and neurodegeneration. *Neuron* **83**(4): 771-787.

Lapiz MD, Morilak DA (2006). Noradrenergic modulation of cognitive function in rat medial prefrontal cortex as measured by attentional set shifting capability. *Neuroscience* **137**(3): 1039-1049.

Laurent V, Westbrook RF (2009). Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learn Mem* **16**(9): 520-529.

LeDoux JE (2000). Emotion circuits in the brain. *Annu Rev Neurosci* **23**: 155-184.

Lemos JC, Wanat MJ, Smith JS, Reyes BA, Hollon NG, Van Bockstaele EJ, *et al* (2012). Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive. *Nature* **490**(7420): 402-406.

Levkovitz Y, Fenchel D, Kaplan Z, Zohar J, Cohen H (2015). Early post-stressor intervention with minocycline, a second-generation tetracycline, attenuates post-traumatic stress response in an animal model of PTSD. *Eur Neuropsychopharmacol* **25**(1): 124-132.

Lian XY, Stringer JL (2004). Astrocytes contribute to regulation of extracellular calcium and potassium in the rat cerebral cortex during spreading depression. *Brain Res* **1012**(1-2): 177-184.

Liberzon I, Krstov M, Young EA (1997). Stress-restress: effects on ACTH and fast feedback. *Psychoneuroendocrinology* **22**(6): 443-453.

Lim MM, Liu Y, Ryabinin AE, Bai Y, Wang Z, Young LJ (2007). CRF receptors in the nucleus accumbens modulate partner preference in prairie voles. *Horm Behav* **51**(4): 508-515.

Lindqvist D, Wolkowitz OM, Mellon S, Yehuda R, Flory JD, Henn-Haase C, *et al* (2014). Proinflammatory milieu in combat-related PTSD is independent of depression and early life stress. *Brain Behav Immun* **42**: 81-88.

Liu H, Wang HT, Han F, Shi YX (2011). Activity of 5-HT1A receptor is involved in neuronal apoptosis of the amygdala in a rat model of post-traumatic stress disorder. *Mol Med Rep* **4**(2): 291-295.

Liu TF, Brown CM, El Gazzar M, McPhail L, Millet P, Rao A, *et al* (2012). Fueling the flame: bioenergy couples metabolism and inflammation. *J Leukoc Biol* **92**(3): 499-507.

Logue MW, Amstadter AB, Baker DG, Duncan L, Koenen KC, Liberzon I, *et al* (2015). The Psychiatric Genomics Consortium Posttraumatic Stress Disorder Workgroup: Posttraumatic

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

Lottspeich F, Kellermann J (2011). ICPL labeling strategies for proteome research. *Methods Mol Biol* **753**: 55-64.

Lu W, Bennett BD, Rabinowitz JD (2008). Analytical strategies for LC-MS-based targeted metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci* **871**(2): 236-242.

László A, Horváth E, Eck E, Fekete M (1994). Serum serotonin, lactate and pyruvate levels in infantile autistic children. *Clin Chim Acta* **229**(1-2): 205-207.

Lüthi A, Lüscher C (2014). Pathological circuit function underlying addiction and anxiety disorders. *Nat Neurosci* **17**(12): 1635-1643.

Maes M (1995). Evidence for an immune response in major depression: a review and hypothesis. *Prog Neuropsychopharmacol Biol Psychiatry* **19**(1): 11-38.

Maes M (2008). The cytokine hypothesis of depression: inflammation, oxidative & nitrosative stress (IO&NS) and leaky gut as new targets for adjunctive treatments in depression. *Neuro Endocrinol Lett* **29**(3): 287-291.

Maes M, Lin AH, Delmeire L, Van Gastel A, Kenis G, De Jongh R, *et al* (1999). Elevated serum interleukin-6 (IL-6) and IL-6 receptor concentrations in posttraumatic stress disorder following accidental man-made traumatic events. *Biol Psychiatry* **45**(7): 833-839.

Maier SF, Watkins LR (1998). Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol Rev* **105**(1): 83-107.

Maki AE, Morris KA, Catherman K, Chen X, Hatcher NG, Gold PE, *et al* (2014). Fibrinogen α-chain-derived peptide is upregulated in hippocampus of rats exposed to acute morphine injection and spontaneous alternation testing. *Pharmacol Res Perspect* **2**(3): e00037.

Maren S, Quirk GJ (2004). Neuronal signalling of fear memory. *Nat Rev Neurosci* **5**(11): 844-852.

Markham CM, Luckett CA, Huhman KL (2012). The medial prefrontal cortex is both necessary and sufficient for the acquisition of conditioned defeat. *Neuropharmacology* **62**(2): 933-939.

Marmar CR, Schoenfeld F, Weiss DS, Metzler T, Zatzick D, Wu R, *et al* (1996). Open trial of fluvoxamine treatment for combat-related posttraumatic stress disorder. *J Clin Psychiatry* **57 Suppl 8**: 66-70; discussion 71-62.

Maroun M (2013). Medial prefrontal cortex: multiple roles in fear and extinction. *Neuroscientist* **19**(4): 370-383.

Martenyi F, Brown EB, Caldwell CD (2007). Failed efficacy of fluoxetine in the treatment of posttraumatic stress disorder: results of a fixed-dose, placebo-controlled study. *J Clin Psychopharmacol* **27**(2): 166-170.

Martenyi F, Brown EB, Zhang H, Koke SC, Prakash A (2002). Fluoxetine v. placebo in prevention of relapse in post-traumatic stress disorder. *Br J Psychiatry* **181**: 315-320.

Martí O, García A, Vallès A, Harbuz MS, Armario A, Vellès A (2001). Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. *Eur J Neurosci* **13**(1): 129-136.

Mattson MP, Gleichmann M, Cheng A (2008). Mitochondria in neuroplasticity and neurological disorders. *Neuron* **60**(5): 748-766.

Maya Vetencourt JF, Sale A, Viegi A, Baroncelli L, De Pasquale R, O'Leary OF, *et al* (2008). The antidepressant fluoxetine restores plasticity in the adult visual cortex. *Science* **320**(5874): 385-388.

McEwen BS (1997). Possible mechanisms for atrophy of the human hippocampus. *Mol Psychiatry* **2**(3): 255-262.

McFarlane AC (1989). The aetiology of post-traumatic morbidity: predisposing, precipitating and perpetuating factors. *Br J Psychiatry* **154**: 221-228.

McFarlane AC, Barton CA, Yehuda R, Wittert G (2011). Cortisol response to acute trauma and risk of posttraumatic stress disorder. *Psychoneuroendocrinology* **36**(5): 720-727.

McFarlane AC, Yehuda R, Clark CR (2002). Biologic models of traumatic memories and post-traumatic stress disorder. The role of neural networks. *Psychiatr Clin North Am* **25**(2): 253-270, v.

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, *et al* (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nat Neurosci* **12**(3): 342-348.

Merali Z, McIntosh J, Anisman H (2004). Anticipatory cues differentially provoke in vivo peptidergic and monoaminergic release at the medial prefrontal cortex. *Neuropsychopharmacology* **29**(8): 1409-1418.

Michels L, Schulte-Vels T, Schick M, O'Gorman RL, Zeffiro T, Hasler G, *et al* (2014). Prefrontal GABA and glutathione imbalance in posttraumatic stress disorder: preliminary findings. *Psychiatry Res* **224**(3): 288-295.

Miles SR, Menefee DS, Wanner J, Teten Tharp A, Kent TA (2015). The Relationship Between Emotion Dysregulation and Impulsive Aggression in Veterans With Posttraumatic Stress Disorder Symptoms. *J Interpers Violence*.

Miller EK, Freedman DJ, Wallis JD (2002a). The prefrontal cortex: categories, concepts and cognition. *Philos Trans R Soc Lond B Biol Sci* **357**(1424): 1123-1136.

Miller GE, Cohen S, Ritchey AK (2002b). Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. *Health Psychol* **21**(6): 531-541.

Miller MM, McEwen BS (2006). Establishing an agenda for translational research on PTSD. *Ann N Y Acad Sci* **1071**: 294-312.

Mitchell P, Tollervey D (2001). mRNA turnover. *Curr Opin Cell Biol* **13**(3): 320-325.

Mitchell W (1885). *Fat and Blood* Lippincott: Philadelphia.

Mitra R, Jadhav S, McEwen BS, Vyas A, Chattarji S (2005). Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proc Natl Acad Sci U S A* **102**(26): 9371-9376.

Moco S, Jacques V, Bino RJ, De Vos RCH (2007). Metabolomics technologies and metabolite identification. *Trends in Analytical Chemistry* **26**(9): 855-866.

Morey RA, Dolcos F, Petty CM, Cooper DA, Hayes JP, LaBar KS, *et al* (2009). The role of trauma-related distractors on neural systems for working memory and emotion processing in posttraumatic stress disorder. *J Psychiatr Res* **43**(8): 809-817.

Morgan MA, Romanski LM, LeDoux JE (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci Lett* **163**(1): 109-113.

Morris MC, Compas BE, Garber J (2012). Relations among posttraumatic stress disorder, comorbid major depression, and HPA function: a systematic review and meta-analysis. *Clin Psychol Rev* **32**(4): 301-315.

Morrow BA, Elsworth JD, Rasmusson AM, Roth RH (1999). The role of mesoprefrontal dopamine neurons in the acquisition and expression of conditioned fear in the rat. *Neuroscience* **92**(2): 553-564.

Munchel SE, Shultzaberger RK, Takizawa N, Weis K (2011). Dynamic profiling of mRNA turnover reveals gene-specific and system-wide regulation of mRNA decay. *Mol Biol Cell* **22**(15): 2787-2795.

Murrough JW, Huang Y, Hu J, Henry S, Williams W, Gallezot JD, *et al* (2011). Reduced amygdala serotonin transporter binding in posttraumatic stress disorder. *Biol Psychiatry* **70**(11): 1033-1038.

Nagalakshmi U, Wang Z, Waern K, Shou C, Raha D, Gerstein M, *et al* (2008). The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science* **320**(5881): 1344-1349.

Naik E, Dixit VM (2011). Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp Med* **208**(3): 417-420.

Neelamekam S, Nurjono M, Lee J (2014). Regulation of interleukin-6 and leptin in schizophrenia patients: a preliminary analysis. *Clin Psychopharmacol Neurosci* **12**(3): 209-214.

Nicholson EL, Bryant RA, Felmingham KL (2014). Interaction of noradrenaline and cortisol predicts negative intrusive memories in posttraumatic stress disorder. *Neurobiol Learn Mem* **112**: 204-211.

O'Donovan A, Sun B, Cole S, Rempel H, Lenoci M, Pulliam L, *et al* (2011). Transcriptional control of monocyte gene expression in post-traumatic stress disorder. *Dis Markers* **30**(2-3): 123-132.

O'Farrell PH (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**(10): 4007-4021.

Oda Y, Huang K, Cross FR, Cowburn D, Chait BT (1999). Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci U S A* **96**(12): 6591-6596.

Ogłodek EA, Szota AM, Just MJ, Moś DM, Araszkiewicz A (2015). The MCP-1, CCL-5 and SDF-1 chemokines as pro-inflammatory markers in generalized anxiety disorder and personality disorders. *Pharmacol Rep* **67**(1): 85-89.

Ohman A, Mineka S (2001). Fears, phobias, and preparedness: toward an evolved module of fear and fear learning. *Psychol Rev* **108**(3): 483-522.

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, *et al* (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* **1**(5): 376-386.

Ongür D, Price JL (2000). The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. *Cereb Cortex* **10**(3): 206-219.

Osuch EA, Willis MW, Bluhm R, Ursano RJ, Drevets WC, Group CNS (2008). Neurophysiological responses to traumatic reminders in the acute aftermath of serious motor vehicle collisions using [15O]-H2O positron emission tomography. *Biol Psychiatry* **64**(4): 327-335.

Ota Y, Zanetti AT, Hallock RM (2013). The role of astrocytes in the regulation of synaptic plasticity and memory formation. *Neural Plast* **2013**: 185463.

Pace TW, Heim CM (2011). A short review on the psychoneuroimmunology of posttraumatic stress disorder: from risk factors to medical comorbidities. *Brain Behav Immun* **25**(1): 6-13.

Papadimitriou A, Priftis KN (2009). Regulation of the hypothalamic-pituitary-adrenal axis. *Neuroimmunomodulation* **16**(5): 265-271.

Papadopoulos A, Chandramohan Y, Collins A, Droste SK, Nutt DJ, Reul JM (2011). GABAergic control of novelty stress-responsive epigenetic and gene expression mechanisms in the rat dentate gyrus. *Eur Neuropsychopharmacol* **21**(4): 316-324.

Pape HC, Pare D (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiol Rev* **90**(2): 419-463.

Pare D, Duvarci S (2012). Amygdala microcircuits mediating fear expression and extinction. *Curr Opin Neurobiol* **22**(4): 717-723.

Parker KJ, Schatzberg AF, Lyons DM (2003). Neuroendocrine aspects of hypercortisolism in major depression. *Horm Behav* **43**(1): 60-66.

Pastural E, Ritchie S, Lu Y, Jin W, Kavianpour A, Khine Su-Myat K, *et al* (2009). Novel plasma phospholipid biomarkers of autism: mitochondrial dysfunction as a putative causative mechanism. *Prostaglandins Leukot Essent Fatty Acids* **81**(4): 253-264.

Pavlides C, Watanabe Y, Magariños AM, McEwen BS (1995). Opposing roles of type I and type II adrenal steroid receptors in hippocampal long-term potentiation. *Neuroscience* **68**(2): 387-394.

Peciña S, Schulkin J, Berridge KC (2006). Nucleus accumbens corticotropin-releasing factor increases cue-triggered motivation for sucrose reward: paradoxical positive incentive effects in stress? *BMC Biol* **4**: 8.

Pederson CL, Maurer SH, Kaminski PL, Zander KA, Peters CM, Stokes-Crowe LA, *et al* (2004). Hippocampal volume and memory performance in a community-based sample of women with posttraumatic stress disorder secondary to child abuse. *J Trauma Stress* **17**(1): 37-40.

Pelton GH, Lee Y, Davis M (1997). Repeated stress, like vasopressin, sensitizes the excitatory effects of corticotropin releasing factor on the acoustic startle reflex. *Brain Res* **778**(2): 381-387.

Peres JF, Newberg AB, Mercante JP, Simão M, Albuquerque VE, Peres MJ, *et al* (2007). Cerebral blood flow changes during retrieval of traumatic memories before and after psychotherapy: a SPECT study. *Psychol Med* **37**(10): 1481-1491.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**(18): 3551-3567.

Perkonigg A, Kessler RC, Storz S, Wittchen HU (2000). Traumatic events and post-traumatic stress disorder in the community: prevalence, risk factors and comorbidity. *Acta Psychiatr Scand* **101**(1): 46-59.

Pezze MA, Feldon J (2004). Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol* **74**(5): 301-320.

Pitkänen A, Pikkarainen M, Nurminen N, Ylinen A (2000). Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Ann N Y Acad Sci* **911**: 369-391.

Pitman RK, Rasmusson AM, Koenen KC, Shin LM, Orr SP, Gilbertson MW, *et al* (2012). Biological studies of post-traumatic stress disorder. *Nat Rev Neurosci* **13**(11): 769-787.

Pitman RK, Sanders KM, Zusman RM, Healy AR, Cheema F, Lasko NB, *et al* (2002). Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biol Psychiatry* **51**(2): 189-192.

Popoli M, Yan Z, McEwen BS, Sanacora G (2012). The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci* **13**(1): 22-37.

Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, *et al* (2004). Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Mol Psychiatry* **9**(7): 684-697, 643.

Price JC, Guan S, Burlingame A, Prusiner SB, Ghaemmaghami S (2010). Analysis of proteome dynamics in the mouse brain. *Proc Natl Acad Sci U S A* **107**(32): 14508-14513.

Quirarte GL, Roozendaal B, McGaugh JL (1997). Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. *Proc Natl Acad Sci U S A* **94**(25): 14048-14053.

Quirk GJ, Mueller D (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology* **33**(1): 56-72.

Radley JJ, Sisti HM, Hao J, Rocher AB, McCall T, Hof PR, *et al* (2004). Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. *Neuroscience* **125**(1): 1-6.

Rajasekaran A, Venkatasubramanian G, Berk M, Debnath M (2015). Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications. *Neurosci Biobehav Rev* **48**: 10-21.

Rauch SL, Shin LM, Phelps EA (2006). Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research--past, present, and future. *Biol Psychiatry* **60**(4): 376-382.

Rauch SL, Whalen PJ, Shin LM, McInerney SC, Macklin ML, Lasko NB, *et al* (2000). Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: a functional MRI study. *Biol Psychiatry* **47**(9): 769-776.

Ravindran LN, Stein MB (2009). Pharmacotherapy of PTSD: premises, principles, and priorities. *Brain Res* **1293**: 24-39.

Reinartz J, Bruyns E, Lin JZ, Burcham T, Brenner S, Bowen B, *et al* (2002). Massively parallel signature sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. *Brief Funct Genomic Proteomic* **1**(1): 95-104.

Resick PA, Schnicke MK (1992). Cognitive processing therapy for sexual assault victims. *J Consult Clin Psychol* **60**(5): 748-756.

Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, *et al* (2011). Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* **470**(7335): 492-497.

Reul JM (2014). Making memories of stressful events: a journey along epigenetic, gene transcription, and signaling pathways. *Front Psychiatry* **5**: 5.

Ritov G, Ardi Z, Richter-Levin G (2014). Differential activation of amygdala, dorsal and ventral hippocampus following an exposure to a reminder of underwater trauma. *Front Behav Neurosci* **8**: 18.

Rittenhouse KD, Pollack GM (2000). Microdialysis and drug delivery to the eye. *Adv Drug Deliv Rev* **45**(2-3): 229-241.

Robbins TW (2000). From arousal to cognition: the integrative position of the prefrontal cortex. *Prog Brain Res* **126**: 469-483.

Robertson SD, Plummer NW, de Marchena J, Jensen P (2013). Developmental origins of central norepinephrine neuron diversity. *Nat Neurosci* **16**(8): 1016-1023.

Robinson OJ, Krimsky M, Lieberman L, Allen P, Vytal K, Grillon C (2014). Towards a mechanistic understanding of pathological anxiety: the dorsal medial prefrontal-amygdala 'aversive amplification' circuit in unmedicated generalized and social anxiety disorders. *Lancet Psychiatry* **1**(4): 294-302.

Rogers MA, Yamasue H, Abe O, Yamada H, Ohtani T, Iwanami A, *et al* (2009). Smaller amygdala volume and reduced anterior cingulate gray matter density associated with history of post-traumatic stress disorder. *Psychiatry Res* **174**(3): 210-216.

Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL (2006). Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proc Natl Acad Sci U S A* **103**(17): 6741-6746.

Roozendaal B, Quirarte GL, McGaugh JL (2002). Glucocorticoids interact with the basolateral amygdala beta-adrenoceptor--cAMP/cAMP/PKA system in influencing memory consolidation. *Eur J Neurosci* **15**(3): 553-560.

Roseboom PH, Nanda SA, Bakshi VP, Trentani A, Newman SM, Kalin NH (2007). Predator threat induces behavioral inhibition, pituitary-adrenal activation and changes in amygdala CRF-binding protein gene expression. *Psychoneuroendocrinology* **32**(1): 44-55.

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, *et al* (2004). Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* **3**(12): 1154-1169.

Sah R, Geracioti TD (2013). Neuropeptide Y and posttraumatic stress disorder. *Mol Psychiatry* **18**(6): 646-655.

Schmidt A, Kellermann J, Lottspeich F (2005). A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics* **5**(1): 4-15.

Schnurr PP, Vielhauer MJ (1999). *Personality as a risk factor for PTSD. In R. Yehuga (Ed.), Risk factors for posttraumatic stress disorder* American Psychiatric Press: Washington, DC.

Schuff N, Meyerhoff DJ, Mueller S, Chao L, Sacrey DT, Laxer K, *et al* (2006). N-acetylaspartate as a marker of neuronal injury in neurodegenerative disease. *Adv Exp Med Biol* **576**: 241-262; discussion 361-243.

Schuff N, Neylan TC, Fox-Bosetti S, Lenoci M, Samuelson KW, Studholme C, *et al* (2008). Abnormal N-acetylaspartate in hippocampus and anterior cingulate in posttraumatic stress disorder. *Psychiatry Res* **162**(2): 147-157.

Schwartzer JJ, Careaga M, Onore CE, Rushakoff JA, Berman RF, Ashwood P (2013). Maternal immune activation and strain specific interactions in the development of autism-like behaviors in mice. *Transl Psychiatry* **3**: e240.

Seamans JK, Yang CR (2004). The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Prog Neurobiol* **74**(1): 1-58.

Segman RH, Cooper-Kazaz R, Macciardi F, Goltser T, Halfon Y, Dobroborski T, *et al* (2002). Association between the dopamine transporter gene and posttraumatic stress disorder. *Mol Psychiatry* **7**(8): 903-907.

Selye H (1974). Stress without distress. Lippincott: New York.

Shadforth IP, Dunkley TP, Lilley KS, Bessant C (2005). i-Tracker: for quantitative proteomics using iTRAQ. *BMC Genomics* **6**: 145.

Shi Q, Qin L, Wei W, Geng F, Fan R, Shin YS, *et al* (2012). Single-cell proteomic chip for profiling intracellular signaling pathways in single tumor cells. *Proc Natl Acad Sci U S A* **109**(2): 419-424.

Shin LM, Liberzon I (2010). The neurocircuitry of fear, stress, and anxiety disorders. *Neuropsychopharmacology* **35**(1): 169-191.

Shin LM, Wright CI, Cannistraro PA, Wedig MM, McMullin K, Martis B, *et al* (2005). A functional magnetic resonance imaging study of amygdala and medial prefrontal cortex responses to overtly presented fearful faces in posttraumatic stress disorder. *Arch Gen Psychiatry* **62**(3): 273-281.

Shiraki T, Kondo S, Katayama S, Waki K, Kasukawa T, Kawaji H, *et al* (2003). Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage. *Proc Natl Acad Sci U S A* **100**(26): 15776-15781.

Siegmund A, Kaltwasser SF, Holsboer F, Czisch M, Wotjak CT (2009). Hippocampal N-acetylaspartate levels before trauma predict the development of long-lasting posttraumatic stress disorder-like symptoms in mice. *Biol Psychiatry* **65**(3): 258-262.

Siegmund A, Wotjak CT (2006). Toward an animal model of posttraumatic stress disorder. *Ann N Y Acad Sci* **1071**: 324-334.

Siegmund A, Wotjak CT (2007). A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. *J Psychiatr Res* **41**(10): 848-860.

Silberman DM, Ayelli-Edgar V, Zorrilla-Zubilete M, Zieher LM, Genaro AM (2004). Impaired T-cell dependent humoral response and its relationship with T lymphocyte

sensitivity to stress hormones in a chronic mild stress model of depression. *Brain Behav Immun* **18**(1): 81-90.

Smith ER, Porter KE, Messina MG, Beyer JA, Defever ME, Foa EB, *et al* (2015). Prolonged Exposure for PTSD in a Veteran group: a pilot effectiveness study. *J Anxiety Disord* **30**: 23-27.

Soeter M, Kindt M (2011). Noradrenergic enhancement of associative fear memory in humans. *Neurobiol Learn Mem* **96**(2): 263-271.

Solati J, Kleehaupt E, Kratz O, Moll GH, Golub Y (2015). Inverse effects of lipopolysaccharides on anxiety in pregnant mice and their offspring. *Physiol Behav* **139**: 369-374.

Solomon Z, Garb R, Bleich A, Grupper D (1987). Reactivation of combat-related posttraumatic stress disorder. *Am J Psychiatry* **144**(1): 51-55.

Song Y, Zhou D, Guan Z, Wang X (2007). Disturbance of serum interleukin-2 and interleukin-8 levels in posttraumatic and non-posttraumatic stress disorder earthquake survivors in northern China. *Neuroimmunomodulation* **14**(5): 248-254.

Southwick SM, Krystal JH, Bremner JD, Morgan CA, Nicolaou AL, Nagy LM, *et al* (1997). Noradrenergic and serotonergic function in posttraumatic stress disorder. *Arch Gen Psychiatry* **54**(8): 749-758.

Spiers T, Harrington G (2001). *A brief history of trauma* Taylor & Francis Group: New York.

Spivak B, Vered Y, Graff E, Blum I, Mester R, Weizman A (1999). Low platelet-poor plasma concentrations of serotonin in patients with combat-related posttraumatic stress disorder. *Biol Psychiatry* **45**(7): 840-845.

Steckler T, Risbrough V (2012). Pharmacological treatment of PTSD - established and new approaches. *Neuropharmacology* **62**(2): 617-627.

Steen H, Mann M (2004). The ABC's (and XYZ's) of peptide sequencing. *Nat Rev Mol Cell Biol* **5**(9): 699-711.

Stein MB, Kerridge C, Dimsdale JE, Hoyt DB (2007). Pharmacotherapy to prevent PTSD: Results from a randomized controlled proof-of-concept trial in physically injured patients. *J Trauma Stress* **20**(6): 923-932.

Stone EA, McEwen BS, Herrera AS, Carr KD (1987). Regulation of alpha and beta components of noradrenergic cyclic AMP response in cortical slices. *Eur J Pharmacol* **141**(3): 347-356.

Strawn JR, Geracioti TD (2008). Noradrenergic dysfunction and the psychopharmacology of posttraumatic stress disorder. *Depress Anxiety* **25**(3): 260-271.

Su J, Tanaka Y, Muratsubaki T, Kano M, Kanazawa M, Fukudo S (2015). Injection of corticotropin-releasing hormone into the amygdala aggravates visceral nociception and induces noradrenaline release in rats. *Neurogastroenterol Motil* **27**(1): 30-39.

Su ZH, Li SQ, Zou GA, Yu CY, Sun YG, Zhang HW, *et al* (2011). Urinary metabonomics study of anti-depressive effect of Chaihu-Shu-Gan-San on an experimental model of depression induced by chronic variable stress in rats. *J Pharm Biomed Anal* **55**(3): 533-539.

Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, *et al* (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* **144**(5): 810-823.

Swanson LW (1982). The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. *Brain Res Bull* **9**(1-6): 321-353.

Szabo DT (2014). *Biomarkers in toxicology: transcriptomic biomarkers in safety and risk assessment of chemicals* Oxford Academic Press.

Söndergaard HP, Hansson LO, Theorell T (2004). The inflammatory markers C-reactive protein and serum amyloid A in refugees with and without posttraumatic stress disorder. *Clin Chim Acta* **342**(1-2): 93-98.

Tachi M, Kondo F, Fukayama M, Yoshikawa K, Matsuura K, Okada S (2014). Mass spectrometric determination of prostanoids in rat hypothalamic paraventricular nucleus microdialysates. *Auton Neurosci* **181**: 49-54.

Takei S, Morinobu S, Yamamoto S, Fuchikami M, Matsumoto T, Yamawaki S (2011). Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. *J Psychiatr Res* **45**(4): 460-468.

Tanda G, Valentini V, De Luca MA, Perra V, Serra GP, Di Chiara G (2015). A systematic microdialysis study of dopamine transmission in the accumbens shell/core and prefrontal cortex after acute antipsychotics. *Psychopharmacology (Berl)* **232**(8): 1427-1440.

Theodoridis GA, Gika HG, Want EJ, Wilson ID (2012). Liquid chromatography-mass spectrometry based global metabolite profiling: a review. *Anal Chim Acta* **711**: 7-16.

Thoeringer CK, Henes K, Eder M, Dahlhoff M, Wurst W, Holsboer F, *et al* (2012). Consolidation of remote fear memories involves Corticotropin-Releasing Hormone (CRH) receptor type 1-mediated enhancement of AMPA receptor GluR1 signaling in the dentate gyrus. *Neuropsychopharmacology* **37**(3): 787-796.

Tirosh O, Sen CK, Roy S, Packer L (2000). Cellular and mitochondrial changes in glutamate-induced HT4 neuronal cell death. *Neuroscience* **97**(3): 531-541.

Totah NK, Jackson ME, Moghaddam B (2013). Preparatory attention relies on dynamic interactions between prelimbic cortex and anterior cingulate cortex. *Cereb Cortex* **23**(3): 729-738.

Tovote P, Fadok JP, Lüthi A (2015). Neuronal circuits for fear and anxiety. *Nat Rev Neurosci* **16**(6): 317-331.

Tsai TH, Cheng FC, Chen YF, Chen CF (2001). On-line microdialysis coupled with microbore liquid chromatography with ultraviolet detection for continuous monitoring of free cefsulodin in rat blood. *J Chromatogr A* **914**(1-2): 83-87.

Tsoory M, Cohen H, Richter-Levin G (2007). Juvenile stress induces a predisposition to either anxiety or depressive-like symptoms following stress in adulthood. *Eur Neuropsychopharmacol* **17**(4): 245-256.

Turck CW, Maccarrone G, Sayan-Ayata E, Jacob AM, Ditzen C, Kronsbein H, *et al* (2005). The quest for brain disorder biomarkers. *J Med Invest* **52 Suppl**: 231-235.

Tylee DS, Chandler SD, Nievergelt CM, Liu X, Pazol J, Woelk CH, *et al* (2015). Blood-based gene-expression biomarkers of post-traumatic stress disorder among deployed marines: A pilot study. *Psychoneuroendocrinology* **51**: 472-494.

Tynan RJ, Naicker S, Hinwood M, Nalivaiko E, Buller KM, Pow DV, *et al* (2010). Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions. *Brain Behav Immun* **24**(7): 1058-1068.

Ungerstedt U, Pycock C (1974). Functional correlates of dopamine neurotransmission. *Bull Schweiz Akad Med Wiss* **30**(1-3): 44-55.

Vaiva G, Thomas P, Ducrocq F, Fontaine M, Boss V, Devos P, *et al* (2004). Low posttrauma GABA plasma levels as a predictive factor in the development of acute posttraumatic stress disorder. *Biol Psychiatry* **55**(3): 250-254.

van der Kolk BA, Dreyfuss D, Michaels M, Shera D, Berkowitz R, Fisler R, *et al* (1994). Fluoxetine in posttraumatic stress disorder. *J Clin Psychiatry* **55**(12): 517-522.

van Dijken HH, de Goeij DC, Sutanto W, Mos J, de Kloet ER, Tilders FJ (1993). Short inescapable stress produces long-lasting changes in the brain-pituitary-adrenal axis of adult male rats. *Neuroendocrinology* **58**(1): 57-64.

van Huijstee AN, Mansvelder HD (2014). Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction. *Front Cell Neurosci* **8**: 466.

van Zuiden M, Geuze E, Willemen HL, Vermetten E, Maas M, Amarouchi K, *et al* (2012). Glucocorticoid receptor pathway components predict posttraumatic stress disorder symptom development: a prospective study. *Biol Psychiatry* **71**(4): 309-316.

van Zuiden M, Kavelaars A, Geuze E, Olff M, Heijnen CJ (2013). Predicting PTSD: pre-existing vulnerabilities in glucocorticoid-signaling and implications for preventive interventions. *Brain Behav Immun* **30**: 12-21.

Vaquero-Lorenzo C, Riaza Bermudo-Soriano C, Perez-Rodriguez MM, Diaz-Hernandez M, López-Castromán J, Fernandez-Piqueras J, *et al* (2009). Positive association between SAT-1 -1415T/C polymorphism and anxiety. *Am J Med Genet B Neuropsychiatr Genet* **150B**(4): 515-519.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW (1995). Serial analysis of gene expression. *Science* **270**(5235): 484-487.

Veldhuis HD, Van Koppen C, Van Ittersum M, De Kloet ER (1982). Specificity of the adrenal steroid receptor system in rat hippocampus. *Endocrinology* **110**(6): 2044-2051.

Vermetten E, Schmahl C, Southwick SM, Bremner JD (2007). Positron tomographic emission study of olfactory induced emotional recall in veterans with and without combat-related posttraumatic stress disorder. *Psychopharmacol Bull* **40**(1): 8-30.

Voloboueva LA, Suh SW, Swanson RA, Giffard RG (2007). Inhibition of mitochondrial function in astrocytes: implications for neuroprotection. *J Neurochem* **102**(4): 1383-1394.

Volpicelli JR, Ulm RR (1990). The influence of control over appetitive and aversive events on alcohol preference in rats. *Alcohol* **7**(2): 133-136.

von Känel R, Begré S, Abbas CC, Saner H, Gander ML, Schmid JP (2010). Inflammatory biomarkers in patients with posttraumatic stress disorder caused by myocardial infarction and the role of depressive symptoms. *Neuroimmunomodulation* **17**(1): 39-46.

Vukojevic V, Kolassa IT, Fastenrath M, Gschwind L, Spalek K, Milnik A, *et al* (2014). Epigenetic modification of the glucocorticoid receptor gene is linked to traumatic memory and post-traumatic stress disorder risk in genocide survivors. *J Neurosci* **34**(31): 10274-10284.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* **22**(15): 6810-6818.

Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB (2005). Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. *J Neurosci* **25**(22): 5389-5396.

Wang Z, Gerstein M, Snyder M (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**(1): 57-63.

Werner NS, Meindl T, Engel RR, Rosner R, Riedel M, Reiser M, *et al* (2009). Hippocampal function during associative learning in patients with posttraumatic stress disorder. *J Psychiatr Res* **43**(3): 309-318.

Westerhout J, Ploeger B, Smeets J, Danhof M, de Lange EC (2012). Physiologically based pharmacokinetic modeling to investigate regional brain distribution kinetics in rats. *AAPS J* **14**(3): 543-553.

Whitehouse CM, Dreyer RN, Yamashita M, Fenn JB (1985). Electrospray interface for liquid chromatographs and mass spectrometers. *Anal Chem* **57**(3): 675-679.

Williams LM, Kemp AH, Felmingham K, Barton M, Olivieri G, Peduto A, *et al* (2006). Trauma modulates amygdala and medial prefrontal responses to consciously attended fear. *Neuroimage* **29**(2): 347-357.

Wilm M, Mann M (1996a). Analytical properties of the nanoelectrospray ion source. *Anal Chem* **68**(1): 1-8.

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, *et al* (1996b). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**(6564): 466-469.

Wilson CB, McLaughlin LD, Nair A, Ebenezer PJ, Dange R, Francis J (2013). Inflammation and oxidative stress are elevated in the brain, blood, and adrenal glands during the progression of post-traumatic stress disorder in a predator exposure animal model. *PLoS One* **8**(10): e76146.

Wingenfeld K, Whooley MA, Neylan TC, Otte C, Cohen BE (2015). Effect of current and lifetime posttraumatic stress disorder on 24-h urinary catecholamines and cortisol: results from the Mind Your Heart Study. *Psychoneuroendocrinology* **52**: 83-91.

Wingenfeld K, Wolf OT (2011). HPA axis alterations in mental disorders: impact on memory and its relevance for therapeutic interventions. *CNS Neurosci Ther* **17**(6): 714-722.

Woodward SH, Kaloupek DG, Streeter CC, Martinez C, Schaer M, Eliez S (2006). Decreased anterior cingulate volume in combat-related PTSD. *Biol Psychiatry* **59**(7): 582-587.

Yamamoto S, Morinobu S, Iwamoto Y, Ueda Y, Takei S, Fujita Y, *et al* (2010). Alterations in the hippocampal glycinergic system in an animal model of posttraumatic stress disorder. *J Psychiatr Res* **44**(15): 1069-1074.

Yang P, Wu MT, Hsu CC, Ker JH (2004). Evidence of early neurobiological alternations in adolescents with posttraumatic stress disorder: a functional MRI study. *Neurosci Lett* **370**(1): 13-18.

Yang R, Daigle BJ, Muhie SY, Hammamieh R, Jett M, Petzold L, *et al* (2013). Core modular blood and brain biomarkers in social defeat mouse model for post traumatic stress disorder. *BMC Syst Biol* **7**: 80.

Yates JR, Cociorva D, Liao L, Zabrouskov V (2006). Performance of a linear ion trap-Orbitrap hybrid for peptide analysis. *Anal Chem* **78**(2): 493-500.

Yau JL, Noble J, Kenyon CJ, Ludwig M, Seckl JR (2015). Diurnal and stress-induced intra-hippocampal corticosterone rise attenuated in 11β-HSD1-deficient mice: a microdialysis study in young and aged mice. *Eur J Neurosci* **41**(6): 787-792.

Yehuda R (2001). Biology of posttraumatic stress disorder. *J Clin Psychiatry* **62 Suppl 17**: 41-46.

Yehuda R, Bell A, Bierer LM, Schmeidler J (2008). Maternal, not paternal, PTSD is related to increased risk for PTSD in offspring of Holocaust survivors. *J Psychiatr Res* **42**(13): 1104-1111.

Yehuda R, Koenen KC, Galea S, Flory JD (2011a). The role of genes in defining a molecular biology of PTSD. *Dis Markers* **30**(2-3): 67-76.

Yehuda R, Pratchett LC, Elmes MW, Lehrner A, Daskalakis NP, Koch E, *et al* (2014). Glucocorticoid-related predictors and correlates of post-traumatic stress disorder treatment response in combat veterans. *Interface Focus* **4**(5): 20140048.

Yehuda R, Seckl J (2011b). Minireview: Stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. *Endocrinology* **152**(12): 4496-4503.

Yehuda R, Siever LJ, Teicher MH, Levengood RA, Gerber DK, Schmeidler J, *et al* (1998). Plasma norepinephrine and 3-methoxy-4-hydroxyphenylglycol concentrations and severity of depression in combat posttraumatic stress disorder and major depressive disorder. *Biol Psychiatry* **44**(1): 56-63.

Yehuda R, Southwick S, Giller EL, Ma X, Mason JW (1992). Urinary catecholamine excretion and severity of PTSD symptoms in Vietnam combat veterans. *J Nerv Ment Dis* **180**(5): 321-325.

Yerys BE, Herrington JD (2014). Multimodal imaging in autism: an early review of comprehensive neural circuit characterization. *Curr Psychiatry Rep* **16**(11): 496.

You JM, Yun SJ, Nam KN, Kang C, Won R, Lee EH (2009). Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. *Can J Physiol Pharmacol* **87**(6): 440-447.

Yuan M, Breitkopf SB, Yang X, Asara JM (2012). A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat Protoc* **7**(5): 872-881.

Zelikowsky M, Hersman S, Chawla MK, Barnes CA, Fanselow MS (2014). Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. *J Neurosci* **34**(25): 8462-8466.

Zhang CL, Houbaert X, Lepleux M, Deshors M, Normand E, Gambino F, *et al* (2014). The hippocampo-amygdala control of contextual fear expression is affected in a model of intellectual disability. *Brain Struct Funct*.

Zhang L, Li H, Benedek D, Li X, Ursano R (2009). A strategy for the development of biomarker tests for PTSD. *Med Hypotheses* **73**(3): 404-409.

Zhang Y, Reckow S, Webhofer C, Boehme M, Gormanns P, Egge-Jacobsen WM, *et al* (2011). Proteome scale turnover analysis in live animals using stable isotope metabolic labeling. *Anal Chem* **83**(5): 1665-1672.

Zhe D, Fang H, Yuxiu S (2008). Expressions of hippocampal mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the single-prolonged stress-rats. *Acta Histochem Cytochem* **41**(4): 89-95.

Zoladz PR, Conrad CD, Fleshner M, Diamond DM (2008). Acute episodes of predator exposure in conjunction with chronic social instability as an animal model of post-traumatic stress disorder. *Stress* **11**(4): 259-281.

Zoladz PR, Diamond DM (2013). Current status on behavioral and biological markers of PTSD: a search for clarity in a conflicting literature. *Neurosci Biobehav Rev* **37**(5): 860-895.

Zoladz PR, Fleshner M, Diamond DM (2012). Psychosocial animal model of PTSD produces a long-lasting traumatic memory, an increase in general anxiety and PTSD-like glucocorticoid abnormalities. *Psychoneuroendocrinology* **37**(9): 1531-1545.

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

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

	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:

Kao CY, Stalla G, Stalla J, Wotjak C, Anderzhanova E. Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice. *European Journal of Neuroscience*, 41(9): 1139-48

Kao CY, Anderzhanova E, Asara JM, Wotjak C, Turck CW. NextGen Brain Microdialysis – applying modern metabolomics technology to the analysis of extracellular fluid. *Molecular Neuropsychiatry*, 1:60-67

List of author contributions

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

Chi-Ya Kao, Zhisong He, Kathrin Henes, John M. Asara, Philipp Khaitovich, Carsten T. Wotjak, Christoph W. Turck (2015). Fluoxetine treatment rescues energy metabolism pathway and myelin sheath protein alterations in a posttraumatic stress disorder mouse model. Submitted.

- 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

Chi-Ya Kao, Zhisong He, Oliver Hahn, Carsten T. Wotjak, Philipp Khaitovich, Christoph W. Turck (2015). Transcriptomic profiling reveals affected inflammatory pathway in a mouse model of posttraumatic stress disorder. Submitted.

- 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

Kao CY, Stalla G, Stalla J, Wotjak C, Anderzhanova E (2015). Norepinephrine and corticosterone in the medial prefrontal cortex and hippocampus predict PTSD-like symptoms in mice. European Journal of Neuroscience 41(9): 1139-48

- 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

Kao CY**,** Anderzhanova E, Asara JM, Wotjak C, Turck CW (2015). NextGen Brain Microdialysis – applying modern metabolomics technology to the analysis of extracellular fluid. Molecular Neurophychiatry 1: 60-67

- 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

June 30, 2015

Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation
'Pathway and biomarker discovery in a posttraumatic stress disorder mouse model'
selbstständig angefertigt habe, mich außer der angegebenen keiner
weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder
annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter
Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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