

Dissertation of the Faculty of Biology  
at the Ludwig-Maximilians-University Munich

Molecular delineation of cellular pathways  
associated with the antidepressant treatment response

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2016





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## Summary

A substantial number of psychiatric patients do not benefit from chronic antidepressant treatment. To identify biochemical pathways that can stratify antidepressant response sub-groups, DBA/2J mice were subjected to paroxetine administration for 28 days and classified into drug responder and non-responder groups based on floating time during the forced swim test (FST). Hippocampal metabolome and proteome profiles were analyzed and integrated to identify significant molecular pathway differences between paroxetine-responding and non-responding animals. I identified metabolites and proteins involved in purine and pyrimidine metabolism pathways whose levels were significantly different between paroxetine responding and non-responding mice. In addition, the glutamate/ubiquitin proteasome system (UPS)-associated pathways were associated with the chronic paroxetine treatment response. Specifically, N-Methyl-D-aspartate (NMDA) receptor, postsynaptic density protein 95 (PSD-95), and neuronal nitric oxide synthase (nNOS) levels significantly correlated with FST floating time suggesting their potential role in the antidepressant treatment response. The results from mice were further corroborated in human peripheral blood mononuclear cells (PBMCs) of major depressive disorder (MDD) patients. Protein signatures including ATIC, CPS2, PM2A, sGC- $\beta$ 1 and protein ubiquitination significantly correlated with clinical antidepressant treatment response. In my thesis project I have identified affected pathways and biomarker candidates related to the heterogeneous antidepressant treatment response using integrated -omics analyses combined with sub-group stratification based on behavioral phenotyping.

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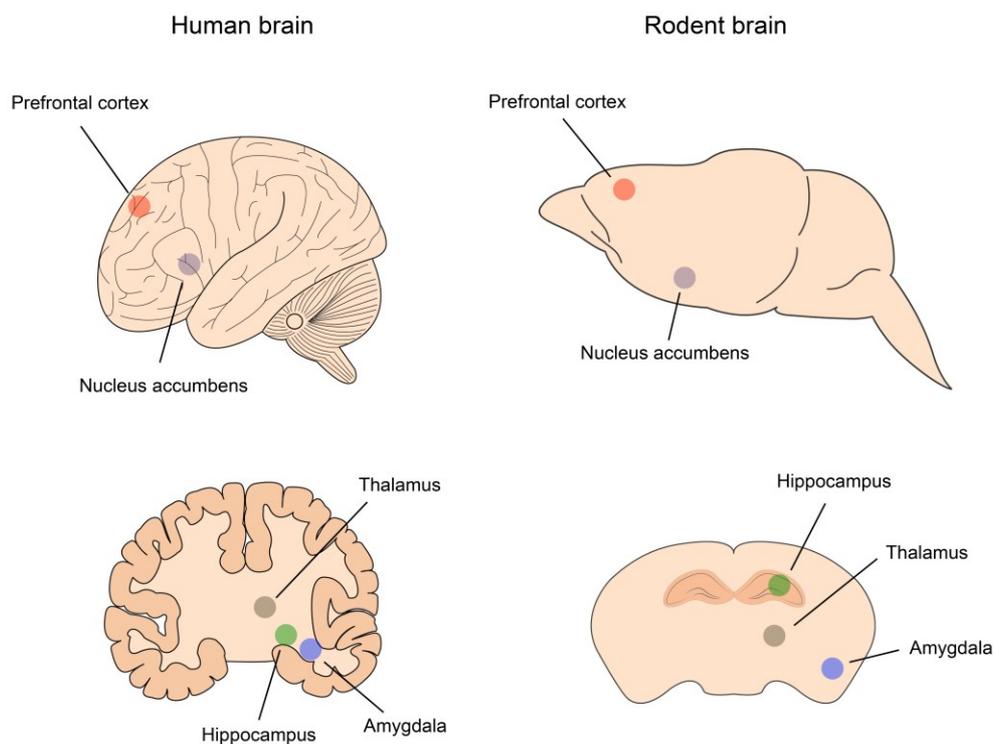


# 1. Introduction

Major depressive disorder (MDD) is one of the most common psychiatric disorders which is characterized by persistent decrease/loss of interest and low mood (Hori et al., 2016). The World Health Organization estimates 350 million people suffer from MDD. MDD is often associated with suicide attempts and ranked second for global disease burden (Beautrais et al., 1996; Murray and Lopez, 1997).

## 1.1. Neuroanatomy of major depressive disorder

Several brain regions involved in cognitive and emotional processing have been shown to be affected in MDD (Figure 1).



**Figure 1.** Brain regions affected in MDD.

The prefrontal cortex is part of the limbic system that controls emotional and cognitive functions. The association of prefrontal cortex with mood disorders has been extensively studied in animals and humans. Chronic stress has been shown to cause dendritic spine atrophy in rodents (Qiao et al., 2016). Prefrontal cortex dysfunction has been implicated in depressed patients (Merriam et al., 1999; Murray et

al., 2011). Structural atrophy has been shown in the prefrontal cortex of depressed patients (Drevets et al., 1997; Bremner et al., 2002).

Hippocampal volume loss and structural atrophy have been frequently reported in MDD patients (Sheline et al., 1996; 1999; Campbell et al., 2004; Opel et al., 2014). In the hippocampus adult neurogenesis takes place and this process plays important roles in various brain functions including synaptic plasticity, learning and memory, and emotional regulation (Jun et al., 2012). Hippocampal adult neurogenesis has been associated with MDD pathology and antidepressant treatment response (Malberg et al., 2000; Anacker et al., 2011; Lee et al., 2013; Mahar et al., 2014; Rotheneichner et al., 2014).

Nucleus accumbens (NAc) has also been strongly associated with stress-related neuropsychiatric conditions. Chronic mild stress was shown to induce decreased dopamine D2 receptor expression in the NAc (Papp et al., 1994). Altered serotonin and dopamine turnover was shown in the Flinders sensitive line, a rat model of depression (Zangen et al., 1999; 2001). Reduced NAc activity and volume have been reported in patients with mood disorders (Baumann et al., 1999; Heller et al., 2009). Its extensive functional connectivity with other brain regions including prefrontal cortex, hippocampus and amygdala that are also significantly associated with depressive disorders implicate a critical role of NAc in MDD pathophysiology (Shirayama and Chaki, 2006).

Volumetric abnormality has been observed in the amygdala of MDD subjects. Unmedicated depression patients showed a decrease of amygdala volume compared to controls (Hamilton et al., 2008). Depressed female individuals were shown to have a smaller amygdala (Hastings et al., 2004) and a hyperactive amygdala has been shown in depressed individuals (Drevets et al., 1992; Yang et al., 2010).

Like other brain regions involved in MDD, a significant volume decrease of the thalamus has also been observed in MDD individuals (Nugent et al., 2013). Thalamic area hyperactivity was shown to correlate with treatment-resistant depression and antidepressant treatment response (Yamamura et al., 2016).

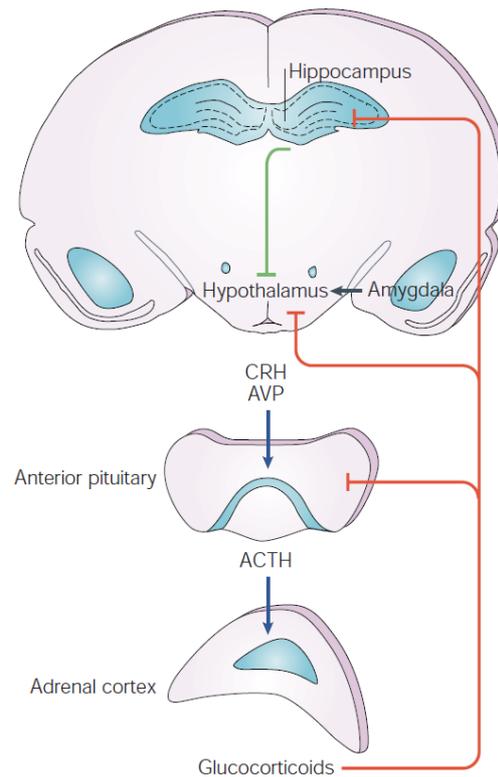
## 1.2. Neurobiology of major depressive disorder

### 1.2.1. Neurotrophic hypothesis of depression

Neurotrophic factors are growth factors that are essential for neuronal cell proliferation and survival. Based on observations in rodents and depression patients low levels of neurotrophic factors have been implicated in the pathobiology of depression. They include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Particularly BDNF has been extensively studied and associated with depressive disorders. BDNF level alterations have also been found in animal models of chronic stress (Murakami et al., 2005; Dwivedi, 2009). Antidepressants increase BDNF expression in brain regions including the hippocampus and prefrontal cortex (Lee and Kim, 2010). BDNF was shown to produce antidepressant-like effects (Shirayama et al., 2002) implying its critical role in the pathology and treatment of depressive disorders.

### 1.2.2. HPA axis hypothesis of depression

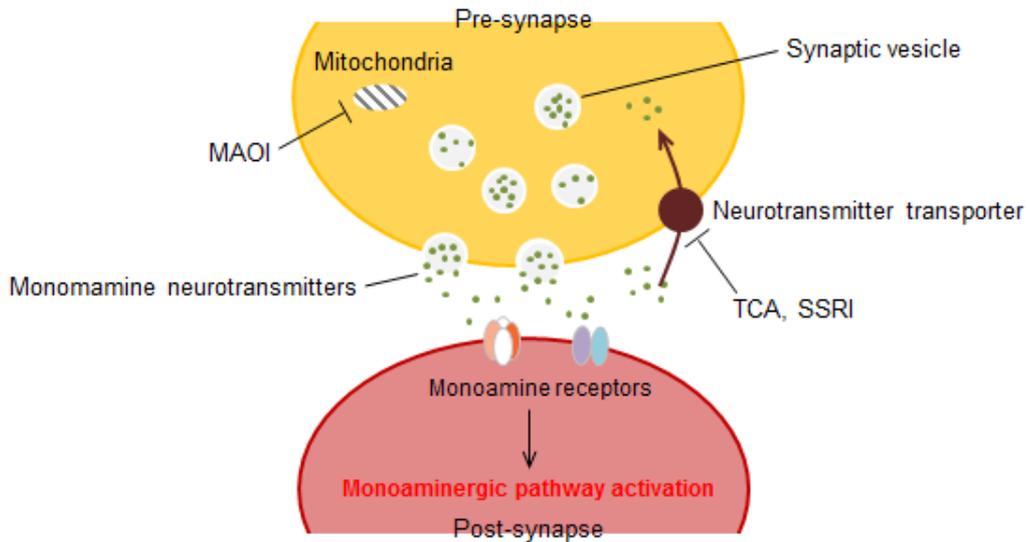
The hypothalamic-pituitary-adrenal (HPA) axis is a network that spans three endocrine systems, hypothalamus, pituitary gland and adrenal gland. In response to a stressor, neuropeptides including corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) are secreted from the paraventricular nucleus of hypothalamus. This causes adrenocorticotrophic hormone (ACTH) release from the anterior pituitary, which stimulates adrenal cortex to synthesize and release glucocorticoids into the blood stream. Negative feedback control of the HPA axis is mediated by glucocorticoid receptor (Figure 2). HPA axis abnormalities have been found in depressive disorders. Depression patients have increased cortisol levels in body fluids including saliva, plasma and urine (Nemeroff and Vale, 2005). An increased volume of the pituitary (MacMaster and Kusumakar, 2004; MacMaster et al., 2006) and adrenal gland (Rubin et al., 1995) have been found in patients with depressive disorder reflecting stress-induced HPA axis dysregulation.



**Figure 2.** Diagram of the HPA axis (adapted from Sandi, 2004).

### 1.2.3. Monoamine hypothesis of depression

Drugs and agents that increase monoamine neurotransmitter levels and availability in the synaptic cleft have been found to be effective for alleviating depressive symptoms (Sangkuhl et al., 2009; Dell'Osso et al., 2011). These observations led to the hypothesis that monoamine deficiency may cause depressive symptoms. In this regard, depressive disorders have been associated with impaired neurotransmission of monoaminergic pathways (D'Aquila et al., 2000; Schmidt and Reith, 2005; Popik et al., 2006). Conventional antidepressants including TCAs, SSRIs and MAOIs affect monoamine levels including serotonin, norepinephrine and dopamine. Drugs of the tricyclic antidepressant (TCA) and selective serotonin reuptake inhibitor (SSRI) type inhibit monoamine transporters that regulate extracellular monoamine levels by reuptake from the synaptic cleft. The resulting increased neurotransmitter levels activate post-synaptic monoaminergic pathways. Monoamine oxidase inhibitor (MAOI) increases neurotransmitter levels by inhibiting monoamine oxidase (MAO) activity that catalyzes the degradation of monoamine neurotransmitters (Figure 3).



**Figure 3.** Monoamine neurotransmission in the synapse.

#### 1.2.4. Neuroplasticity theory of depression

Neuroplasticity defines neuronal adaptation which includes structural and functional activity changes. Hippocampal synaptic plasticity in the form of long-term potentiation (LTP) persistently strengthens electrical signals in the synapse. Calcium,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartate (NMDA) receptors, cyclic adenosine monophosphate (cAMP), calcium-calmodulin-dependent kinase II (CaMKII) and cAMP response element binding protein (CREB) are important for the regulation of neuronal plasticity (Pittenger and Duman, 2008). Stress was shown to impair LTP in the rodent hippocampus (Kim and Diamond, 2002). High levels of glucocorticoid, a stress hormone, suppress hippocampal LTP (De Kloet, 2004). In the prefrontal cortex, chronic stress not only induces pyramidal cell dendrite atrophy (Cook and Wellman, 2004; Radley et al., 2004), but also leads to glia and endothelial cell number reduction in animal models of stress (Banasr et al., 2007). Depressed individuals show decreased neuroplasticity, which was measured by motor cortical excitability in response to transient brain stimulation (Ford and Erlinger, 2004; Liukkonen et al., 2006; Elovainio et al., 2009; Vogelzangs et al., 2012; Player et al., 2013; Valkanova et al., 2013; Hickman et al., 2014b).

### 1.3. Biomarkers for major depressive disorder

Research using animal models and patient specimens has resulted in several biomarker candidates for major depressive disorder (MDD).

C-reactive protein (CRP) is an acute phase protein circulating in blood whose concentration rises rapidly in response to physiological changes including infection and inflammation (Thompson et al., 1999). Several studies have found that elevated CRP level are associated with MDD (Ford and Erlinger, 2004; Liukkonen et al., 2006; Elovainio et al., 2009; Howren et al., 2009; Vogelzangs et al., 2012; Valkanova et al., 2013; Hickman et al., 2014a).

The HPA axis activity has been suggested as a potential biomarker of MDD. The dexamethasone suppression test (DST) has been used to test adrenal gland activity. Dexamethasone, a synthetic analogue of cortisol, induces negative feedback control to the pituitary gland and suppresses cortisol release from the adrenal gland. Plasma cortisol levels in response to dexamethasone injection can be used as a measure for HPA axis dysfunction (Targum et al., 1983; Dam et al., 1985; Fountoulakis et al., 2008).

BDNF serum levels have been proposed as an MDD biomarker in the clinic (Karege et al., 2002; Shimizu et al., 2003; Karege et al., 2005). FK506-binding protein 51 (FKBP51) is a co-chaperone protein that interacts with glucocorticoid receptor and regulates its activity. FKBP51 has been found to be a risk factor for stress-related neuropsychiatric disorders including MDD (Lekman et al., 2008; Binder, 2009). Several *FKBP5* gene polymorphisms have been found to be associated with depressive disorders (Appel et al., 2011; Szczepankiewicz et al., 2014). Reduced p11 mRNA and protein expression in NAc and hippocampus have been reported in depressed patients and suicide victims (Svenningsson et al., 2006; Anisman et al., 2008; Alexander et al., 2010). Despite all the promising research findings none of these biomarkers have made it to the clinic to guide diagnosis of depressive disorders.

## 1.4. Antidepressant drugs

### 1.4.1. First generation of antidepressants

The first generation of antidepressants was developed in 1950s. Isoniazid and iproniazid were used as anti-tuberculosis agents and found to be potent inhibitors of MAO and to have psycho-stimulant effects (Healy, 2000). The TCA imipramine was subsequently developed by the Swiss psychiatrist Ronald Kuhn. Tricyclics were first found to inhibit norepinephrine reuptake and later also shown to block the serotonin transporter (López-Muñoz and Alamo, 2009). Hence TCAs act as serotonin-norepinephrine reuptake inhibitors (SNRIs) resulting in elevated neurotransmitter levels in the synaptic cleft.

### 1.4.2. Second generation of antidepressants

MAOIs and TCAs mode of action led to the hypothesis that the drugs' antidepressant activities are related to elevated neurotransmitter levels (Pletscher, 1991). Novel SSRIs and selective norepinephrine reuptake inhibitors (SNRIs) were subsequently developed and introduced on the market. SSRIs and SNRIs have fewer adverse side effects compared to other types of antidepressants. Currently they are used as first line medications for the treatment of a wide range of psychiatric disorders.

### 1.4.3. Antidepressant treatment response

Approximately 50% of MDD patients do not respond adequately to conventional antidepressant treatment (Trivedi et al., 2006; Papakostas, 2009). The patients who fail to achieve remission after two or more of antidepressant trials are diagnosed to have treatment resistant depression (TRD) (Malhi et al., 2005). The development of novel more targeted antidepressant drugs has not been very successful. According to several meta-analyses with SSRIs, patients' response rate remains at around 47% (Nelson, 1998).

To overcome this high rate of drug non-response, several clinical strategies have been conducted. Augmentation therapy uses a second non-antidepressant agent with antidepressant medication. Augmentation treatment with lithium has shown a significant increase of response rate (de Montigny et al., 1983; Heninger et al., 1983;

Schöpf et al., 1989; Joffe and Schuller, 1993; Stein and Bernadt, 1993). Augmentation therapies with thyroid hormone and 5-HT<sub>2A</sub> receptor antagonists such as trazodone have been reported to be more effective than antidepressant monotherapy (Maes et al., 1996; Joffe, 1997). Various other substances including pindolol, omega-3 fatty acids, modafinil, bupropion/bupropion and methylphenidate, testosterone, mecamylamine and inositol have been used for augmentation therapy (Papakostas, 2009).

Combination therapy uses multiple antidepressants. The combination of desipramine (noradrenergic TCA) with fluoxetine has been shown to be more effective for achieving a response in MDD patients (Fava et al., 1994; Nemeroff et al., 1996). Using mirtazapine in combination with SSRIs was also found to be effective for TRD treatment (Carpenter et al., 1998). The adjunctive effect of mianserin was also found superior compared to SSRI monotherapy (Maes et al., 1999; Ferreri et al., 2001).

Electric and magnetic brain stimulations induce an antidepressant-like effect in TRD patients. Deep brain stimulation (DBS) is a surgical method that implants electrodes and stimulates certain brain regions (Mayberg et al., 2005; Kennedy et al., 2011). Transcranial magnetic stimulation (TMS) applies electro-magnetic fields to stimulate a population of nerve cells. Because of its non-invasive and safe nature, TMS is a promising therapeutic strategy to treat TRD (George et al., 1995; Pascual-Leone et al., 1996; Lee et al., 2012). Electroconvulsive therapy is a procedure that applies electric current through the brain to induce biochemical and functional activity changes. This method has been shown to be effective for the treatment of TRD (Khalid et al., 2008; Dierckx et al., 2012; Kellner et al., 2012).

Novel fast-acting antidepressant-like agents have also been investigated for the treatment of TRD. They include scopolamine and ketamine. Scopolamine, an antagonist for muscarinic cholinergic receptors has rapid antidepressant-like effects in TRD patients (Furey and Drevets, 2006; Drevets et al., 2013; Jaffe et al., 2013). Ketamine, an NMDA receptor blocker, also produces a rapid antidepressant effect (Murrough et al., 2013; Lally et al., 2014) in TRD patients (Murrough et al., 2013; Lally et al., 2014).

Recent research has delineated potential candidates associated with antidepressant treatment resistance. Serotonin transporter (Huezo-Diaz et al., 2009) and serotonin autoreceptors (Malagié et al., 2001; Samuels et al., 2015) have been found to be critical for the antidepressant response. In addition, alterations and abnormalities of the HPA axis have been associated with antidepressant treatment outcome (Binder et

al., 2008; Ventura-Juncá et al., 2014). BDNF gene Val66Met polymorphism has been also studied with regard to the antidepressant treatment response and was shown to result in antidepressant treatment resistance in rodents and humans (Chen et al., 2006; Zou et al., 2010a; 2010b; Kocabas et al., 2011). A link between inflammatory cytokines and antidepressant response has been documented. Cerebrospinal fluid IL-1, IL-6 and TNF- $\alpha$  blood levels in MDD patients were significantly correlated with depression severity (Martinez et al., 2012). High cytokine concentrations have been found in antidepressant treatment resistant depression patients (Sluzewska et al., 1997; Lanquillon et al., 2000; Fitzgerald et al., 2006).

### 1.5. Personalized medicine strategy

The high heterogeneity of the antidepressant treatment response requires a tailored treatment to improve therapeutic efficacy. Several studies have been conducted to identify biological markers that predict and/or evaluate the antidepressant treatment response. Genetic polymorphism studies have found polymorphisms in tryptophan hydroxylase, serotonin transporter and serotonin 5-HT<sub>2</sub> receptor to be statistically associated with SSRI treatment outcome (Serretti et al., 2001; Zanardi et al., 2001; Arias et al., 2003; Serretti et al., 2006; Ham et al., 2007; Serretti et al., 2007).

Neuroimaging measurements have identified neurophysiological markers for the antidepressant response. Mayberg et al. found that brain region-specific blood glucose metabolism changes were observed only in 6-week fluoxetine treatment responder MDD patients (Mayberg et al., 2000; 2002). Several studies have reported that a decrease of theta cordance from prefrontal electroencephalography during the first weeks with either SSRI or SNRI treatment is able to predict symptom improvement for the following weeks of continued treatment (Cook et al., 2002; 2005; Bares et al., 2007).

Omics data promise to have great potential for the personalized medicine approach in psychiatry. Psychiatric patient sub-group stratification based on omics profiling data allows a more precise and tailored treatment (Guest et al., 2013; Sethi and Brietzke, 2015).

## 1.6. Omics analyses

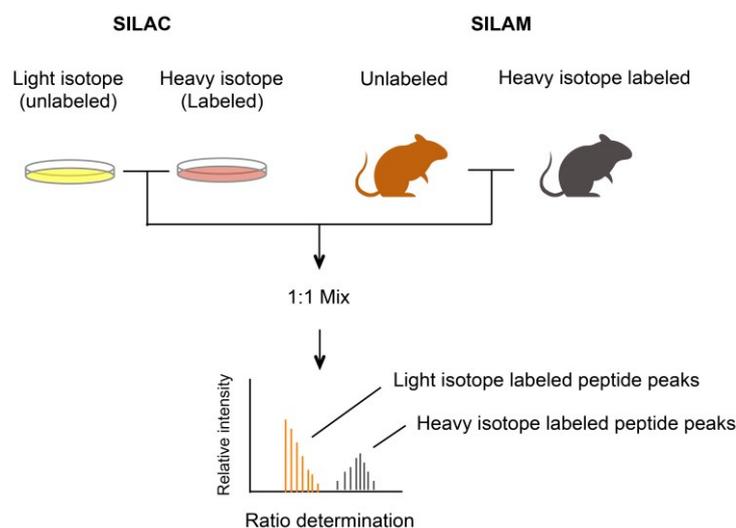
### 1.6.1. Quantitative proteomics

The shotgun proteomics approach has been very successful for the high-throughput analysis of complex protein mixtures (Domon and Aebersold, 2010; Meissner and Mann, 2014). The method involves enzymatic digestion of proteins into peptides that are subjected to tandem mass spectrometry. With the help of stable isotopes proteomes can be profiled and compared by quantitative mass spectrometry.

#### 1.6.1.1. Metabolic labeling

Stable isotopes can be incorporated into proteins during cellular synthesis *in vitro* and *in vivo* (Figure 4). For the stable isotope labeling of amino acids in cell culture (SILAC) method labeled essential amino acids (arginine, lysine and methionine) are added to the cell culture medium (Ong et al., 2002). Due to the mass difference between light and heavy isotopes, mass spectrum signals of unlabeled and labeled peptides have different mass-to-charge ratio ( $m/z$ ) values and their intensities can be used for quantification.

Stable isotope labeling in mammals (SILAM) refers to *in vivo* labeling of the entire mammalian proteome with stable isotopes.  $^{13}\text{C}$ - or  $^{15}\text{N}$ -containing diets are used for partial or full proteome labeling in rodents (Kruger et al., 2008; Zhang et al., 2011b). Labeled rodent tissues and organs can serve as reference material to investigate *in vivo* proteome turnover and expression changes (Filiou et al., 2011; Zhang et al., 2011b; Webhofer et al., 2013).



**Figure 4.** SILAC and SILAM metabolic labeling methods.

### 1.6.1.2. Post-synthesis labeling

Chemical probes have been used for post-synthesis labeling of peptides and proteins. Whereas metabolic labeling methods require cellular protein synthesis, non-metabolic labeling with chemical probes is applied to investigate body fluids.

Isotope-coded affinity tag (ICAT) consists of three domains, a reactive group for labeling amino acids, an isotopically coded linker region and a tag for affinity isolation. The ratio of signal intensities between light and heavy ICATs is used for the relative quantification of two samples. Four sets of isotope-coded protein label (ICPL) probes, ICPL0, ICPL4, ICPL6 and ICPL10, are available for the comparison of four different samples. Each probe has a different mass by replacing  $^1\text{H}$  or  $^{12}\text{C}$  with deuterium or  $^{13}\text{C}$ , respectively.

Isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tag (TMT) are used for the comparison of multiple biological conditions (Tonack et al., 2013; Núñez Galindo et al., 2015; Yao et al., 2015). Isobaric tags consist of three components, an amine reactive group that allows covalent binding to amino acids, a reporter group that includes a differential mass, and balance group between the other two components. The different reporter masses from multiple samples can be analyzed in one mass spectrometry run. TMT isobaric tags with various combinations of  $^{13}\text{C}$  and  $^{15}\text{N}$  allow comparison of 10 different samples.

### 1.6.1.3. Label-free quantitation

Label-free quantitation is a method based on measuring peptide peak areas, intensities or spectral counts. In contrast to protein labeling methods that combine labeled and unlabeled samples, during label-free quantitation samples are subjected separately to mass spectrometry analysis (Zhu et al., 2010).

## 1.6.2. Targeted metabolomics

Metabolomics has been an important method to investigate biological pathway and metabolism changes (Sato et al., 2012; Shah et al., 2012; Inoue et al., 2013). It has been extensively applied for biomarker research and the identification of affected biological pathway (Griffiths et al., 2010; Armitage and Barbas, 2014).

Metabolomics analysis has been conducted with several platforms including nuclear magnetic resonance, liquid chromatography coupled to mass spectrometry and gas chromatography coupled to mass spectrometry.

Two different strategies can be utilized for metabolomics analysis – untargeted and targeted. Untargeted metabolomics assesses all measurable analytes including the ones with unknown identity, which requires follow-ups for their identification and characterization. Targeted metabolomics captures biochemically characterized small molecules. By using internal standards or metabolite signal intensity, targeted metabolomics data can be quantitative. Relevant biochemical pathways can be enriched by a list of identified metabolites. Quantified metabolite levels also reflect pathway activity.

## 1.7. Aim of the thesis

Using quantitative -omics analyses and *in silico* data integration this thesis aims at identifying biosignatures and molecular pathways relevant for the stratification of antidepressant treatment sub-groups and antidepressant efficacy. The study represents an attempt to address the high rate of antidepressant non-response, one of the major problems in psychiatry, and to bridge the translational gap between preclinical and clinical studies.

The separation of antidepressant responder and non-responder sub-groups is a prerequisite for biomarker identification. My -omics analyses were carried out with specimens from an animal model that reflects clinical bimodal distribution of patient sub-groups, which was established in Prof. Marianne Müllers laboratory during her tenure at the *Max Planck Institute of Psychiatry*.

Resulting from -omics profiles of the mouse model, protein signatures that are part of the identified pathways were further corroborated in mice and MDD patients' peripheral blood mononuclear cells (PBMCs) with the aim of identifying biomarker candidates relevant for assessing antidepressant treatment response.

## 2. Materials and Methods

### 2.1. Animal housing and husbandry

The experiments were performed with male DBA/2J mice (Charles River Laboratories, Chatillon-sur-Chalaronne, France). All animals were between 8-10 weeks old and single-housed for at least one week prior to the beginning of the experiments. Mice were held under normal light and temperature conditions (12 light: 12 dark light cycle, lights on at 7 pm, temperature at  $23 \pm 2^\circ\text{C}$ , and humidity at  $55 \pm 5\%$ ) with standard bedding and nesting material, in polycarbonate cages (21 x 15 x 14 cm). Water and Altromin 1324 standard mouse chow (Altromin GmbH, Lage, Germany) were provided *ad libitum*. All procedures were carried out in accordance with the European Communities Council Directive 2010/63/EU and approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

### 2.2. Drug administration

Mice were treated with either vehicle or 5 mg/kg paroxetine pills (Paroxetine hydrochloride Carbone Scientific, London, UK) for 28 days twice a day. Animals were randomly assigned to the vehicle- or paroxetine-treated groups. Either vehicle or paroxetine was voluntarily self-administered via customized palatable pellets (40mg PQPellets, Phenoquest AG, Martinsried, Germany). Animals that did not take the pills properly were excluded from further analyses.

### 2.3. Mouse brain and blood collection

Blood was collected at least one month before commencing paroxetine treatment from retro-orbital puncture and after 28 days of paroxetine treatment through cardiac puncture or trunk blood. On day 29, the animals were subjected to a forced swim test (FST) and sacrificed. Trunk blood and brains of the animals were collected and stored at  $-80^\circ\text{C}$  until further use. Blood was centrifuged to separate plasma and erythrocytes (1300g, 10 min,  $4^\circ\text{C}$ ) before storage.

## 2.4. Behavioral analysis

### 2.4.1. Forced Swim Test

The forced swim test was performed to evaluate antidepressant-like activity of chronic paroxetine treatment in DBA2/J mice, and to further stratify paroxetine-treated mouse sub-groups based on behavior profiles. After 28 days of paroxetine pill administration, mice were subjected to the forced swim test on day 29. Mice were placed into a glass beaker (height 24 cm, diameter 13 cm) filled with water ( $21 \pm 1^\circ\text{C}$ ) up to a height of 15 cm, so that the animals were unable to reach the ground or escape for 6 min testing. After the test, animals were immediately dried and returned to home cage. Main parameter of interest is floating time scored by an experienced observer blind to treatment.

### 2.4.2. Female urine sniffing test

The female urine sniffing test was conducted to assess anhedonia-like behavior of animals before and after chronic paroxetine treatment. The test was performed prior to commencing paroxetine treatment and 28 days after treatment. Mice were habituated to a cotton swab inserted into their home cage for 1 h prior to testing. Mice were exposed to a sterile cotton swab dipped into water for 3 min and after a 45 min inter trial interval. Then they were exposed to a sterile cotton swab dipped in estrous female urine from the same strain. Total sniffing time was recorded.

## 2.5. Paroxetine measurements

Mouse whole brains were homogenized in a fivefold volume of phosphate buffered saline containing protease inhibitor cocktail tablets (Roche, Penzberg, Germany) using a Dispomix Drive (Medic Tools AG, Zug, Switzerland). All samples were prepared using Ostro protein precipitation and phospholipid removal plates (Waters, Eschborn, Germany). Plasma and brain homogenates were analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) using an Agilent 1100 Series (Agilent, Waldbronn, Germany) liquid chromatograph interfaced with an Applied Biosystems API 4000 (ABSciex, Darmstadt, Germany) triple quadrupole mass spectrometer. Deuterated paroxetine (Paro-D6) was used as internal

standard. Five  $\mu\text{l}$  samples were loaded and gradient eluted from an Accucore RP-MS 2.6  $\mu\text{m}$  column (2.1 x 50 mm, Thermo Scientific, Dreieich, Germany) at a flow rate of 0.3 ml/min and 30°C (eluent A: methanol, 10 mM ammonium formate, 0.1% formic acid eluent B: 10 mM ammonium formate, 0.1% formic acid). Gradient: 0-0.5 min 20% A, 0.5-2 min 20- 90% A, 1 min held at 90% A, 3-3.5 min 90-20% A and 3.5-8 min 20% A. The ion source was operated in positive mode at 500°C and multiple reaction monitoring collision-induced dissociation was performed using nitrogen collision gas. The collision energy was set to 29 V for paroxetine and 33 V for Paro-D6. The transitions monitored during analysis were  $m/z$  330  $\rightarrow$  192 for paroxetine and  $m/z$  336  $\rightarrow$  198 for Paro-D6.

## 2.6. Omics analyses

### 2.6.1. Proteomics analysis

Mouse hippocampus was homogenized in a buffer containing 2M NaCl, 10mM HEPES/NaOH, 1mM EDTA and protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Homogenates were sonicated with an ultra-sonicator (Branson, Danbury, CT, USA) and centrifuged (16100g, 20 min, 4°C). The protein concentration was quantified by Bradford assay.

Protein extracts were mixed with equal amounts of  $^{15}\text{N}$ -labeled DBA/2 mouse hippocampal protein extract (Sato et al., 2012; Shah et al., 2012; Inoue et al., 2013). Forty  $\mu\text{g}$  of  $^{14}\text{N}$ -  $^{15}\text{N}$  hippocampal protein mixture was separated in a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250 (BioRad, Hercules, CA, USA) overnight. After destaining and cutting the gel lane into slices, gel bands were further destained 3 times with 25 mM  $\text{NH}_4\text{HCO}_3$ /50% acetonitrile (Merck, Darmstadt, Germany). The gel slices were then reduced with 10 mM dithiothreitol (BioRad, Hercules, CA, USA) for 30 min at 56°C and carboxyaminomethylated with 50 mM iodoacetamide (Biorad, Hercules, CA, USA) for 30 min at room temperature, followed by additional twice of washing with with 25 mM  $\text{NH}_4\text{HCO}_3$ /50% acetonitrile. The gel slices were subjected to tryptic digestion to produce peptides (overnight, 37°C). Tryptic peptides were extracted with 2% formic acid/50%

acetonitrile (Merck, Darmstadt, Germany) with shaking. The peptides were lyophilized and dissolved in 1% formic acid (Merck, Darmstadt, Germany). The extracted peptides were analyzed by LC-MS/MS using a nanoflow HPLC-2D system (Eksigent, Dublin, California) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Proteins were identified by Sequest (Thermo Fischer, Scientific, Bremen, Germany) search using a decoy Uniprot mouse protein  $^{14}\text{N}$  and  $^{15}\text{N}$  database. Peptide search results were filtered and combined with the help of Trans-Proteomic Pipeline (TPP). Based on protein group detection data from TPP, protein quantitation was carried out using ProRata software (version 1.0).

### 2.6.2. Metabolomics analysis

A 30-fold excess (w/v) of 80% cold methanol was added to the hippocampus and prefrontal cortex. Brain tissues were homogenized ( $1200\text{ min}^{-1}$ , 2 min, Potter-S homogenizer, Sartorius, Göttingen, Germany) on ice and centrifuged (14000g, 10 min,  $4^{\circ}\text{C}$ ). Supernatants were transferred and a 6-fold excess (w/v) of 80% cold methanol was added to the pellets. Pellets were sonicated to further extract metabolites and combined with the previous supernatants. Combined samples were vortexed, centrifuged (14000g, 10 min,  $4^{\circ}\text{C}$ ) and lyophilized.

Mouse plasma metabolites were extracted with a 4-fold excess (v/v) of 100% cold methanol. After vortexing for 2 min, samples were incubated on dry ice for 2 h and centrifuged (2053 g, 10 min,  $4^{\circ}\text{C}$ ). Supernatants were filtered using a  $0.22\text{ }\mu\text{m}$  ultrafiltration tube (1105g, 2 min,  $4^{\circ}\text{C}$ ) and the filtrates were lyophilized. The lyophilized metabolites were stored at  $-80^{\circ}\text{C}$  until further use. Samples were dissolved in  $20\text{ }\mu\text{l}$  liquid chromatography-mass spectrometry grade water. Ten microliters were injected and analyzed using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX, Framingham, MA, USA) coupled to a Prominence UFLC high-performance liquid chromatography system (Shimadzu, Columbia, MD, USA) via selected reaction monitoring of a total of 280 endogenous water-soluble metabolites for steady-state analyses of samples. Samples were delivered to the mass spectrometer via normal phase chromatography using a  $4.6\text{-mm i.d} \times 10\text{ cm}$  Amide Xbridge HILIC column (Waters, Milford, MA, USA) at  $350\text{ }\mu\text{l min}^{-1}$ . Gradients were run starting from 85% buffer B (high-performance liquid chromatography grade acetonitrile) to 42% B from 0 to 5 min 42% B to 0% B from 5 to 16 min 0% B was

held from 16 to 24 min 0% B to 85% B from 24 to 25 min 85% B was held for 7 min to re-equilibrate the column. Buffer A comprised 20mM ammonium hydroxide/20mM 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 291 selected reaction monitoring transitions using positive/negative polarity switching. Electrospray ionization voltage was +4900 V in positive ion mode and – 4500 V in negative ion mode. The dwell time was 4ms per selected reaction monitoring 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-selected reaction monitoring transition were integrated using the MultiQuant v2.0 software (AB/SCIEX). Animals from the same cohort were used for all metabolomics analyses. Animals from the same cohort were used for all metabolomics analyses.

## 2.7. Molecular techniques

### 2.7.1. qRT-PCR

Hippocampal total RNA was isolated with TRIzol reagent (Invitrogen, Karlsruhe, Germany) as previously described (Schmidt et al., 2010). RNA levels were quantified using NanoPhotometer (IMPLEN, Munich, Germany). One ug of RNA was subjected to reverse transcription using Omniscript RT kit according to manufacturer's protocol (Quiagen, Santa Clarita, CA, USA). QuantFast SYBR Green PCR kit (Quiagen, Santa Clarita, CA, USA) was used for Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). The reaction was performed using LightCycler 480 (Roche Diagnostics, Penzberg, Germany). The cycling condition used was as follows: denaturation step at 95°C for 10min, followed by 45 cycles of amplification step (95°C for 10sec, 60°C for 30sec, for each cycle). Each set of primer was used for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), nNOS, NR1, NR2A, NR2B and PSD95 (Eurofins MWG Operon, Ebersberg, Germany) (Table 1). Each sample was analyzed in duplicate and normalized with GAPDH level. Relative quantitation was performed based on crossing points value (Pfaffl, 2001).

Gene	Primer sequence (5' to 3')
NR1	Forward: CTGCGACCCCAAGATTGTCAA
	Reverse: TATTGGCCTGGTTTACTGCCT
NR2A	Forward: ACGTGACAGAACGCGAACTT
	Reverse: TCAGTGCGGTTTCATCAATAACG
NR2B	Forward: CAGCAAAGCTCGTTCCCAAAA
	Reverse: GTCAGTCTCGTTCATGGCTAC
PSD-95	Forward: TCCGGGAGGTGACCCATTC
	Reverse: TTTCCGGCGCATGACGTAG
nNOS	Forward: AGCTCCTGGAACGACTACCTG
	Reverse: CCGGCACACAGCTCTAGTG
GAPDH	Forward: AACTTTGGCATTGTGGAAGG
	Reverse: GGATGCAGGGATGATGTT

**Table 1.** List of primers used for qRT-PCR analysis.

### 2.7.2. Immunoprecipitation

Hippocampal proteins were extracted and immunoprecipitated using Pierce Direct IP kit (Thermo Fisher Scientific, Rockford, IL, USA). Mouse hippocampus was homogenized with IP lysis/wash buffer containing 2% SDS (Sigma, St. Louis, MO, USA), protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Lowry assay was performed to measure protein concentration with DC Protein Assay kit (Bio-Rad Laboratories, Munich, Germany). 10ug of Ub antibody (Santa Cruz, Dallas, TX, USA) was covalently bound to the resin according to manufacturer's protocol. 500ug of hippocampal lysates were boiled (95°C, 10 min) and diluted with 5 volumes of IP lysis/wash buffer containing 2% Triton X-100, protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma, St. Louis, MO, USA). The lysates were incubated with Ub antibody-coupled resin (4°C, overnight). The resin was washed three times with IP lysis/wash buffer and was boiled with 1×SDS loading buffer for elution (95°C, 10 min). Immunoprecipitates were separated in a 10% SDS-PAGE gel, and Western blot analysis was performed with NR1, NR2A and PSD-95 antibodies. Ubiquitinated protein levels were normalized by total ubiquitination intensity.

### 2.7.3. Western blot analysis

Mouse hippocampus, prefrontal cortex and erythrocytes were homogenized with the same buffer used for proteomics sample preparation, or RIPA buffer containing protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Patient PBMCs were homogenized with RIPA buffer containing protease and phosphatase inhibitors. Homogenates were sonicated and centrifuged (16100g, 20 min, 4°C). Bradford assay was used to quantify extracted protein concentration. Proteins were separated in 10-15% gradient SDS-PAGE gels. Subsequently, they were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blotting, the membrane was blocked with 5% skim milk solution for 1 h at room temperature and incubated with  $\beta$ -actin (1:4000, Sigma, St. Louis, MO, USA), aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP cyclohydrolase (ATIC) (1:500, Santa Cruz, Dallas, TX, USA),  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK) II (1:2000, Abcam, Cambridge, UK), carboxy-terminal PDZ ligand of *nNOS* (CAPON) (1:500, Santa Cruz, Dallas, TX, USA), carbamoyl phosphate synthase 2 (CPS2) (1:500, Santa Cruz, Dallas, TX, USA), extracellular signal-regulated kinase (ERK) (1:1000, Cell Signaling, Danvers, MA, USA), glutamate dehydrogenase 1 (GDH1) (1:1000, Aviva System Biology, San Diego, CA, USA), glutamine synthetase (GS) (1:1000, Sigma, St. Louis, MO, USA), glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) (1:1000, Cell Signaling, Danvers, MA, USA), soluble guanylate cyclase- $\beta$ 1 (sGC- $\beta$ 1) (1:500, Santa Cruz, Dallas, TX, USA), hypoxanthine-guanine phosphoribosyltransferase (HPRT) (1:500, Sigma, St. Louis, MO, USA), mitochondrial aspartate transaminase (mAST) (1:500, Sigma, St. Louis, MO, USA), mitogen-activated protein kinase kinase (MEK) (1:1000, Cell Signaling, Danvers, MA, USA), neuronal nitric oxide synthase (nNOS) (1:1000, Cell Signaling, Danvers, MA, USA), N-Methyl-D-aspartate (NMDA) receptor subunit (NR) 1 (1:500, Santa Cruz, Dallas, TX, USA), NR2A (1:500, Santa Cruz, Dallas, TX, USA), NR2B (1:500, Santa Cruz, Dallas, TX, USA), phospho-CaMKII (P-CaMKII) (1:1000, Cell Signaling, Danvers, MA, USA), phospho-ERK (P-ERK) (1:1000, Cell Signaling, Danvers, MA, USA), phospho-GSK-3 $\beta$  (Ser9) (P-GSK-3 $\beta$ ) (1:1000, Cell Signaling, Danvers, MA, USA), phospho-MEK (P-MEK) (1:1000, Cell Signaling, Danvers, MA, USA), phospho-NR1 (P-NR1) (1:500, Santa Cruz, Dallas, TX, USA), phospho-NR2A (P-NR2A) (1:500, Santa Cruz, Dallas, TX, USA), phospho-NR2B (P-NR2B)

(1:500, Santa Cruz, Dallas, TX, USA), postsynaptic density protein 95 (PSD-95) (1:1000, GeneTex, Irvine, CA, USA), proteasome subunit  $\alpha$  type-2 (PMSA2), synapsin (1:1000, Cell Signaling, Danvers, MA, USA), synaptic vesicle glycoprotein 2A (SV2A), synaptojanin 1 (SYNJ1), syntaxin binding protein1 (STXBP1) or ubiquitin (Ub) (1:500, Santa Cruz, Dallas, TX, USA) antibody at 4°C overnight. PMSA2, SYNJ1, STXBP1 and SV2A antibodies were provided by the Human Protein Atlas (HPA) program (Albanova University Center, Royal Institute of Technology, Sweden).

The membranes were washed and then incubated with horseradish peroxidase (HRP) conjugated-secondary antibodies. The blots were developed with Luminata<sup>TM</sup> Forte Western HRP Substrate (Millipore, Billerica, MA, USA). Images were acquired by ChemiDoc<sup>TM</sup> MP imaging system (Bio-Rad Laboratories, Munich, Germany). Densitometric data analyses were carried out with ImageJ software (National Institute of Health, USA).

## 2.8. Patient samples

For *in vivo* and *ex vivo* studies, two distinct PBMC batches from different individuals were chosen. PBMCs obtained from 17 participants of the Munich Antidepressant Response Signature (MARS) study were included for assessing protein expression levels (Table 2). PBMCs from 32 individuals were subjected to *ex vivo* cultivation and paroxetine treatment (Table 3). Diagnosis was conducted according to DSM-IV criteria, and all participants were diagnosed as having MDD. Depression severity was evaluated using the 21-item Hamilton Depression Rating Scale (HDRS). Responder and non-responder patients were classified based on clinical antidepressant treatment response corresponding to minimal 50% reduction in HDRS score between baseline (T0) and after 6 weeks of admission (T6). The Munich Antidepressant Response Signature (MARS) project was approved by the ethics committee of the Medical Faculty at *Ludwig Maximilians University* of Munich, Germany (submission number 318/00). Participants included in the study gave oral and written consents after receiving a complete description of the study.

Characteristic	Responders	Non-responders	Statistics
	Mean ( $\pm$ SD)	Mean ( $\pm$ SD)	
Age	49 (14)	49 (11.6)	$p = 0.58$
Males/Females	6/4	3/4	$\chi^2 = 0.4857, p = 0.49$
HDRS at admittance	23.8 (5.7)	22.5 (4.2)	$p = 0.35$
HDRS at T6	7 (3.6)	17.1 (4.2)	$p = 2.0 \times E^4$

HDRS, Hamilton Depression Rating Score  
T6, after six weeks of admission

**Table 2.** Demographic features of antidepressant treatment responder and non-responder patients.

Characteristic	Responders	Non-responders	Statistics
	Mean ( $\pm$ SD)	Mean ( $\pm$ SD)	
Age	47.1 (16.1)	51.5 (16.1)	$p = 1.0$
Males/Females	5/10	6/11	$\chi^2 = 0.136, p = 0.91$
HDRS at admittance	23.4 (5.3)	22.5 (4.2)	$p = 0.52$
HDRS at T6	4.6 (3.7)	18.9 (5.2)	$p < 0.0001$

HDRS, Hamilton Depression Rating Score  
T6, after six weeks of admission

**Table 3.** Demographic and clinical characteristics of antidepressant responders and non-responders included in *ex vivo* PBMCs cultivation and paroxetine treatment.

### 2.8.1. Paroxetine treatment of PBMCs

Blood of patients with MDD was collected between 08:00 and 09:00 h within 5 days after admittance. PBMCs were prepared as described previously (Gassen et al., 2014). Blood was collected from depression patients via venepuncture, and centrifuged (800g, 20 min) to separate PBMCs. Using Biocoll separating solution, PBMCs were enriched and washed with ice-cold PBS. PBMCs were plated at  $4 \times 10^5$  cells/cm<sup>2</sup>. After 6 hours, cells were treated with 120ng/ml paroxetine for 2 days according to the consensus guidelines for therapeutic drug monitoring in psychiatry (Lotrich and Pollock, 2005; Hiemke et al., 2011).

### 2.8.2. Protein level quantitation in cultivated PBMCs

In *ex vivo* cultivated PBMCs, ATIC, CPS2, HPRT and  $\beta$ -actin protein levels were detected and quantitated with an automated capillary immunoassay system, Simple Western<sup>TM</sup> (ProteinSimple, Santa Clara, CA, USA). PBMCs lysates were prepared according to manufacturer's instruction. Fluorescent master mix was prepared with 400mM DTT, 5 $\times$  fluorescent master mix solution and biotinylated protein ladder. Protein lysates were incubated with master mix solution and denatured at 95°C for 5 min. Protein samples, primary and secondary antibodies were loaded on 96 well plate. All following steps were fully automated. Protein levels were quantified using antibodies for  $\beta$ -actin (1:150, Sigma, St.Louis, MO, USA), ATIC (1:25, Santa Cruz, Dallas, TX, USA) and CPS2 (1:50, Santa Cruz, Dallas, TX, USA). Protein quantitation data were normalized with  $\beta$ -actin. Quantitative analysis was performed using Compass software (ProteinSimple, Santa Clara, CA, USA).

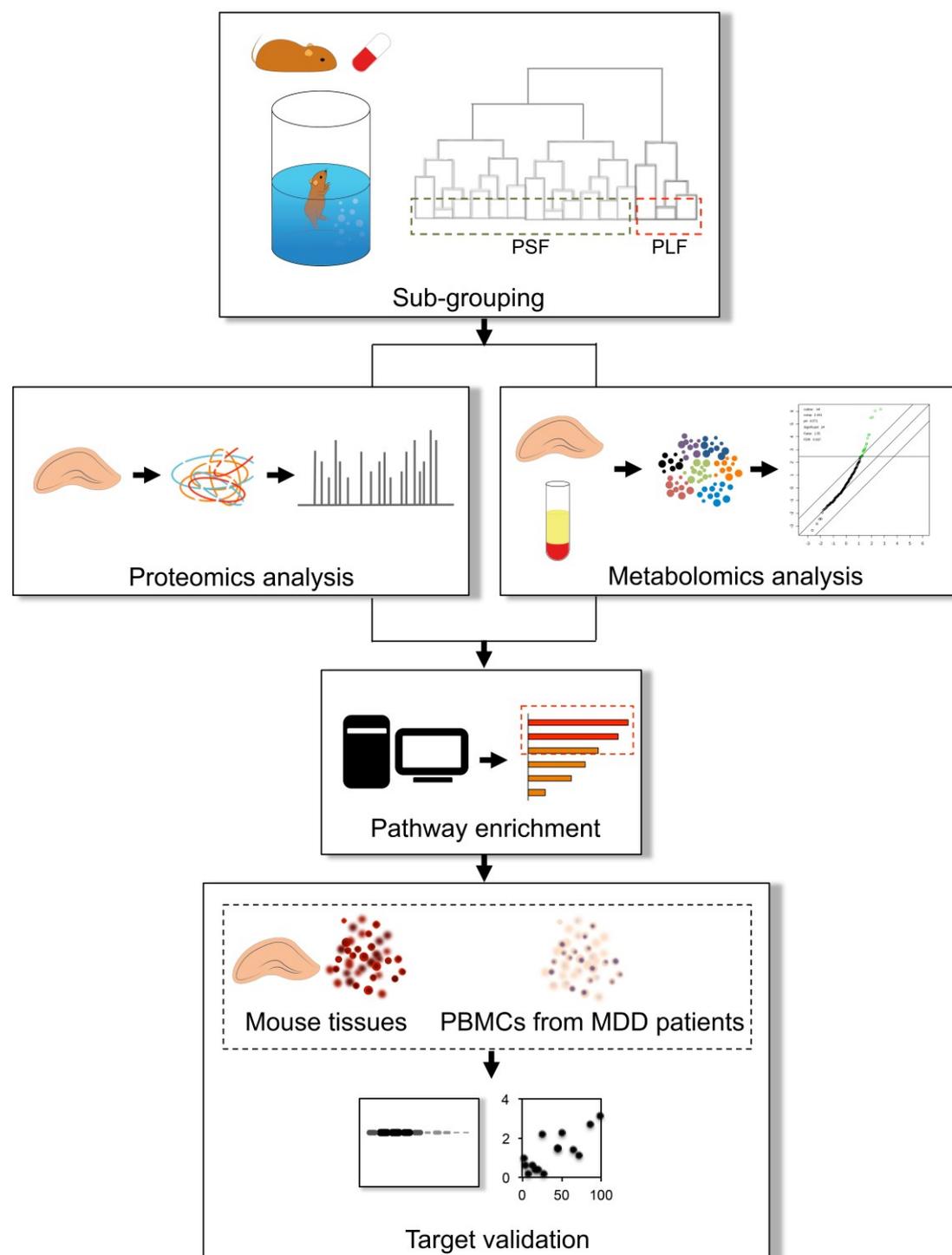
## 2.9. Statistical Analysis

Hierarchical clustering analysis (HCA) was performed to separate paroxetine-treated sub-groups of mice, using SPSS (SPSS version 21, IBM SPSS Inc., Chicago, IL, USA). Statistical analysis of behavioral data (FST and FUST) and covariates were performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Student *t*-test, one-way or two-way ANOVA was used to evaluate statistical significance between groups. Pearson correlation coefficients (*r*) with *p* values were used to evaluate correlation between floating time and body weight gain. For the identification of significantly altered metabolites, metabolite peak intensities were median and auto-scaled normalized. Metabolites with missing values, 30 for the hippocampus and 27 for the prefrontal cortex in all replicates, were excluded from data analysis. Significant analysis of microarrays (and metabolites) (SAM) method was used to identify significantly altered metabolites ( $q < 0.05$ , FDR  $< 0.1$ ). Significantly altered metabolites were subjected to pathway enrichment analysis of MetaboAnalyst (<http://www.metaboanalyst.ca>) to identify differentially affected pathways between the PLF and PSF groups. Pathways with Holm adjusted  $p < 0.05$  and FDR  $< 0.05$  were considered significantly affected. To identify sub-pathways interacting with a differentially affected pathway between the PLF and PSF groups, correlates of each SAM signature were combined and used to enrich relevant

metabolic pathways using MetaboAnalyst (Pearson correlation coefficients ( $r$ )  $> 0.7$ , FDR  $< 0.1$ ). Pathways with Holm adjusted  $p < 0.05$  and FDR  $< 0.05$  were considered significant. Proteomics pathway enrichment was performed using DAVID bioinformatics resources 6.7 according to a Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Huang et al., 2007). Enriched pathways were considered significant at Bonferroni adjusted  $p < 0.05$  and FDR  $< 0.01$ . Proteins common to several pathways were further extracted from the enriched pathways using a Venn diagram comparison. Protein interaction network was created using STRING database. Western blot data were analyzed with GraphPad Prism 5. Two-tailed  $t$ -test was used to evaluate the difference between the groups. Data were expressed as the mean  $\pm$  the standard error of the mean (SEM). Correlations between pathway protein levels and FST floating time/HDRS change were assessed using Pearson correlation coefficients ( $r$ ) with  $p$  values. Statistical data were considered significant at  $p < 0.05$ . D'Agostino & Pearson omnibus normality test was used to check normal distribution.

### 3. Results

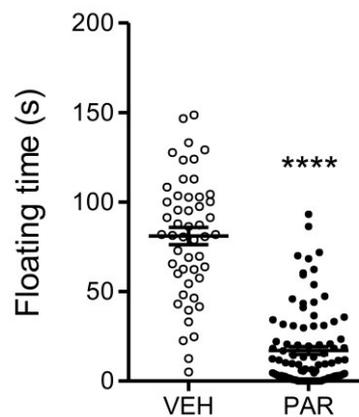
Figure 5 shows a schematic overview of the workflow.



**Figure 5.** A schematic overview of the workflow.

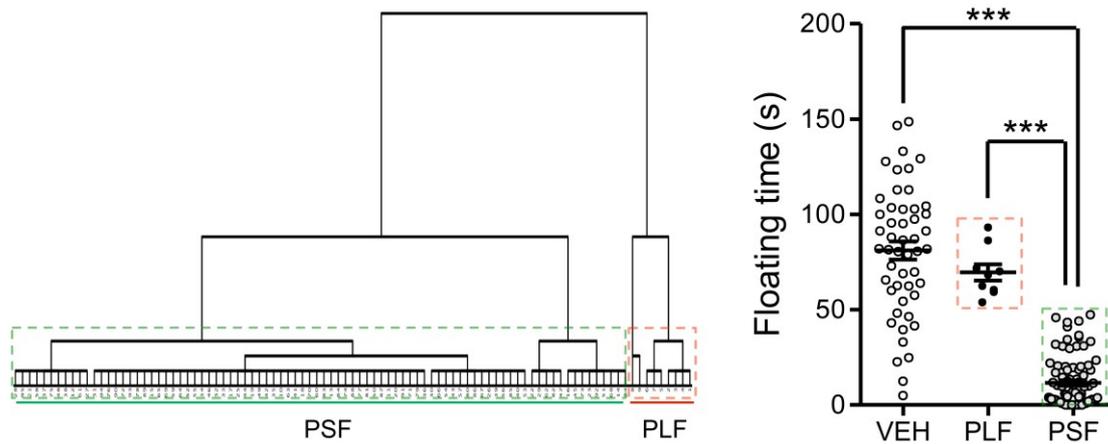
### 3.1. Sub-grouping of paroxetine responder and non-responder mice

DBA/2J mice were administered with vehicle or paroxetine pills (2 x 5 mg/kg/day) for 28 days. The paroxetine-treated group (PAR) showed significantly reduced FST floating time compared to vehicle-treated (VEH) mice ( $t = 13.90$ ,  $df = 143$ ,  $p < 0.0001$ ) (Figure 6).

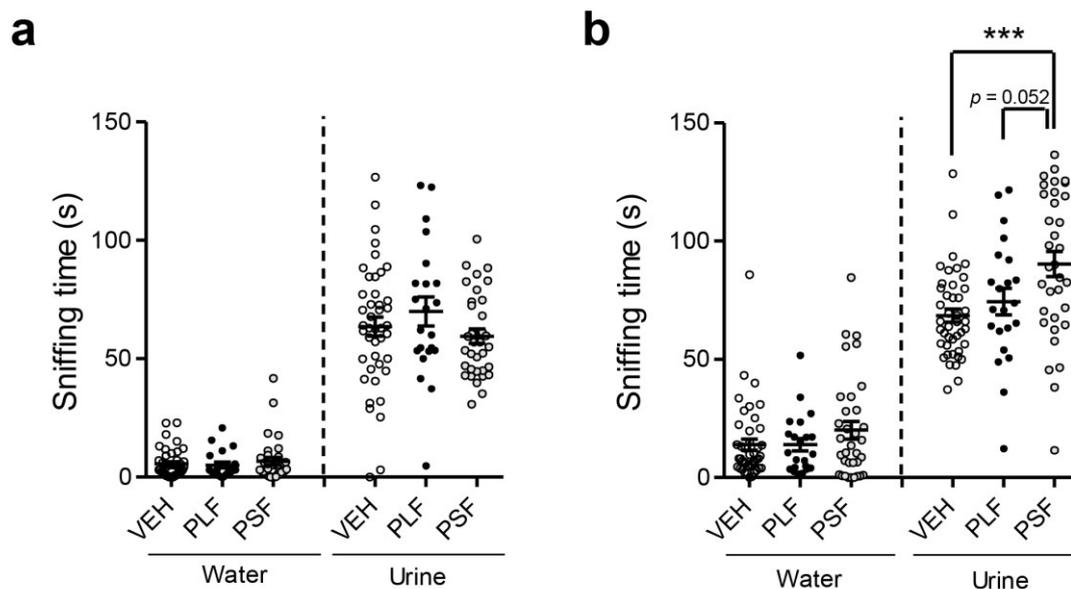


**Figure 6.** The effect of chronic paroxetine treatment on FST floating time. Male DBA/2J mice received paroxetine (5 mg/kg, twice a day, 28 days) and floating time was recorded for 6 min. PAR mice displayed significantly shorter floating time compared to vehicle-treated group (VEH).  $n(\text{VEH}/\text{PAR})=50/95$ . Data are expressed as mean  $\pm$  SEM. \*\*\*\*  $p < 0.0001$  (two-tailed  $t$ -test).

I was able to separate paroxetine-treated mice into long-time floating (PLF) and short-time floating (PSF) groups according to their FST floating time using hierarchical cluster analysis (HCA) ( $F = 159.5$ ,  $df = 144$ ,  $p < 0.001$ ) (Figure 7). PSF mice floating time was significantly lower than for VEH mice ( $p < 0.001$ ) while no floating time difference was observed between PLF and VEH mice. During the female urine sniffing test (FUST) PLF and PSF mice did not show differential sniffing time prior to paroxetine treatment ( $F_{(5,192)} = 104.5$ ,  $p > 0.05$ ) (Figure 8a). A slightly different sniffing time became apparent after 28 days of paroxetine treatment ( $p = 0.052$ ). The time for sniffing female urine was longer for PSF mice compared to VEH and PLF mice ( $F_{(5,192)} = 84.24$ ,  $df = 197$ ,  $p < 0.0001$ ) (Figure 8b). Two-way ANOVA identified no interaction between time points and treatment.



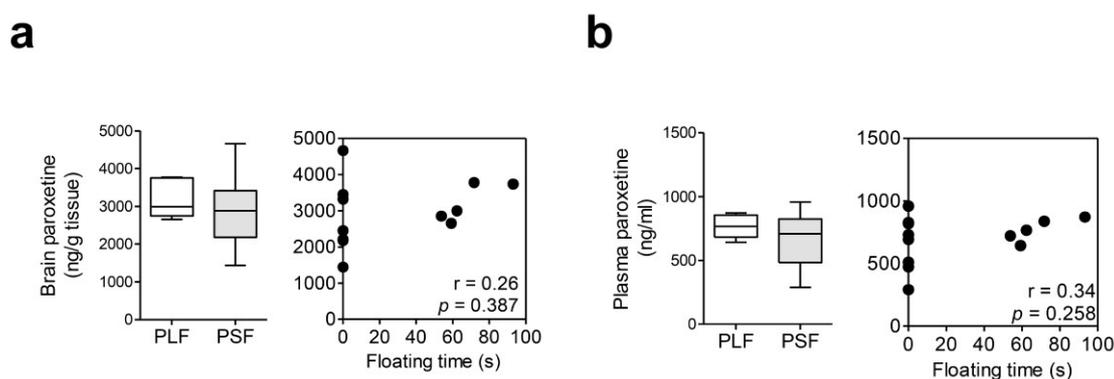
**Figure 7.** Sub-grouping of paroxetine-treated mice. Dendrogram of paroxetine-treated mice. Mice treated with paroxetine were separated into PLF and PSF groups using hierarchical clustering analysis (HCA).  $n(\text{VEH/PLF/PSF})=50/9/86$ . Data are expressed as mean  $\pm$  SEM. \*\*\* $p < 0.001$  (one-way ANOVA with Tukey's test for multiple comparisons).



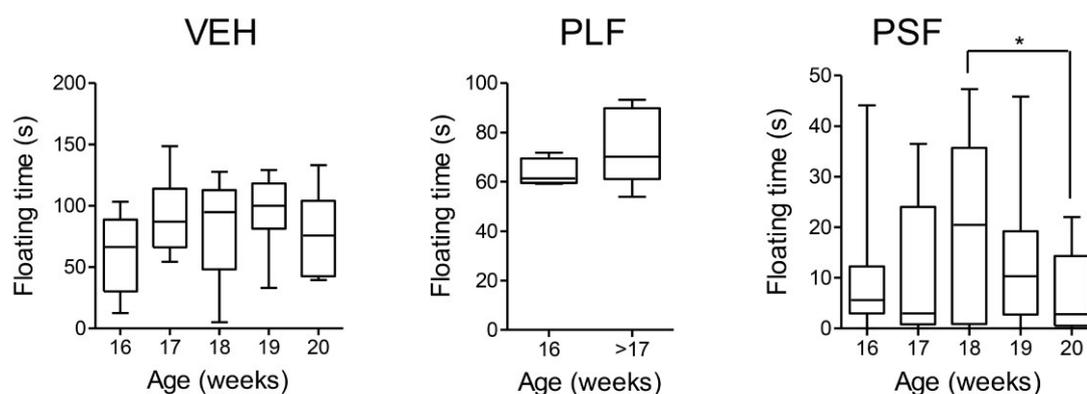
**Figure 8.** The effect of chronic paroxetine treatment on female urine sniffing test (FUST). The effect of chronic paroxetine treatment on female urine sniffing test (FUST). Sniffing time at (a) baseline and (b) after 28 days of paroxetine treatment. Chronic paroxetine treatment induced a slightly significant difference of sniffing time between paroxetine-treated long- time floating (PLF) and paroxetine-treated short-time floating (PSF) groups.  $n(\text{VEH/PLF/PSF}) = 43/22/34$ . \* $p < 0.05$  (two-tailed  $t$ -test), \*\*\* $p < 0.001$  (one-way ANOVA with Tukey's test for multiple comparisons).

### 3.2. Covariate analysis

Covariates that may be relevant for paroxetine treatment response were examined. Paroxetine concentrations in whole brain and plasma did not differ between groups ( $p > 0.05$ ) and did not correlate with FST floating time ( $r = 0.26$ ,  $p = 0.387$ ) (Figure 9a and b). I also analyzed mouse age and body weight gain. A significant age effect on FST floating time was not observed ( $F_{(4,81)} = 2.184$ ,  $p > 0.05$ ) (Figure 10).

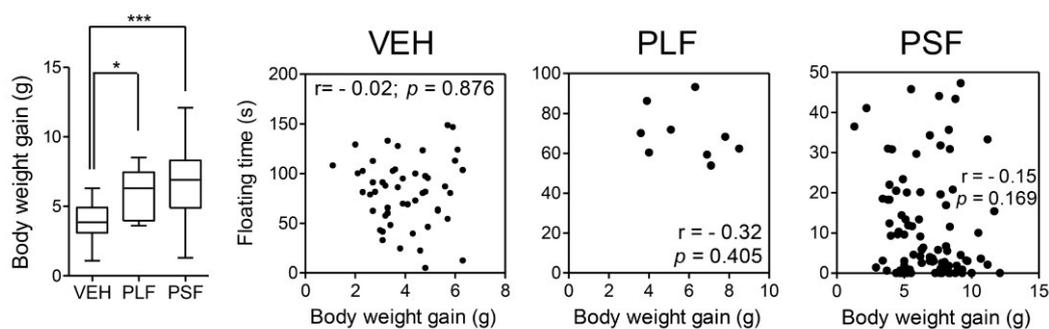


**Figure 9.** Paroxetine levels in (a) whole brain and (b) plasma. PLF and PSF groups did not show significant paroxetine level differences,  $n(\text{PLF/PSF})=5/8$ . Pearson correlation coefficients ( $r$ ) with  $P$  values are indicated in the correlation graphs.



**Figure 10.** The effect of age on FST floating time,  $n(\text{VEH/PLF/PSF})=50/9/86$ .  $*p < 0.05$  (one-way ANOVA with Tukey's test for multiple comparisons).

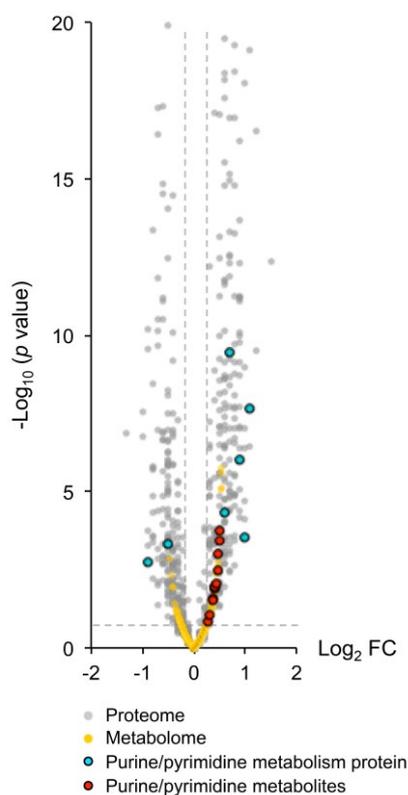
Body weight gain did not vary between PLF and PSF mice. Both groups had a significantly higher body weight gain after chronic paroxetine treatment compared to VEH mice ( $F_{(2,42)} = 29.51$ ,  $p < 0.0001$ ). Correlation between body weight gain and FST floating time was not significant (VEH:  $r = -0.02$ ,  $p = 0.876$  PLF:  $r = -0.32$ ,  $p = 0.405$  PSF:  $r = -0.15$ ,  $p = 0.169$ ) (Figure 11).



**Figure 11.** The effect of body weight gain on FST floating time,  $n(\text{VEH/PLF/PSF})=50/9/86$ . Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.001$  (one-way ANOVA with Tukey's test for multiple comparisons). Pearson correlation coefficients ( $r$ ) with  $P$  values are indicated in the correlation graphs.

### 3.3. Identification of purine and pyrimidine metabolism

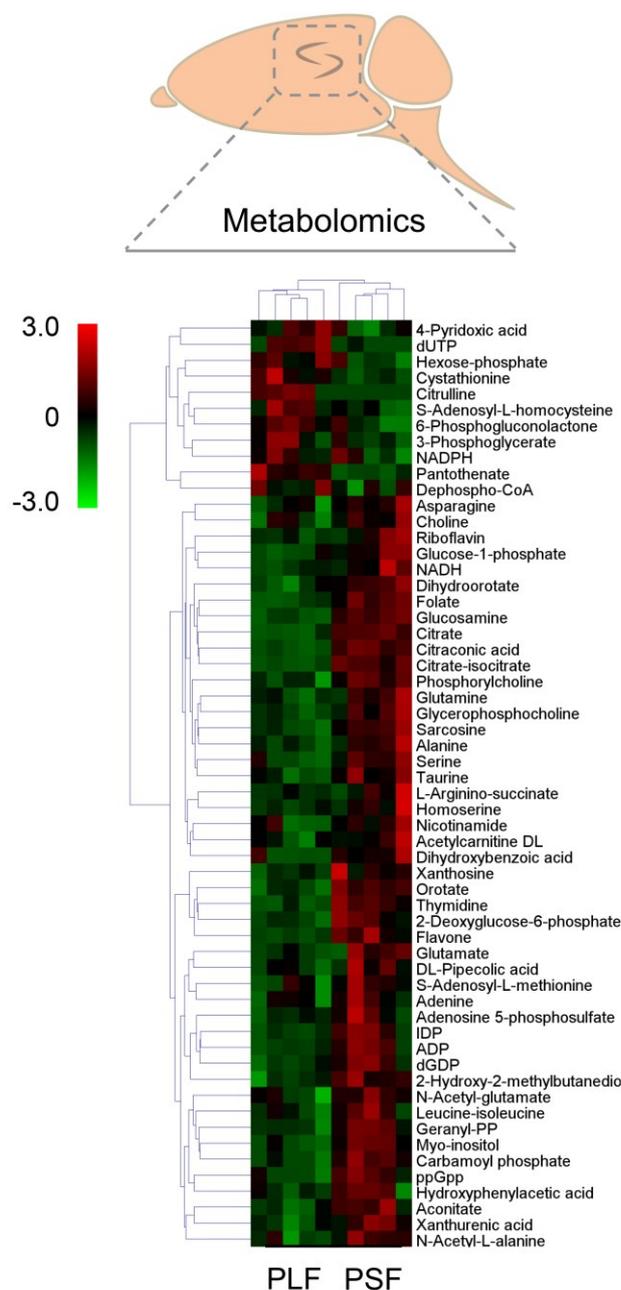
After clustering paroxetine-treated mice into long-floating and short-floating groups, I performed metabolomics and proteomics analyses of the hippocampus. Both -omics analyses showed purine/pyrimidine metabolites and proteins with significant differences between the two groups ( $\log_2|\text{FC}| > 0.3$ ,  $-\log_{10}(p \text{ value}) > 1.3$ ) (Figure 12).



**Figure 12.** A volcano plot of hippocampal metabolome and proteome. Metabolites and proteins with  $\log_2|\text{FC}| > 0.3$  and  $-\log_{10}(p \text{ value}) > 1.3$  were considered significant. Purine/pyrimidine

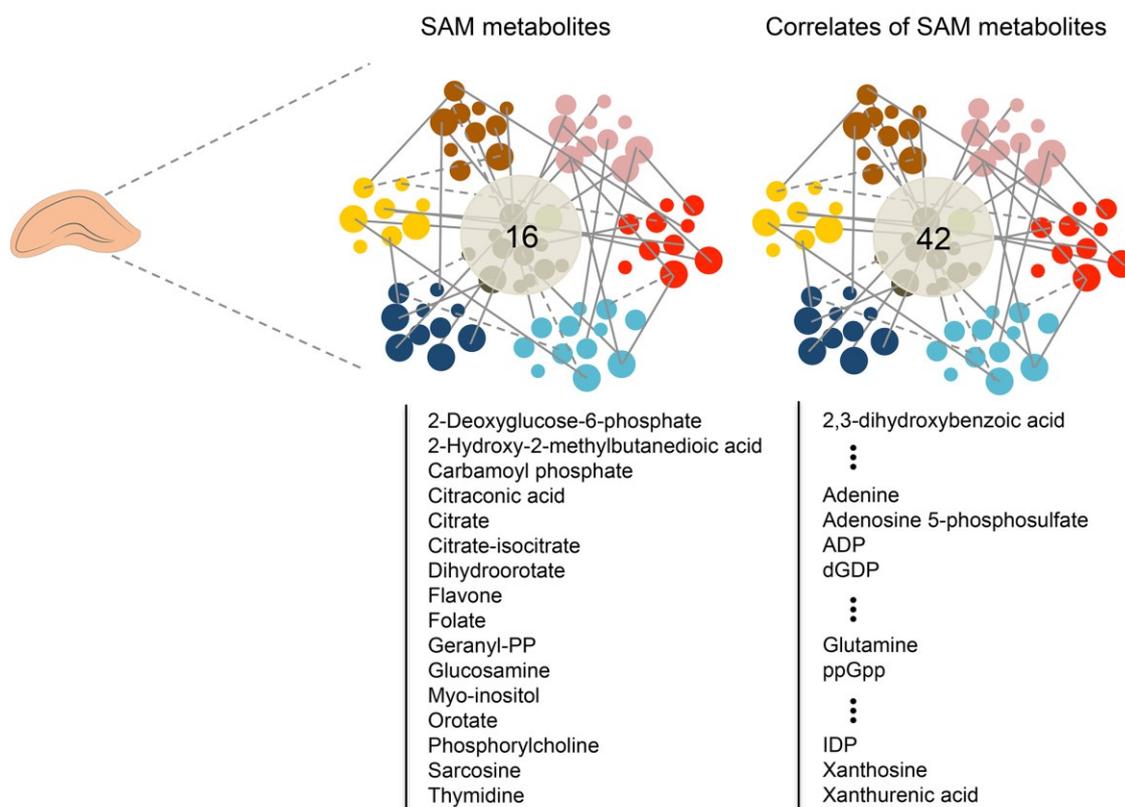
metabolites and metabolism-related proteins were found to be significantly different between the sub-groups,  $n=3-5$ /group.

In metabolomics analysis, significant analysis of microarrays (and metabolites) (SAM) and SAM-driven correlation analysis revealed that chronic paroxetine treatment differentially affected the hippocampal metabolome of the PLF and PSF mice (Figure 13).



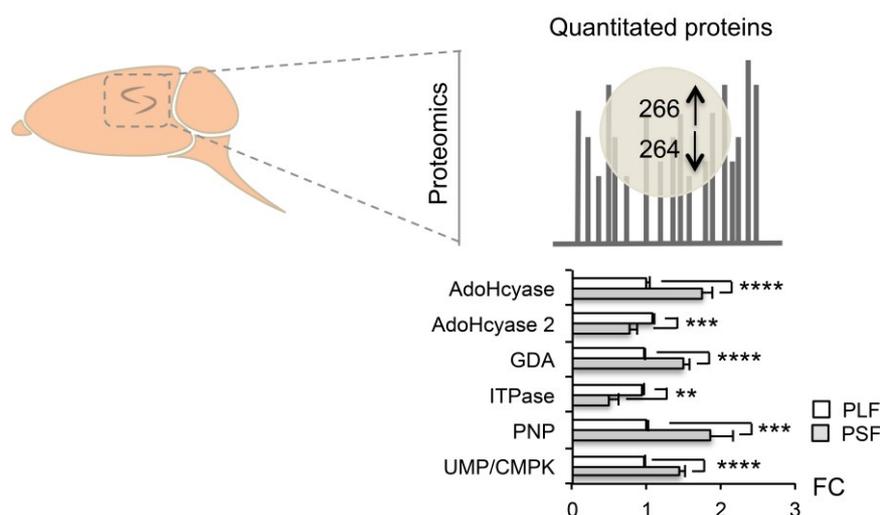
**Figure 13.** A heat map with combined profiles of SAM signatures ( $q < 0.05$ ,  $FDR < 0.1$ ) and their significant correlates ( $r > 0.7$ ,  $p < 0.05$ ). Heat map colors denote normalized metabolite intensity,  $n=5$ /group.

Sixteen metabolites were differentially regulated showing significantly higher levels in PSF compared to PLF mice ( $q < 0.05$ ,  $FDR < 0.1$ ). In addition, I found significant correlations with 42 other hippocampal metabolites ( $r > 0.7$ ,  $FDR < 0.1$ ) (Figure 14).



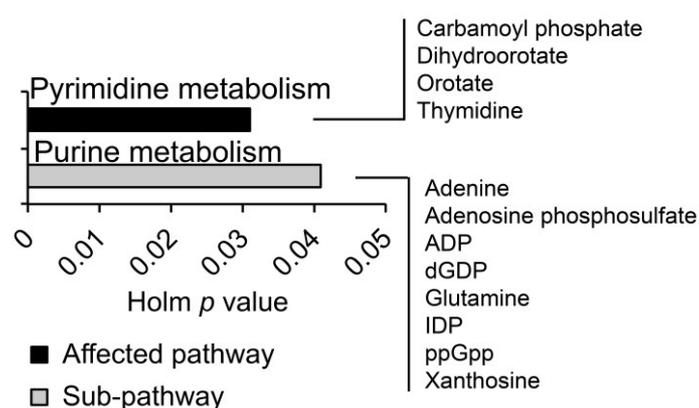
**Figure 14.** Hippocampal SAM metabolites ( $q < 0.05$ ,  $FDR < 0.1$ ) and their significant correlates ( $r > 0.7$ ,  $p < 0.05$ )

In proteomics analysis, I found that purine/pyrimidine metabolism proteins including S-adenosyl-L-homocysteine hydrolase (AdoHcyase), S-adenosyl-L-homocysteine hydrolase 2 (AdoHcyase 2), guanine deaminase (GDA), inosine triphosphate pyrophosphatase (ITPase), purine nucleoside phosphorylase (PNP) and UMP-CMP kinase (UMP/CMPK) were differentially expressed between the PLF and PSF groups (Figure 15).



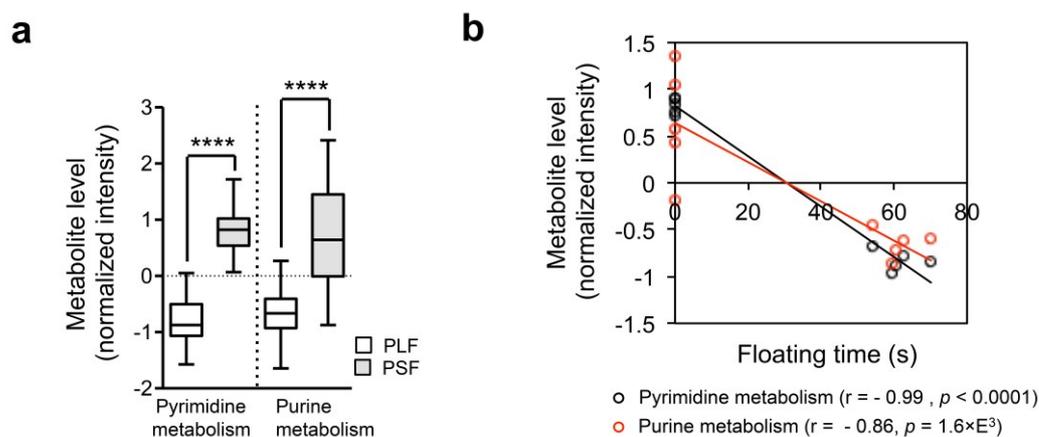
**Figure 15.** Identification of purine and pyrimidine metabolism pathway proteins in proteomics analysis. 266 and 264 proteins were found to be up- and down-regulated between PLF and PSF mice. Purine and pyrimidine metabolisms protein levels were significantly different between the PLF and PSF mice ( $p < 0.05$ ),  $n=3/\text{group}$ . Data are expressed as the mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (two-tailed  $t$ -test).

The pyrimidine metabolism pathway was enriched with four metabolites from hippocampal SAM analysis (carbamoyl phosphate, dihydroorotate, orotate and thymidine). Eight correlates of SAM signatures (adenine, adenosine 5-phosphosulfate, ADP, dGDP, glutamine, IDP, ppGpp, xanthosine) enriched the purine metabolism pathway (Holm adjusted  $p < 0.05$ , FDR  $< 0.05$ ) (Figure 16).

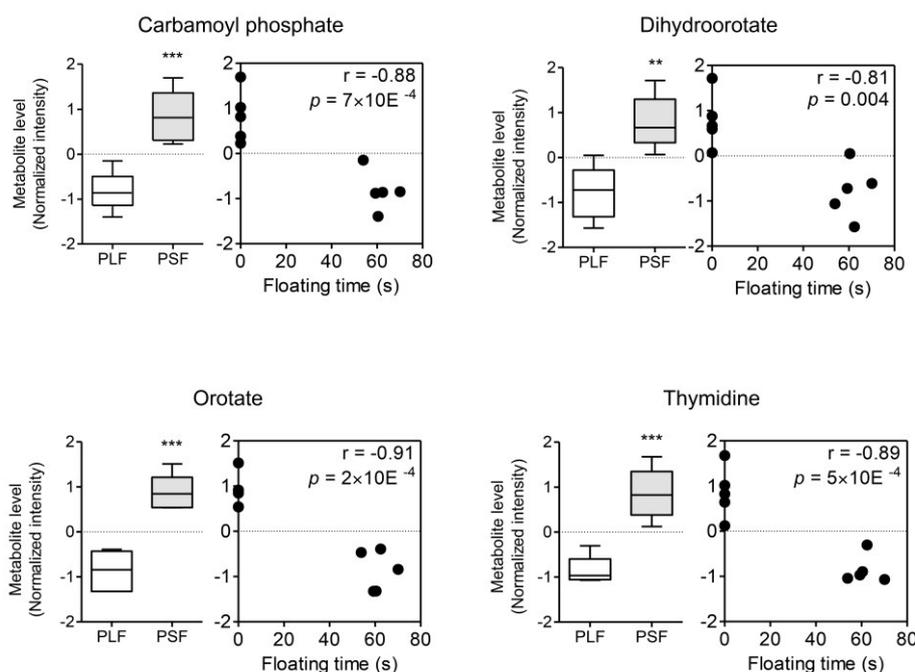


**Figure 16.** Metabolic pathway analysis in the hippocampus. Metabolomics analysis identified purine and pyrimidine metabolisms as sub-pathway and affected pathway, respectively (Holm adjusted  $p < 0.05$ , FDR  $< 0.05$ ).

Average levels of purine and pyrimidine metabolites were significantly higher in PSF than PLF mice and were strongly correlated with FST floating time (Figures 17a and b).

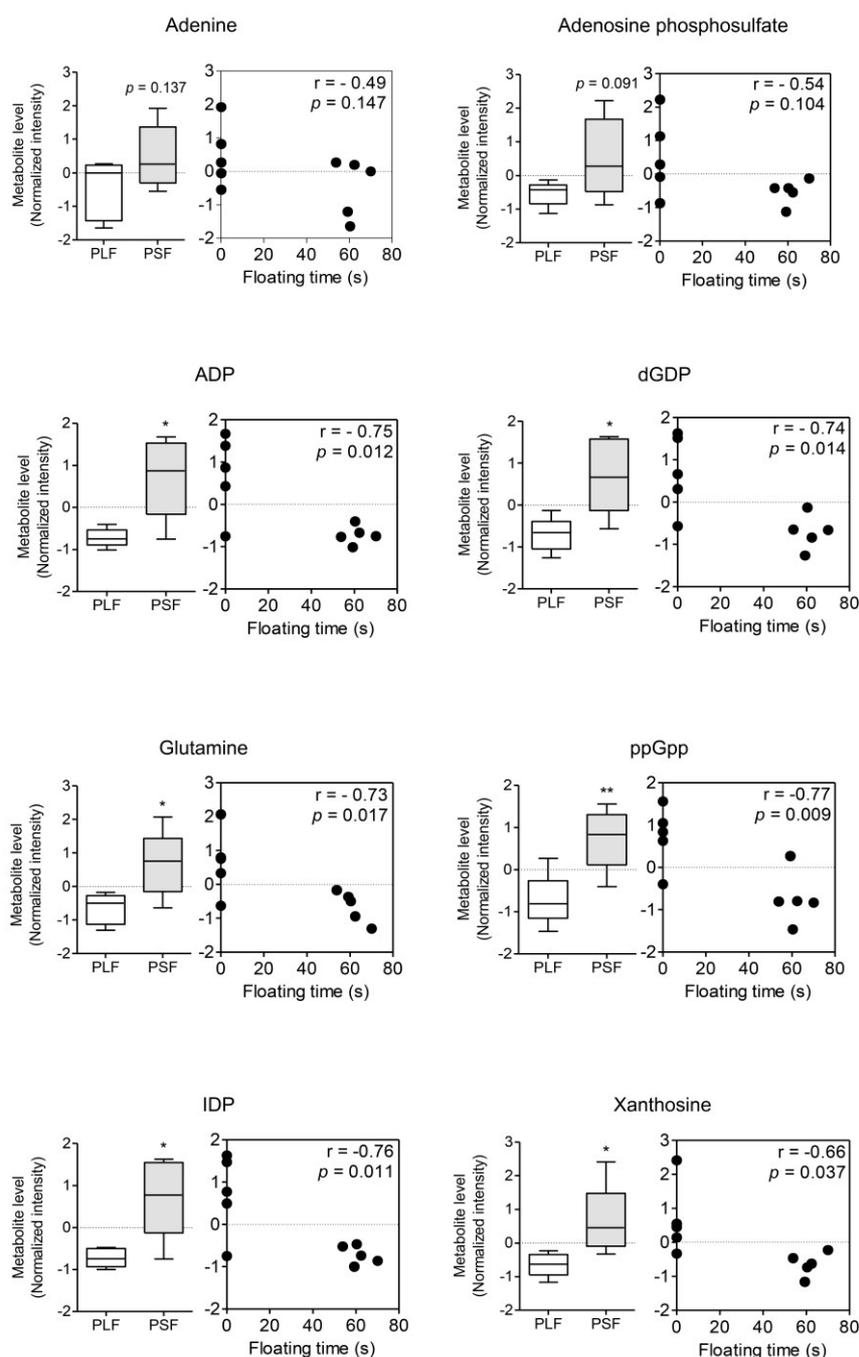


**Figure 17.** Purine and pyrimidine metabolite average levels and correlation with FST floating time. **(a)** Average level difference of purine and pyrimidine metabolites between PLF and PSF group was shown by box plots with whiskers min to max. **(b)** Average purine and pyrimidine pathways metabolite levels were strongly correlated with FST floating time. Data are expressed as the mean  $\pm$  SEM. \*\*\*\* $p < 0.0001$  (two-tailed  $t$ -test). Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated under the correlation graphs.



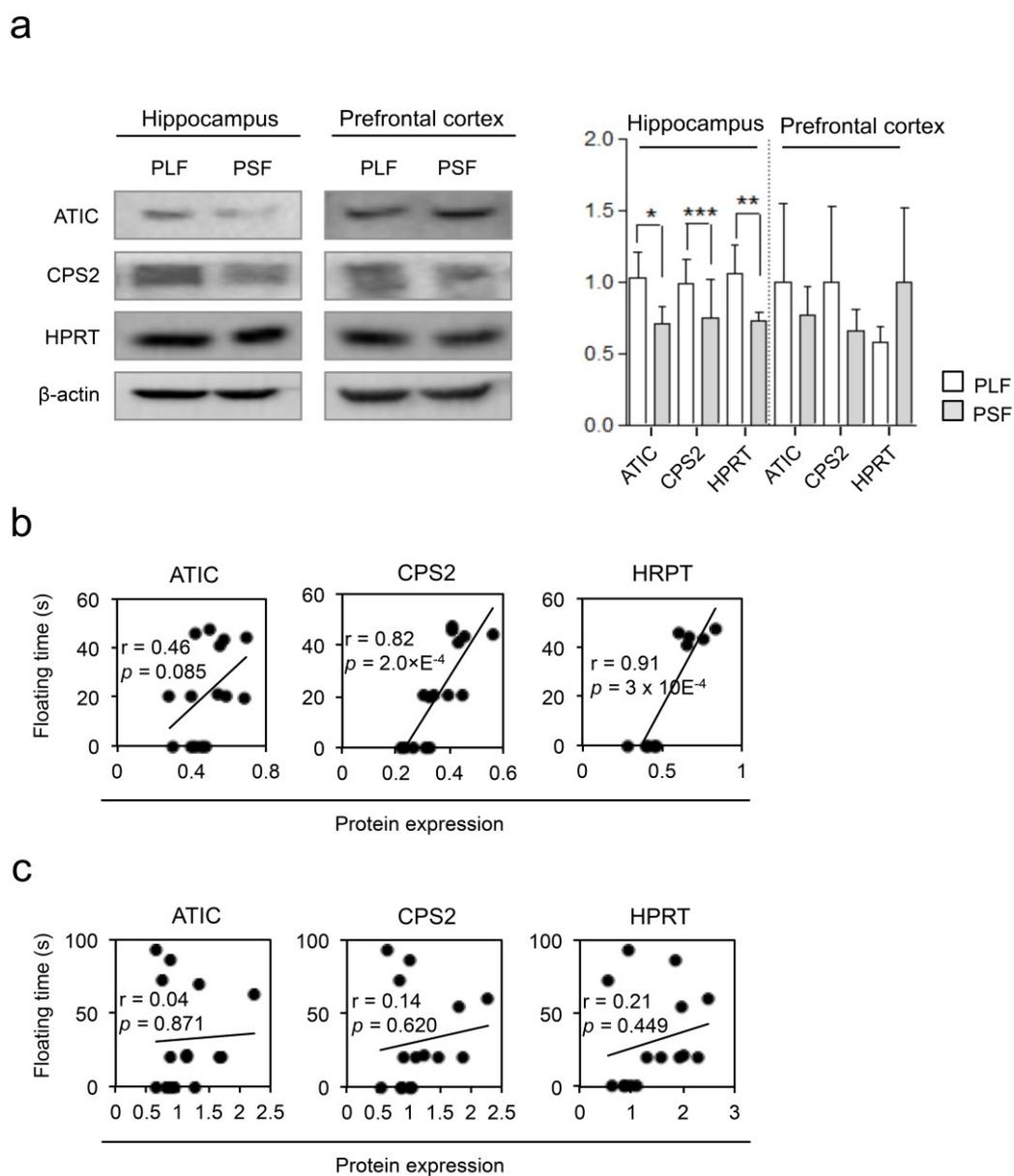
**Figure 18.** Levels of hippocampal metabolites that are part of pyrimidine metabolism pathway and correlation with FST floating time.  $n = 5$ /group. Bars represent mean  $\pm$  SEM. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs PLF (two-tailed  $t$ -test). Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

Purine and pyrimidine metabolite levels were higher in the PSF compared to PLF groups and with the exception of adenine and adenosine phosphosulfate showed a significant negative-correlation with FST floating time (Figures 18 and 19). Metabolomics analysis of the prefrontal cortex did not result in any metabolite and pathway differences distinguishing the PLF and PSF groups.



**Figure 19.** Levels of hippocampal metabolites that are part of purine metabolism pathway and correlation with FST floating time.  $n = 5/\text{group}$ . Bars represent mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  vs

PLF (two-tailed *t*-test). Pearson correlation coefficients (*r*) with *p* values are indicated in the correlation graphs.



**Figure 20.** The effect of chronic paroxetine treatment on ATIC, CPS2 and HPRT protein expressions in the mouse hippocampus and prefrontal cortex. **(a)** Western blot and densitometry analyses of the pathway protein levels in the hippocampus and prefrontal cortex. Hippocampal ATIC, CPS2 and HPRT proteins showed significant expression level differences between the PLF and PSF groups,  $n=5/\text{group}$ . **(b)** Correlation of hippocampal CPS2 and HPRT protein levels with FST floating time was significant in the hippocampus. ATIC protein level showed moderate correlation with FST floating time.  $n=15$ . **(c)** Correlation of FST floating time with the pathway protein levels in the prefrontal cortex. None of the pathway protein showed significant correlation with FST floating time.  $n=15$ . Protein expression levels were normalized with  $\beta$ -actin. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (two-

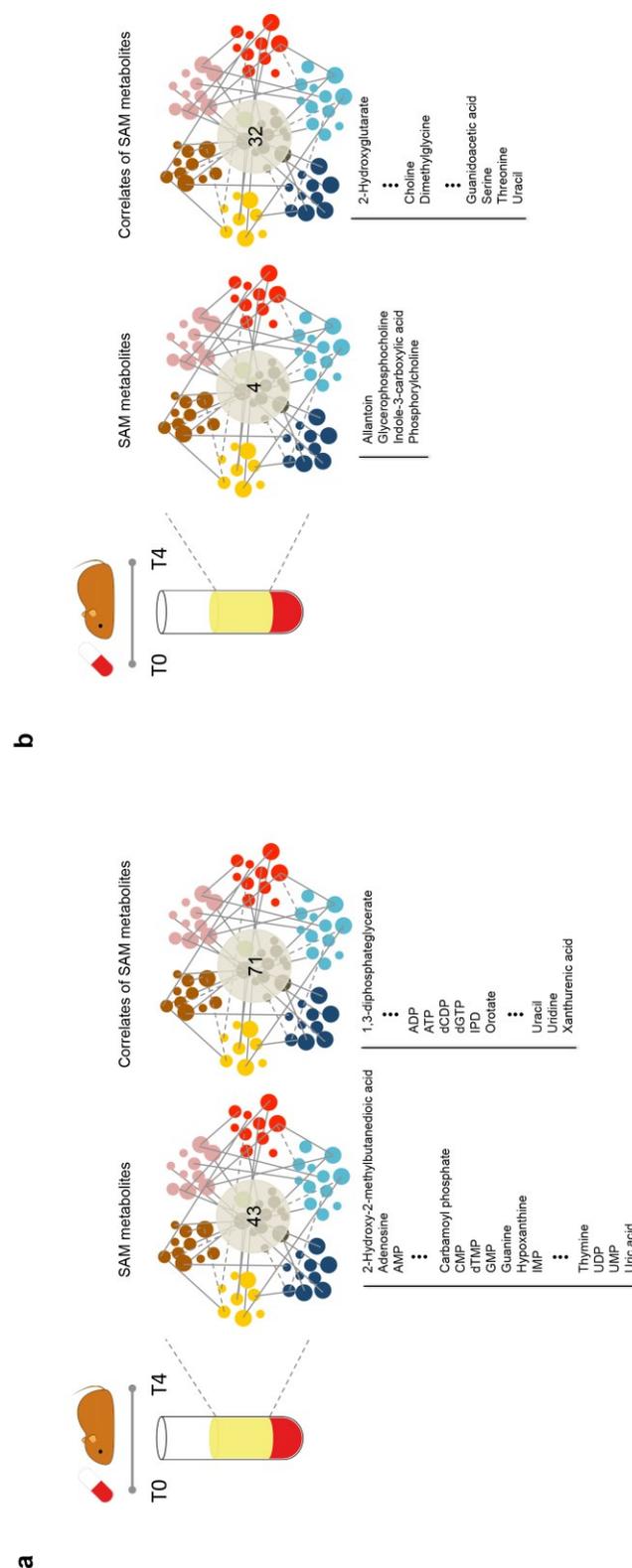
tailed *t*-test). Pearson correlation coefficients (*r*) with *p* values are indicated in the correlation graphs.

Aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP cyclohydrolase (ATIC), carbamoyl phosphate synthase 2 (CPS2) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) protein expression in the hippocampus and prefrontal cortex were assessed to validate differentially affected pathways between the PLF and PSF groups (Figure 20a). In the hippocampus, ATIC, CPS2 and HPRT protein levels showed significant differences between groups. Compared to the PLF group the PSF group had significantly reduced ATIC, CPS2 and HPRT protein expression levels (ATIC:  $t = 3.304$ ,  $df = 8$ ,  $p < 0.05$ , CPS2:  $t = 1.702$ ,  $df = 8$ ,  $p < 0.001$ , HPRT:  $t = 3.488$ ,  $df = 8$ ,  $p < 0.01$ ). In the prefrontal cortex protein expression showed no difference between the two groups. FST floating time significantly correlated with CPS2 and HPRT protein expression levels in the hippocampus, but not in the prefrontal cortex. Correlation between FST floating time and ATIC protein levels also showed strong tendency in the hippocampus (Figures 20b and c).

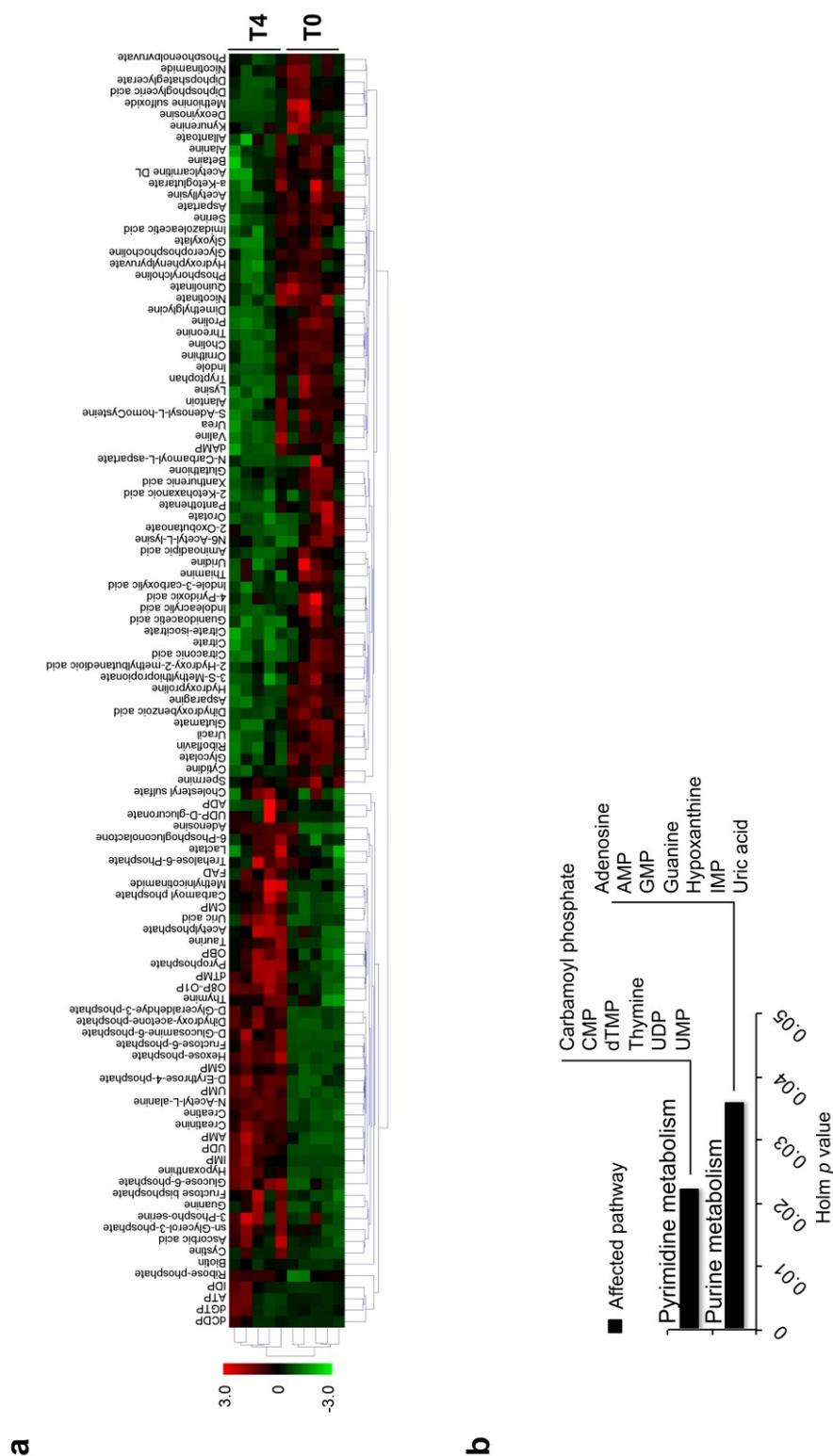
### 3.4. Metabolomics analysis of mouse plasma

To delineate peripheral metabolome changes related to chronic paroxetine treatment response, plasma metabolite levels were investigated in the PLF and PSF groups at baseline (T0) and following 28 days of drug treatment (T4). While metabolomic profiles between PLF and PSF groups showed no significant differences both at T0 and T4, the PSF group exhibited profound differences with 43 significant metabolite level changes between T0 and T4 and another 71 metabolites that were strongly correlated ( $r > 0.7$ ,  $FDR < 0.1$ ) (Figure 21a). In the PSF group pyrimidine metabolism was enriched with 6 metabolites (carbamoyl phosphate, CMP, dTMP, thymine, UDP, UMP) and another 7 metabolites (adenosine, AMP, GMP, guanine, hypoxanthine, IMP, uric acid) enriched the purine metabolism pathway (Holm adjusted  $p < 0.05$ ,  $FDR < 0.05$ ) (Figure 22). The PLF group exhibited smaller metabolome changes compared to the PSF group. Only 4 metabolites were significantly altered after chronic paroxetine treatment according to SAM with another 32 metabolites highly correlated ( $r > 0.7$ ,  $FDR < 0.1$ ) (Figure 21b). While SAM signatures revealed no significantly affected pathways for the PLF group, glycine, serine and threonine

metabolism was enriched for 5 correlates (choline, dimethylglycine, guanidoacetic acid, serine and threonine) (Holm adjusted  $p < 0.05$ , FDR  $< 0.05$ ) (Figure 23).

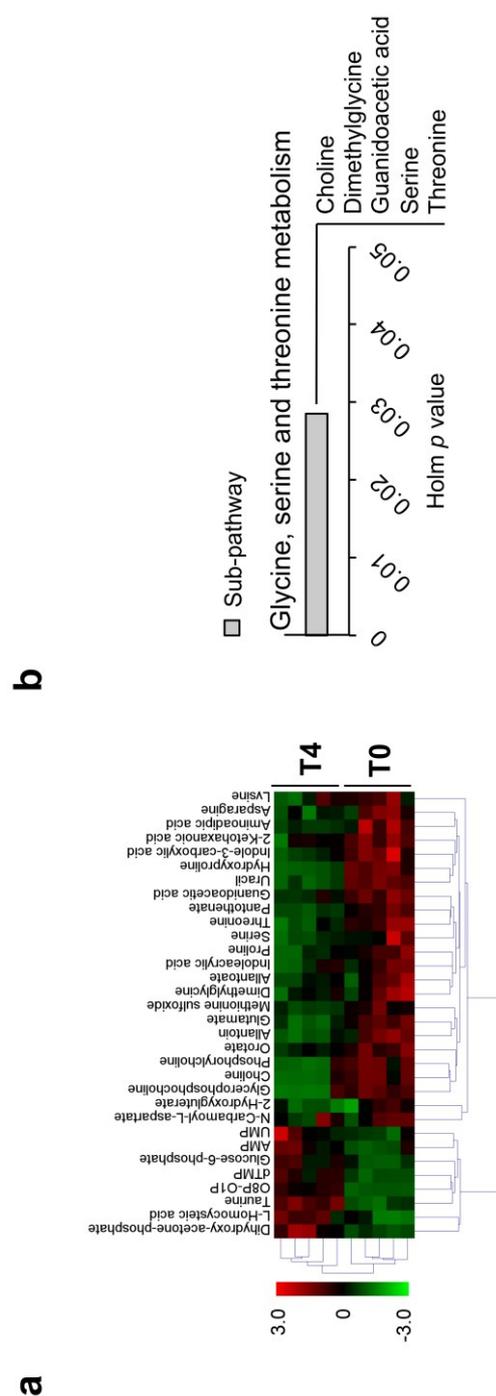


**Figure 21.** Significant plasma metabolite level changes after chronic paroxetine treatment. Plasma SAM metabolites ( $q < 0.05$ , FDR  $< 0.1$ ) and their significant correlates ( $r > 0.7$ ,  $p < 0.05$ ) in **(a)** PSF and **(b)** PLF groups.

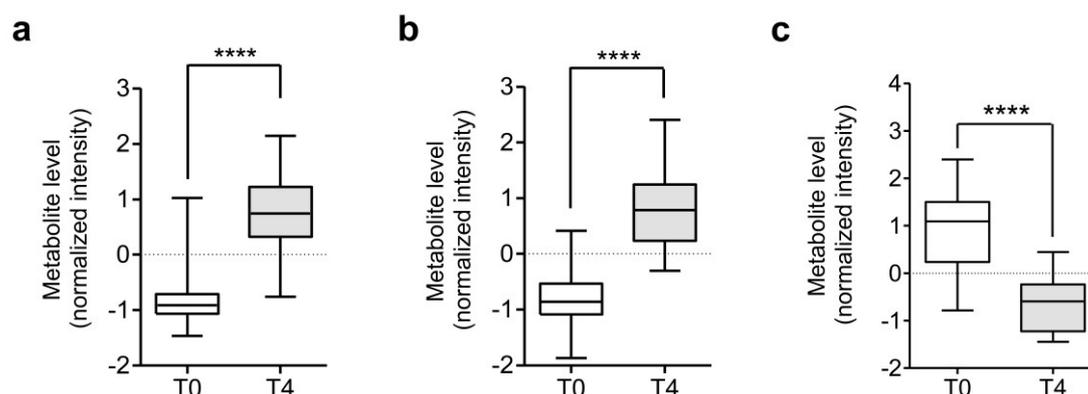


**Figure 22.** Chronic paroxetine treatment induced differential metabolome alterations in PSF mouse plasma. (a) Heat maps and (b) identified pathways of PSF groups comparing metabolome at baseline (T0) and following 28 days of treatment (T4). Purine and pyrimidine

metabolisms were the only affected pathways in the PSF group (Holm adjusted  $p < 0.05$ , FDR  $< 0.05$ ). Heat map colors denote normalized metabolite intensity,  $n=5$ /group.

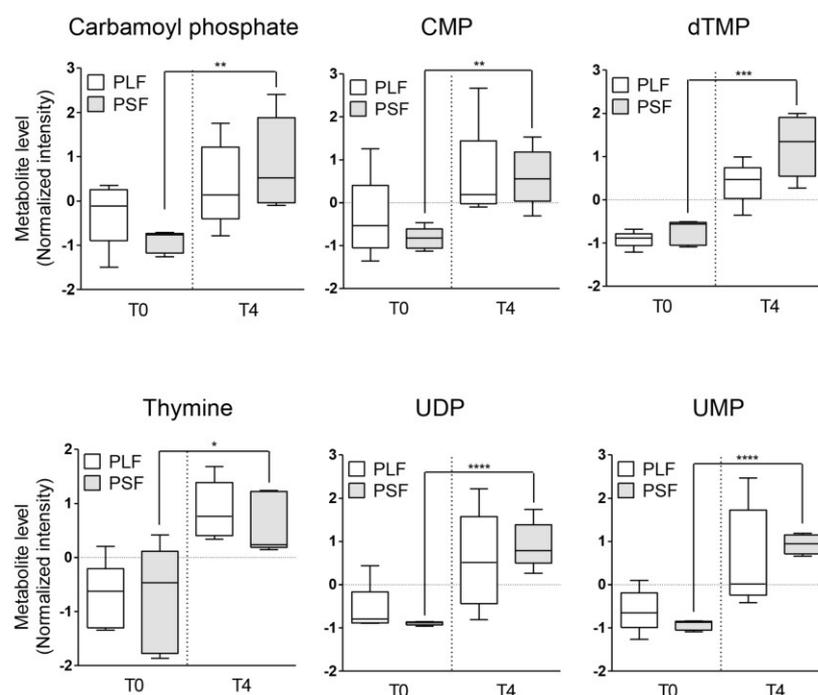


**Figure 23.** (a) Heat maps and (b) identified pathways of PLF groups comparing metabolome at baseline (T0) and following 28 days of treatment (T4). Correlates of SAM signatures identified glycine, serine and threonine metabolism as a sub-pathway (Holm adjusted  $p < 0.05$ , FDR  $< 0.05$ ). Heat map colors denote normalized metabolite intensity,  $n=5$ /group.

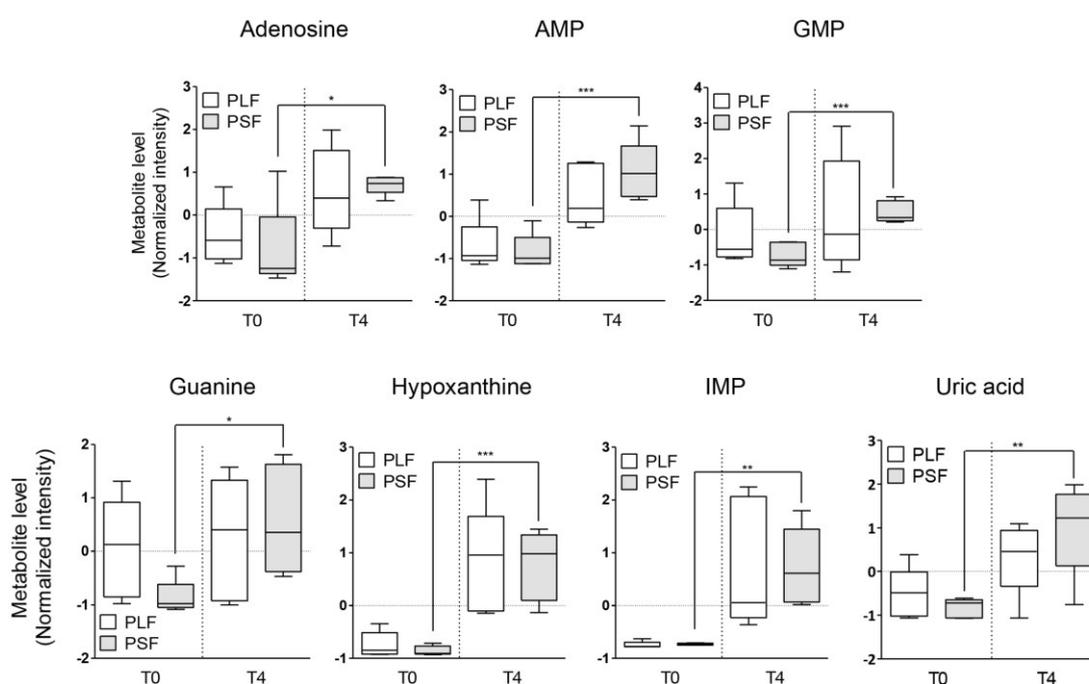


**Figure 24.** Plasma metabolite average level changes in the identified pathways. Chronic paroxetine treatment induced average level changes of (a) purine and (b) pyrimidine pathway metabolites in the PSF group, and (c) glycine/serine/threonine pathway metabolites in PLF group between T0 and T4,  $n=5/\text{group}$ . Metabolite levels are expressed with Box plots with whiskers min to max. \*\*\*\* $p < 0.0001$  (two-tailed paired  $t$ -test).

Plasma metabolite levels of identified pathways were significantly altered after mice had been treated chronically with paroxetine. Purine and pyrimidine metabolism pathway average levels were significantly upregulated by chronic paroxetine treatment in PSF mouse plasma (Figures 24a and b). In PLF mouse plasma, glycine, serine and threonine metabolism pathway was significantly downregulated by chronic paroxetine treatment (Figure 24c). Significant purine and pyrimidine metabolite level changes between T0 and T4 were observed only in the PSF mice. In PSF mice, chronic paroxetine treatment induced significant upregulation of pyrimidine pathway metabolites (carbamoyl phosphate, CMP, dTMP, thymine, UDP, UMP, Figure 25) and purine pathway metabolites (adenosine, AMP, GMP, guanine, hypoxanthine, IMP, uric acid, Figure 26). Glycine, serine and threonine pathway metabolite level changes occurred both in PLF and PSF mice (Figure 27). Plasma levels of 2,3-dihydroxybenzoic acid, aminoadipic acid, choline, pantothenate, taurine, threonine and uracil were found to be regulated to a similar extent in both PLF and PSF groups (Figure 28).

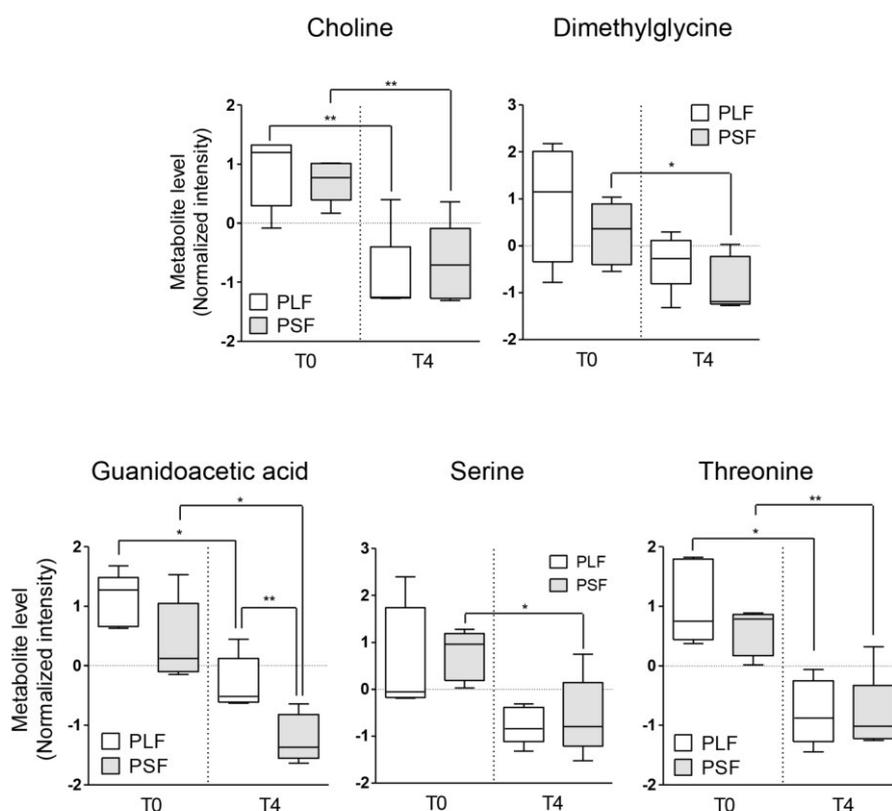


**Figure 25.** Plasma pyrimidine pathway metabolite levels. The metabolite levels in the PSF group were elevated by chronic paroxetine treatment. The PLF and PSF groups exhibited similar metabolite levels both at T0 and T4.  $n = 5/\text{group}$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (two-tailed paired  $t$ -test).



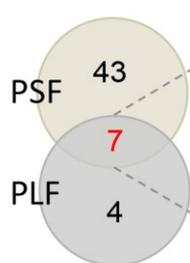
**Figure 26.** Plasma purine pathway metabolite levels. The metabolite levels in the PSF group were elevated by chronic paroxetine treatment. The PLF and PSF groups exhibited similar

metabolite levels both at T0 and T4.  $n = 5/\text{group}$ .  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  (two-tailed paired  $t$ -test).



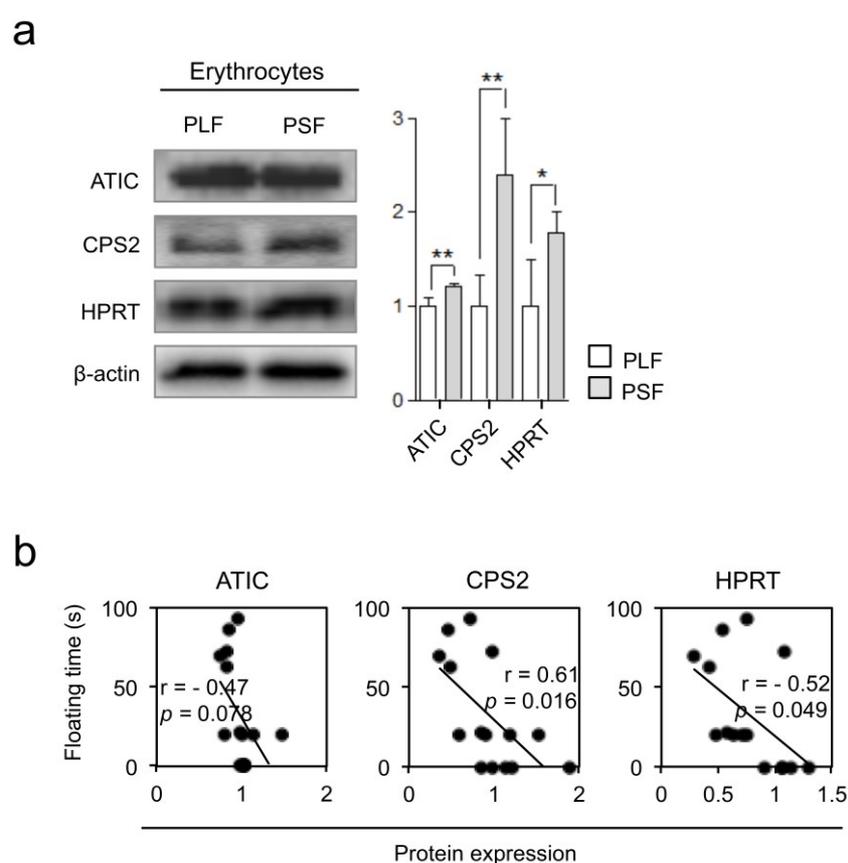
**Figure 27.** Plasma glycine, serine and threonine metabolism pathway metabolite levels. Glycine, serine and threonine metabolite levels in PLF and PSF mice. Both PLF and PSF mice showed that glycine, serine and threonine metabolite levels were significantly down-regulated by chronic paroxetine treatment.  $n = 5/\text{group}$ .  $*p < 0.05$ ,  $**p < 0.01$  (two-tailed paired  $t$ -test).

Metabolite	PLF		PSF	
	$q$ value	FC	$q$ value	FC
2,3-Dihydroxybenzoic acid	5.19E-02	0.55	2.04E-02	0.61
Amino adipic acid	5.19E-02	0.58	3.27E-02	0.63
Choline	4.48E-02	0.54	2.40E-02	0.62
Pantothenate	4.83E-02	0.60	3.14E-02	0.60
Taurine	2.29E-02	1.94	2.21E-02	1.69
Threonine	4.42E-02	0.55	2.40E-02	0.61
Uracil	1.97E-02	0.50	1.69E-02	0.58



**Figure 28.** Common plasma SAM signatures between the PLF and PSF groups.  $q$  value  $< 0.05$  in either PLF or PSF groups,  $\text{FDR} < 0.1$ .

For validation of peripheral pathways identified in plasma, erythrocytes were chosen as source. Interestingly, erythrocytic ATIC, CPS2 and HPRT proteins were differentially expressed between the PLF and PSF groups. Erythrocytic ATIC and CPS2 protein expression in the PSF mice was 1.2-fold and 2.4-fold higher than in the PLF mice, respectively (ATIC:  $t = 4.991$ ,  $df = 8$ ,  $p < 0.01$ , CPS2:  $t = 4.484$ ,  $df = 8$ ,  $p < 0.01$ ). HPRT protein levels were 1.7-fold higher in the PSF compared to the PLF group ( $t = 3.145$ ,  $df = 8$ ,  $p < 0.05$ ) (Figure 29a). Furthermore, CPS2 and HPRT protein levels were significantly correlated to FST floating time. Correlation between FST floating time and ATIC protein levels showed tendency (Figure 29b).



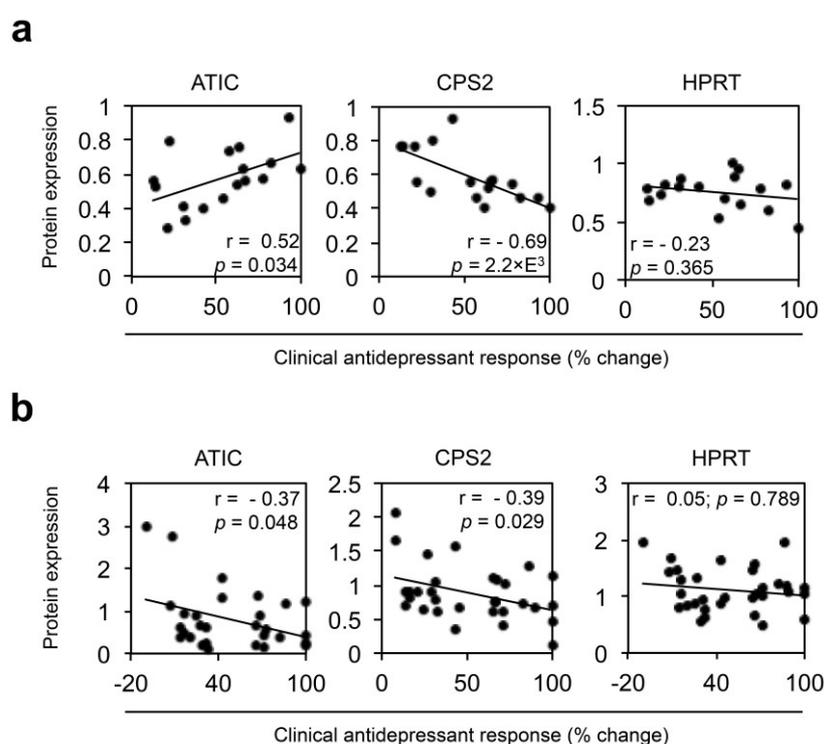
**Figure 29.** The effect of chronic paroxetine treatment on ATIC, CPS2 and HPRT protein expressions in the mouse erythrocytes. **(a)** Western blot and densitometry analyses of CPS2 and HPRT protein levels in the erythrocytes. Erythrocytic ATIC, CPS2 and HPRT proteins showed significant expression level differences between the PLF and PSF groups,  $n=5$ /group. **(b)** Correlation of the pathway protein levels with FST floating time. CPS2 and HPRT protein levels with FST floating time was significant. ATIC protein moderately correlated with FST floating time,  $n=15$ . Protein expression levels were normalized with  $\beta$ -actin. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  (two-tailed  $t$ -test). Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

### 3.5. Analysis of peripheral patient specimens

Next, I sought to corroborate my findings on pyrimidine and purine metabolism in patients chronically treated with antidepressants. For this purpose I carried out experiments with PBMCs isolated from patients.

First, the same pyrimidine and purine pathway proteins analyzed in mice, ATIC, CPS2 and HPRT, were assessed in PBMCs obtained from antidepressant responder and non-responder patients of the MARS study. In PBMCs collected after 4-6 weeks of antidepressant treatment, ATIC and CPS2 protein levels were significantly correlated with clinical antidepressant treatment response (ATIC:  $r = 0.52$ ,  $p = 0.034$  CPS2:  $r = -0.69$ ,  $p = 0.002$  HPRT:  $r = -0.23$ ,  $p = 0.365$ ) (Figure 30a).

To further investigate pharmacological effects of paroxetine I also performed *ex vivo* experiments with patients' PBMCs. Cells collected from patients upon admittance were cultivated and treated with paroxetine for 2 days. As had been the case for the *in vivo* PBMCs analysis, ATIC and CPS2 protein levels also significantly correlated with patients' clinical antidepressant response when their cultured PBMCs were treated with paroxetine (ATIC:  $r = -0.37$ ,  $p = 0.048$  CPS2:  $r = -0.39$ ,  $p = 0.029$  HPRT:  $r = 0.05$ ,  $p = 0.789$ ) (Figure 30b).

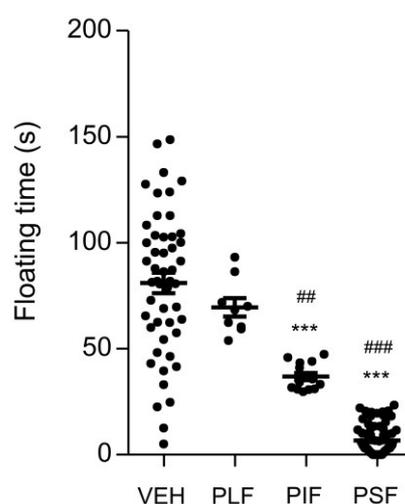


**Figure 30.** Correlation of ATIC, CPS2 and HPRT protein levels with clinical antidepressant treatment response. (a) Depression patients' PBMCs collected after 4-6 weeks of antidepressant

treatment were analyzed for ATIC, CPS2 and HPRT protein expression. ATIC and CPS2 protein levels significantly correlated with clinical antidepressant response,  $n=17$ . **(b)** PBMCs from inpatients with depression were collected at admission. Cells were *ex vivo* cultivated and treated with paroxetine for 2 days. After treatment, ATIC and CPS2 protein levels significantly correlated with clinical antidepressant response,  $n=32$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

### 3.6. Identification of glutamatergic and ubiquitin proteasome system (UPS) pathways

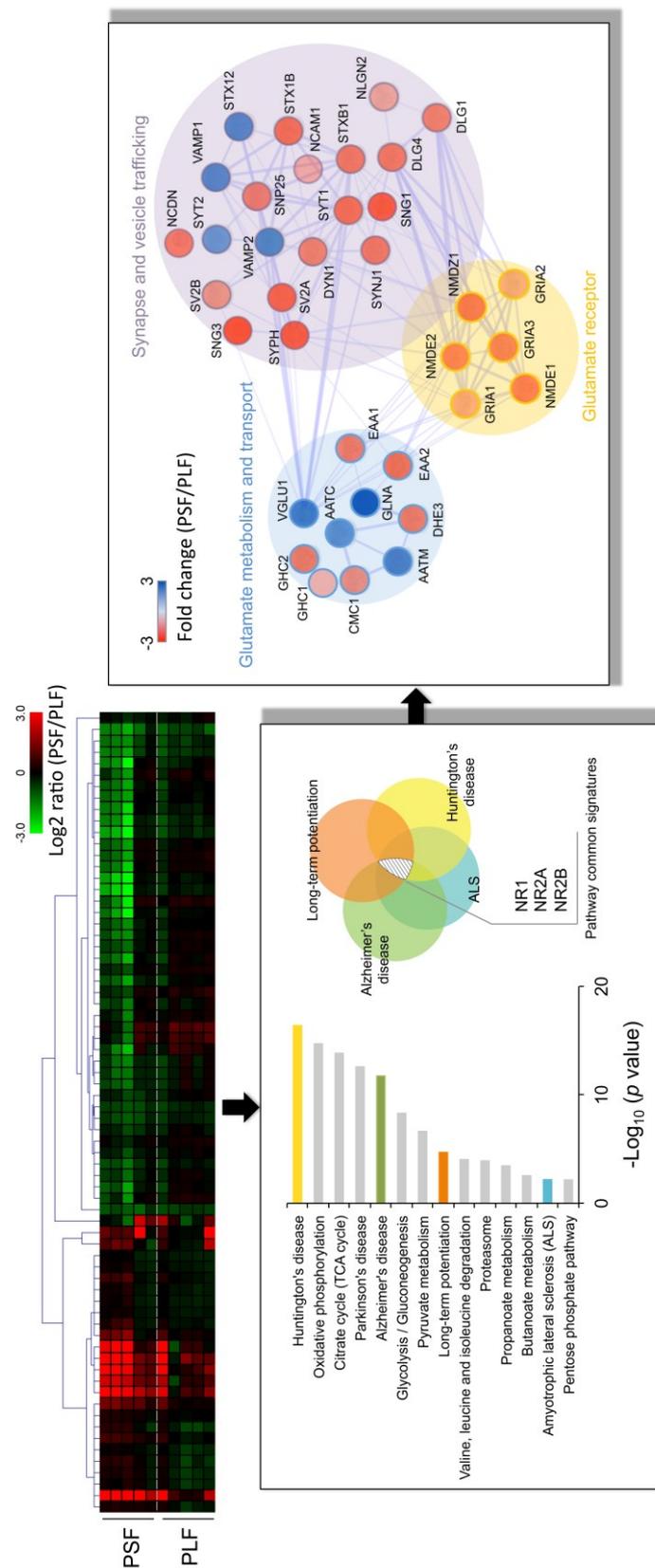
Mice were treated with paroxetine (5 mg/kg, twice a day) for 28 days. Three animal sub-groups, paroxetine-treated long floating (PLF), paroxetine-treated intermediate floating (PIF) and paroxetine-treated short floating (PSF) groups were identified according to FST floating time ( $F_{(3,141)} = 132.1, p < 0.0001$ ) (Figure 31).



**Figure 31.** Sub-grouping of mice treated with paroxetine. Paroxetine-treated mice were categorized into PLF, PIF and PSF groups based on FST floating time,  $n(\text{VEH/PLF/PIF/PSF})=50/9/14/72$ . Data are expressed as the mean  $\pm$  SEM. \*\*\* $p < 0.001$  (two-tailed  $t$ -test). ## $p < 0.01$  vs. PLF, ### $p < 0.001$  vs. PLF and PIF (one-way ANOVA with Tukey's test).

To investigate the systemic effect of chronic paroxetine treatment on hippocampal molecular pathways, proteomic analyses were performed of the two extreme groups (PLF and PSF groups), which resulted in significant protein expression differences. Significantly affected pathways related to Amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease and long-term potentiation commonly enriched NR1, NR2A and NR2B proteins. Based on the common protein signatures,

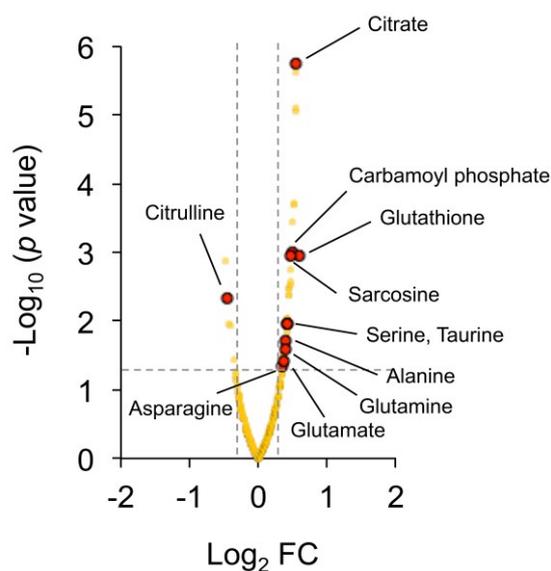
glutamate receptors, glutamate metabolism and transport, and synapse and vesicle trafficking pathways were also altered (Figure 32).



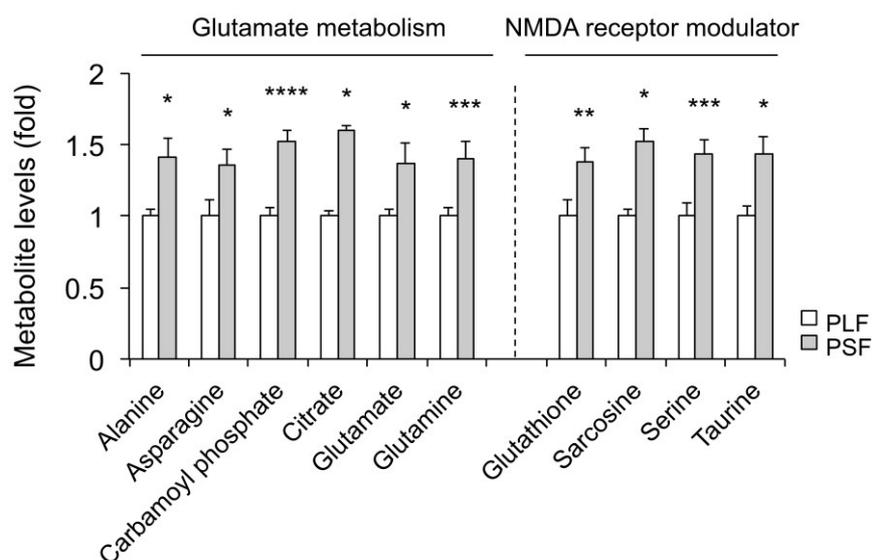
**Figure 32.** Proteomics profiles and enriched pathways between the PLF and PSF groups. The

common protein signatures among pathways were obtained using Venn diagram analysis and further subjected to protein interaction network analysis. In the heatmap, colors denote  $\log_2$  ratio. In the interaction pathway map, colors denote fold difference between the two groups. Proteins with  $|\log_2FC| > 0.3$  and adjusted  $p$  value  $< 0.05$  were considered significant,  $n=5/\text{group}$ .

Hippocampal metabolite profiling data showed altered levels of relevant NMDA receptor modulators and metabolites that are part of the glutamate metabolism pathway (Figure 33). All metabolites were at significantly higher levels in PSF compared to PLF mice (Figure 34).



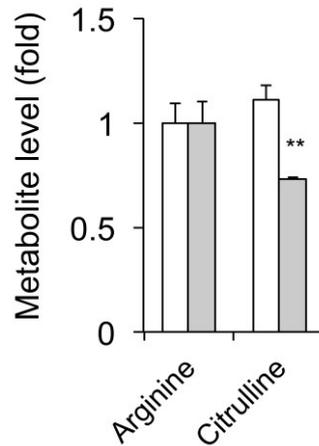
**Figure 33.** A volcano plot comparing PLF and PSF metabolites. Metabolites with  $|\log_2FC| > 0.3$  and  $-\log_{10}(p \text{ value}) > 1.3$  were considered significant,  $n=5/\text{group}$ .



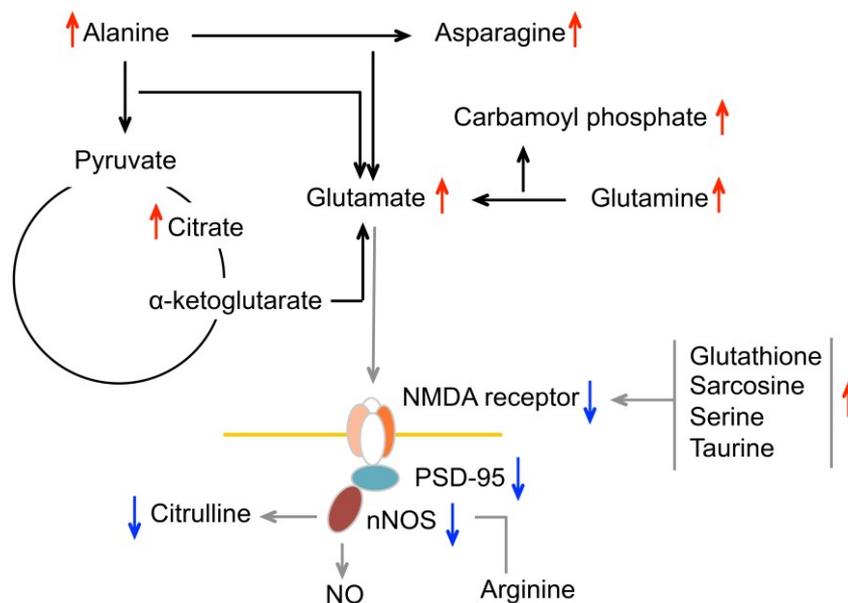
**Figure 34.** Glutamate-related metabolite differences between PLF and PSF mice,  $n=5/\text{group}$ .

Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (two-tailed  $t$ -test).

Altered levels of citrulline whose conversion from arginine is catalyzed by nNOS protein were detected as well (Figure 35). Integration of proteomic and metabolomic data sets identified systemic metabolite-protein network differences between the PLF and PSF groups (Figure 36).



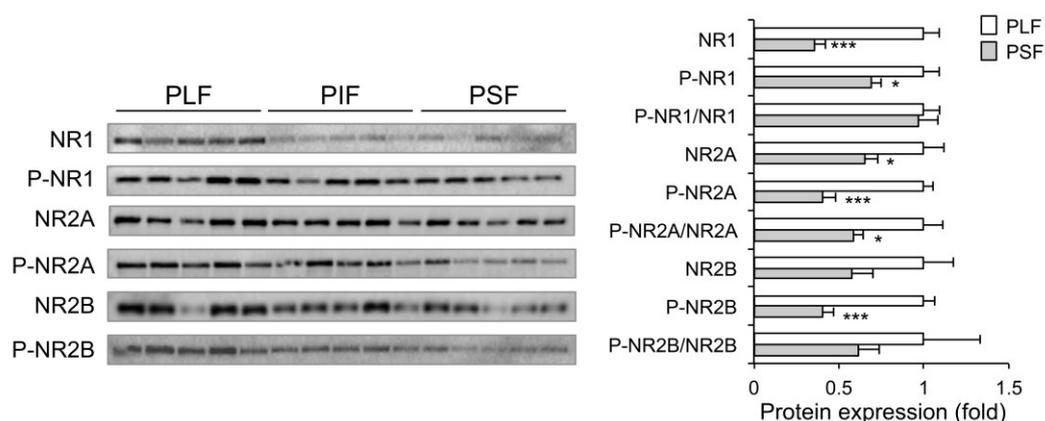
**Figure 35.** Arginine and citrulline levels in PLF and PSF mice,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM. \*\* $p < 0.01$  (two-tailed  $t$ -test).



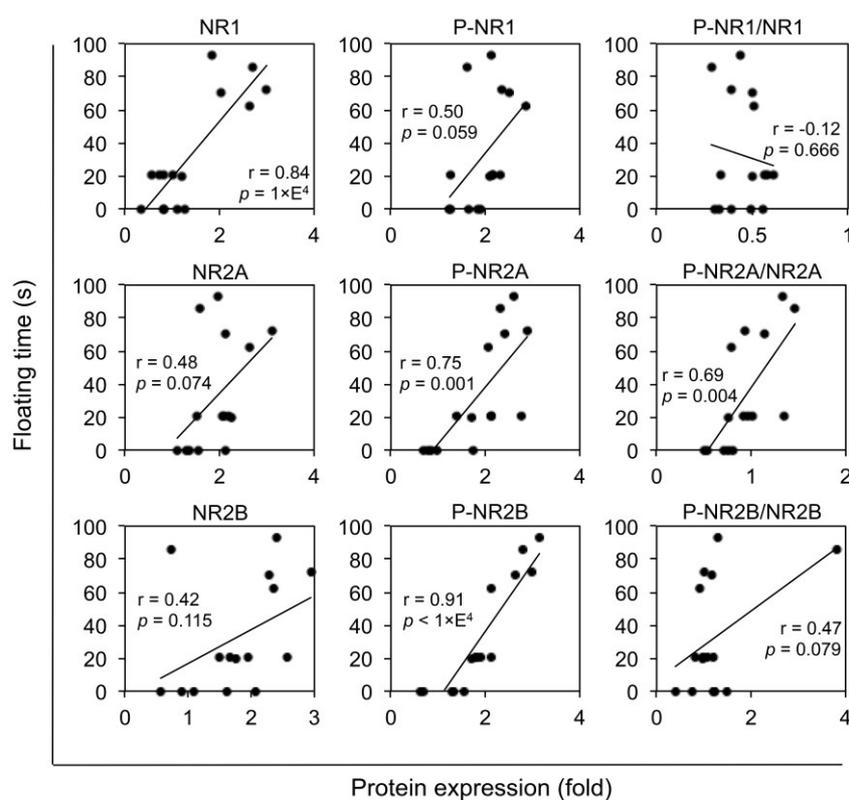
**Figure 36.** Affected protein-metabolite network following chronic paroxetine treatment. Upward-pointing red arrow indicates higher biosignature level in PSF compared to PLF mice. Downward-pointing blue arrow indicates lower biosignature level in PSF compared to PLF mice.

### 3.7. Validation of glutamatergic and UPS pathways

Hippocampal NRs and their phosphorylation levels were compared between PLF and PSF mice. Except for P-NR1/NR1 and P-NR2B/NR2B ratios, all NRs and P-NRs levels were significantly different between the two groups (Figure 37). In addition, NR levels were significantly correlated with FST floating time (Figure 38).

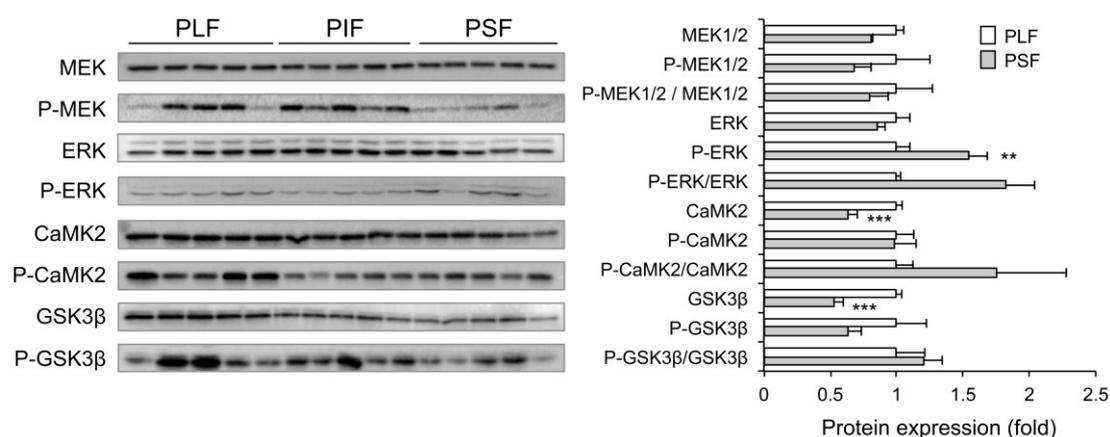


**Figure 37.** NR protein level differences between the sub-groups,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. PLF (two-tailed  $t$ -test).

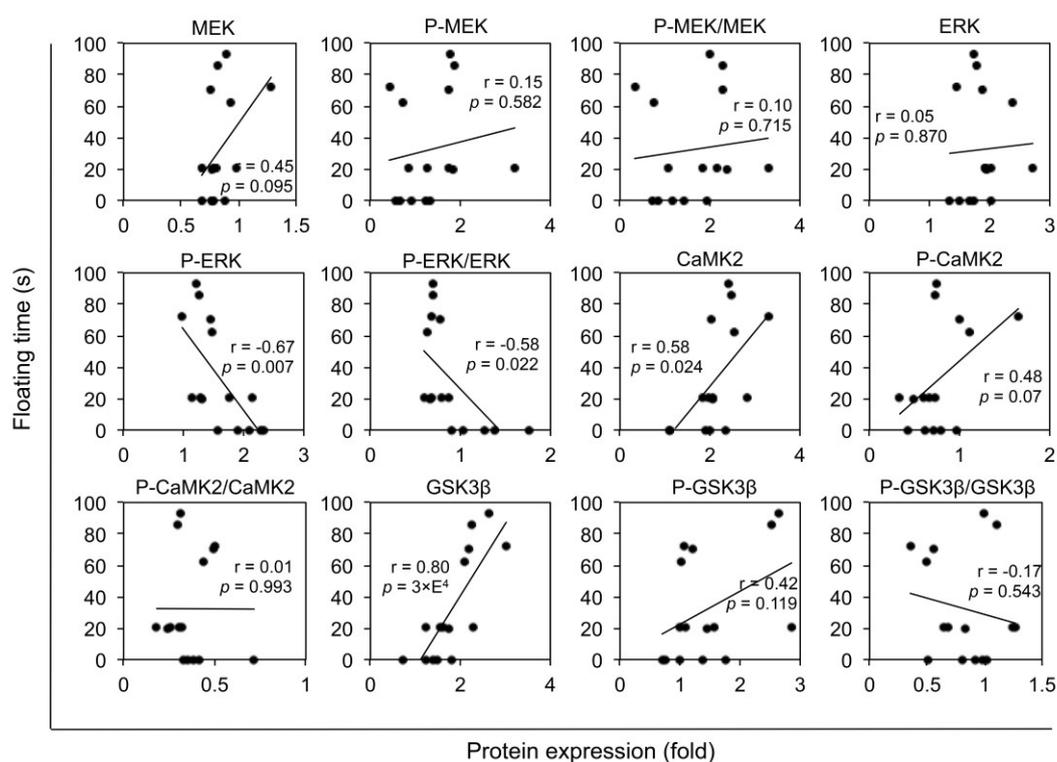


**Figure 38.** Correlation of NR protein levels with FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

Hippocampal NMDA receptor signaling proteins and their phosphorylation status were also investigated between PLF and PSF groups. Especially, P-ERK, CaMK2 and GSK3 $\beta$  levels were significantly different between the two groups (Figure 39) and correlated with FST floating time (Figure 40).

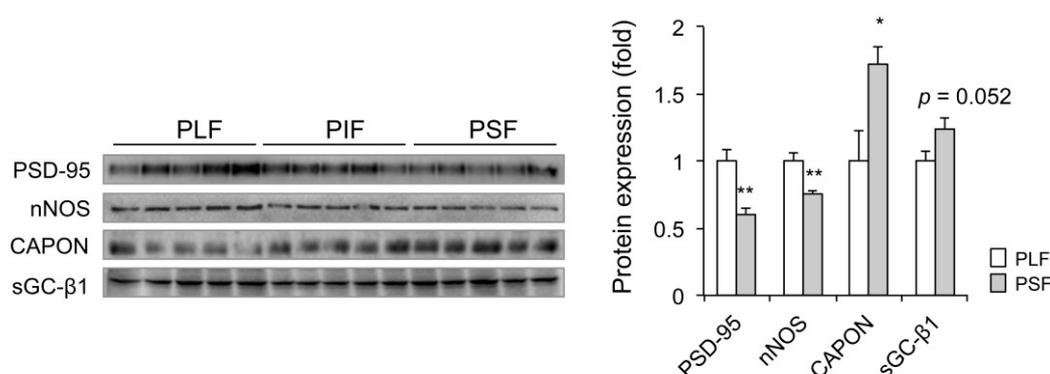


**Figure 39.** NMDA receptor signaling protein level differences between the sub-groups,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. PLF (two-tailed  $t$ -test).

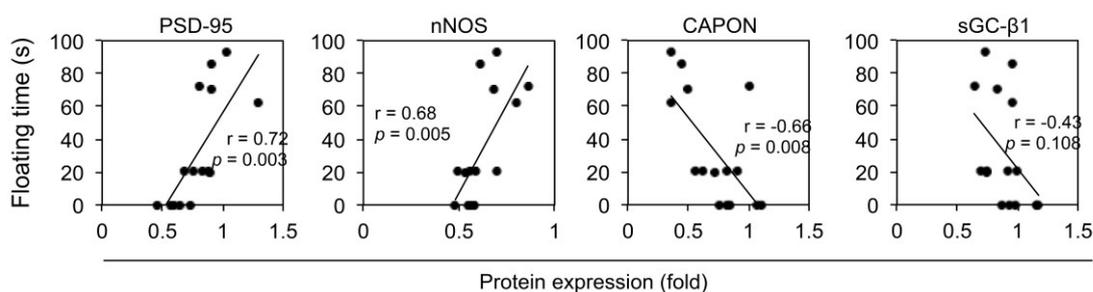


**Figure 40.** Correlation of NMDA receptor signaling protein levels with FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

Nitric oxide (NO) production-related proteins were also significantly different between PLF and PSF mice (Figure 41). PSD-95, nNOS and CAPON protein levels showed significant correlation with FST floating time (Figure 42).

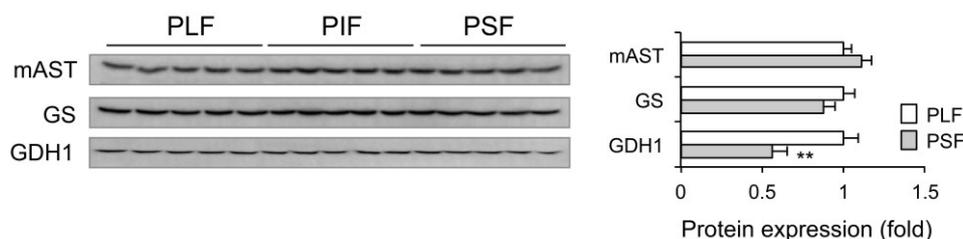


**Figure 41.** Differential effect of chronic paroxetine treatment on PSD-95/nNOS complex. PSD-95, nNOS, CAPON and sGC-β1 protein level differences between PLF and PSF mice,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$  vs. PLF (two-tailed  $t$ -test).

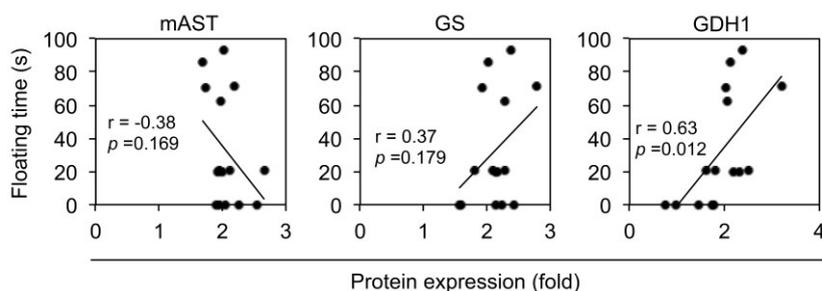


**Figure 42.** Correlation of PSD-95/nNOS complex with FST floating time. PSD-95, nNOS, CAPON and sGC-β1 protein levels with FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

Glutamate metabolism-related protein levels between PLF and PSF mice were further investigated in the hippocampus. Particularly GDH1 showed significant level differences between the two groups and correlated with FST floating time (Figures 43 and 44).

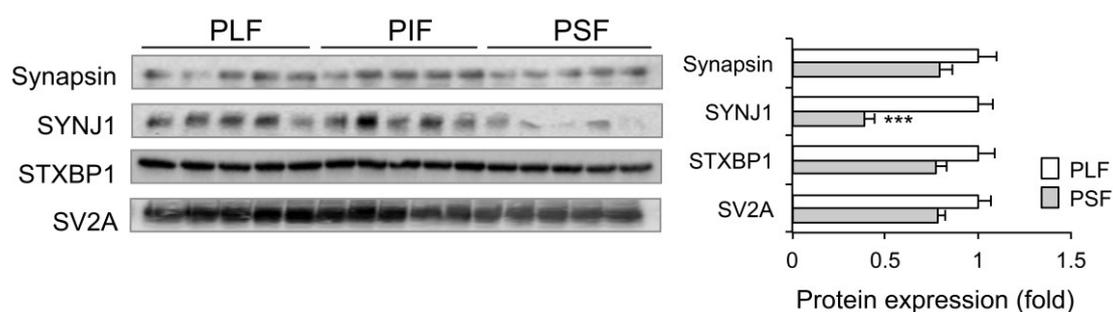


**Figure 43.** Glutamate metabolism protein level differences between PLF and PSF mice,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM.  $**p < 0.01$  vs. PLF (two-tailed  $t$ -test).

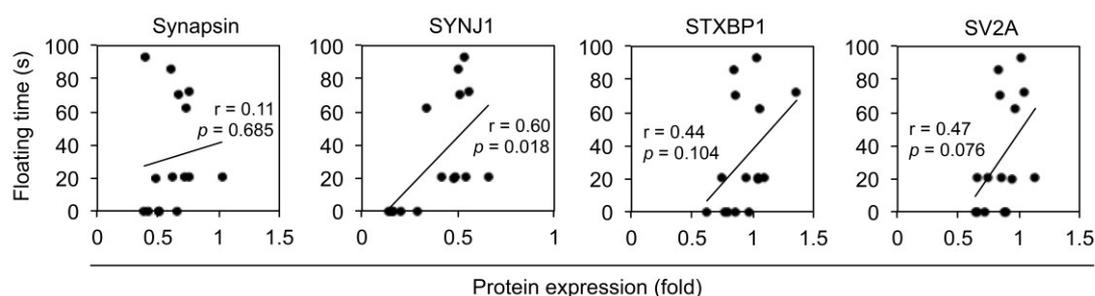


**Figure 44.** Correlation of glutamate metabolism protein levels of FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

Synapse and vesicle trafficking-associated protein level differences were also assessed. Only SYNJ1 protein was found to be significantly different between the groups, and correlated with FST floating time (Figures 45 and 46).

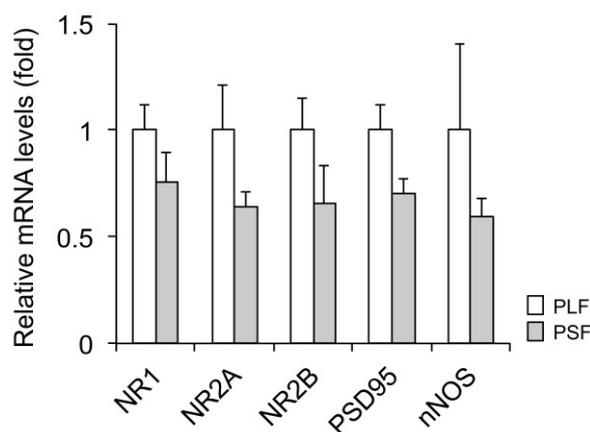


**Figure 45.** Synapse and vesicle trafficking-associated protein level differences between PLF and PSF mice,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM.  $***p < 0.001$  vs. PLF (two-tailed  $t$ -test).

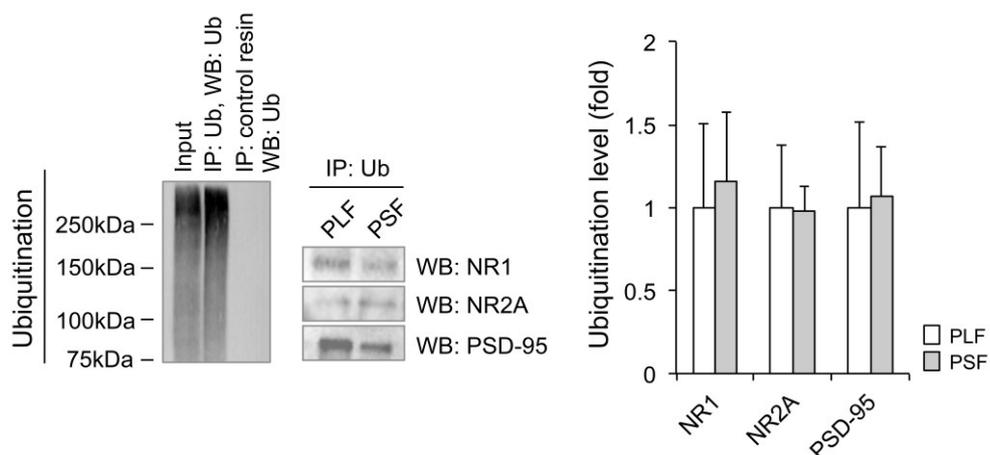


**Figure 46.** Correlation of Synapse and vesicle trafficking-associated protein levels with FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

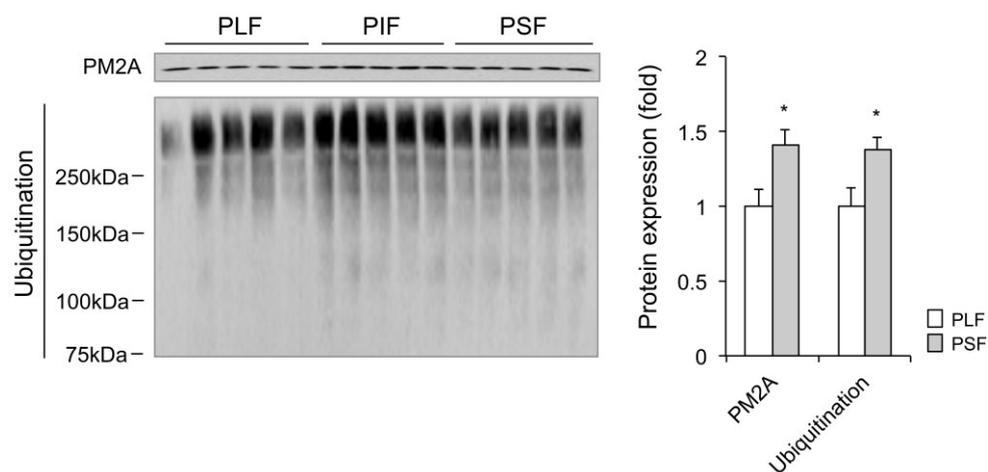
Since I did not see any NRs, PSD-95 and nNOS transcript level differences between PLF and PSF groups (Figure 47) I next examined the possible involvement of the UPS protein degradation pathway in the observed protein expression differences. Whereas no ubiquitinated NR1, NR2A and PSD-95 differences were detected (Figure 48), PSF mice showed greater PM2A and ubiquitination levels compared to PLF mice (Figure 49), which correlated with FST floating time (Figure 50).



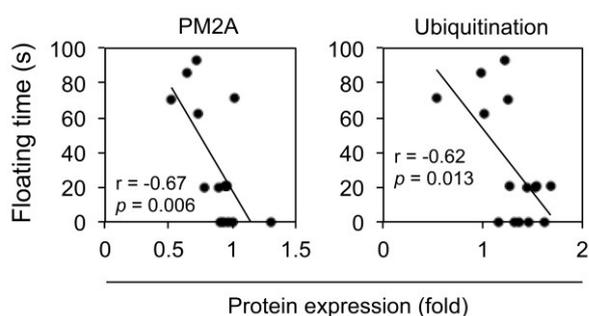
**Figure 47.** qRT-PCR data of NRs, PSD95 and nNOS. No transcription differences were found between PLF and PSF groups,  $n=6$ /group. Data are expressed as the mean  $\pm$  SEM.



**Figure 48.** Ubiquitinated NR1, NR2A and PSD-95 protein level differences between PLF and PSF groups. No differences were found between PLF and PSF groups,  $n=3$ /group. Data are expressed as the mean  $\pm$  SEM.



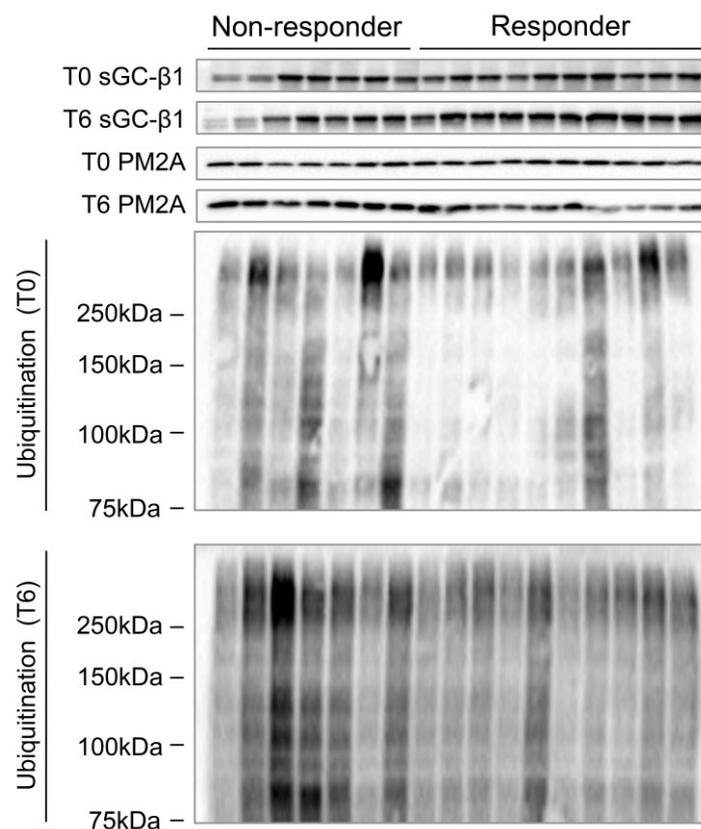
**Figure 49.** PM2A and ubiquitination level differences between PLF and PSF mice,  $n=5$ /group. Data are expressed as the mean  $\pm$  SEM. \* $p < 0.05$  vs. PLF (two-tailed  $t$ -test).



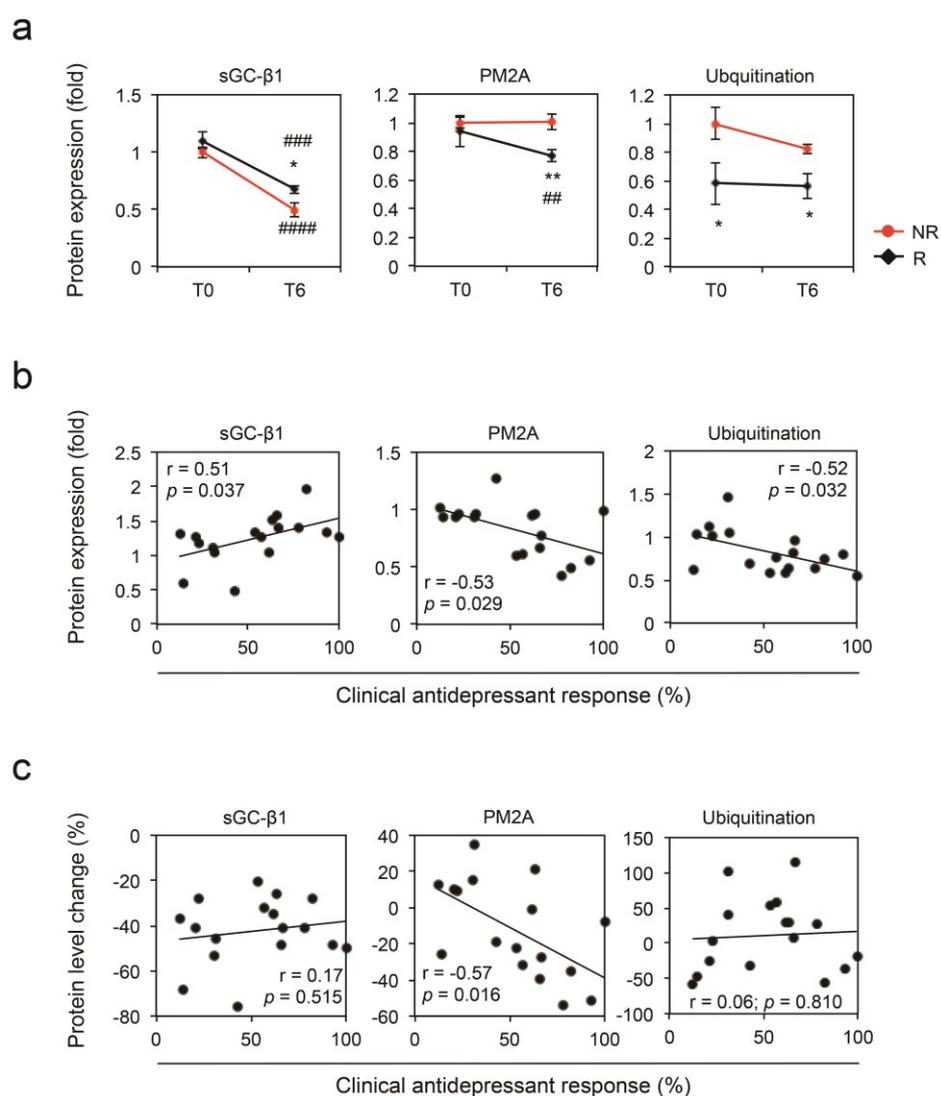
**Figure 50.** Correlation of PM2A and ubiquitination levels with FST floating time,  $n=15$ . Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

### 3.8. Candidate biomarker validation in human PBMCs from MDD patients

To investigate the relevance of the identified biosignatures, sGC- $\beta$ 1, PM2A and ubiquitination levels were analyzed in MDD patients' PBMCs (Figures 51 and 52). All three proteins were differentially expressed between the antidepressant responder and non-responder patient groups, especially 6 weeks after admission (T6) (Figure 52a). Whereas sGC- $\beta$ 1 protein levels were significantly reduced in both groups at T6, PM2A protein levels were significantly reduced only in responder patients' PBMCs at T6. Ubiquitination levels were not altered by chronic antidepressant treatment in either group. However, they were lower in responder compared to non-responder patients. All three protein levels significantly correlated with clinical antidepressant response at T6 (Figure 52b). Only PM2A protein level changes between baseline (T0) and T6 samples significantly correlated with the clinical antidepressant treatment response (Figure 52c).



**Figure 51.** Western blot analysis in MDD patient's PBMCs.



**Figure 52.** sGC-β1, PM2A and ubiquitination levels in human PBMCs from antidepressant responder and non-responder patients. **(a)** sGC-β1, PM2A and ubiquitination level differences between antidepressant non-responder (NR) and responder patients (R) at baseline (T0) and after 6-weeks treatment (T6),  $n=17$ . **(b)** Correlation of protein levels at T6 with clinical antidepressant treatment response,  $n=17$ . **(c)** Correlation of protein level changes (between T0 and T6) with clinical antidepressant treatment response,  $n=17$ . \* $p < 0.05$ , \*\* $p < 0.01$  vs. NR (two-tailed  $t$ -test). ## $p < 0.01$ , ### $p < 0.001$ , #### $p < 0.0001$  vs. T0 (two-tailed paired  $t$ -test). Data are expressed as the mean  $\pm$  SEM. Pearson correlation coefficients ( $r$ ) with  $p$  values are indicated in the correlation graphs.

## 4. Discussion

### 4.1. Purine and pyrimidine metabolism pathway

In my thesis project I have attempted to integrate quantitative proteomics and metabolomics data to improve our understanding of biological pathway changes relevant for the antidepressant treatment response. Integrated -omics data coupled with *in silico* analysis has delineated several molecular pathways and biomarker candidates involved in the differential antidepressant treatment response in mice, which were further validated in human MDD patients' PBMCs.

Using an inbred mouse strain I was able to stratify paroxetine response sub-groups based on animals' behavioral phenotype. For the unbiased separation of paroxetine responder and non-responder mice, I carried out HCA based on FST floating time. The HCA has been a method to build and split different hierarchies of clusters. It has been applied to identify sub-groups of cells and animals based on marker protein expression or behavioral parameters (Droy-Dupré et al., 2015; Muehlmann et al., 2015).

The FST is a behavioral test commonly used to evaluate antidepressant-like effects in mice (Webhofer et al., 2011; Doucet et al., 2013; Kaster et al., 2013; Weckmann et al., 2014). I submit that paroxetine-treated mice that exhibit no FST floating time difference compared with vehicle-treated mice are drug non-responders. My results indicate that 15-40% of the mice are antidepressant non-responders, similar to what is observed for MDD patients.

To further characterize the PLF and PSF sub-groups I also assessed female urine sniffing time, a behavioral parameter pertinent to evaluate SSRI treatment effect in mice (Malkesman et al., 2010; Wagner et al., 2012). While the PLF and PSF groups did not show any difference of female urine sniffing time prior to being treated with paroxetine, chronic paroxetine treatment induced a differential behavioral effect between the groups. This finding indicates that the different behavior between mice is not inherent, but is the result of chronic paroxetine treatment.

Stratification of sub-groups and tailored treatment has been suggested to result in a more favorable outcome and increased treatment efficiency (O'Donnell, 2013; Landeck et al., 2016). Sub-group stratification of patients with multifactorial diseases

like psychiatric and neurodegenerative disorders need to be assessed for molecular and phenotypic signatures. In the current study I only took FST floating time into account for the stratification to simplify data processing and interpretation. Including other behavioral parameters such as FST swimming and struggling time may add further information with regard to the heterogeneous individual response to chronic antidepressant treatment. Associated physiological signatures can also be used towards this goal. ACTH, corticosterone, CRH and other biomolecules associated with psychiatric disorders and treatment, as well as phenotypic markers including cognitive function, specific brain region activity and body mass index (BMI) would qualify in this regard. Integration of other biological dimensions such as the microbiome, lipidome and glycome can also be informative to enhance the systemic understanding of depressive disorders and determine sub-groups more precisely.

To examine the relevance of covariates, drug levels, age and body weight gain were assessed. Paroxetine concentrations in whole brain and plasma were analyzed to check for a possible association of drug levels with antidepressant-like activity. Previous studies have reported a significant relationship between plasma levels and therapeutic response towards paroxetine (Yoshimura and Nakano, 2009). An association of genetic variants of CYP2D6 and ABCB1, which are involved in drug metabolism and permeability, with heterogeneous paroxetine treatment response has been suggested previously (Gex-Fabry et al., 2008; Preskorn, 2014). In my study I did not find any paroxetine concentration differences between PLF and PSF mice in either whole brain or plasma and drug levels did not correlate with FST floating time. This suggests that paroxetine levels in whole brain and plasma are irrelevant for the observed differential drug treatment response in our long-term treatment setting. I also considered animal age and its relationship with paroxetine response. Age-dependent outcome of SSRI treatment has been assessed with regard to adverse effects of drug treatment by comparing antidepressant-induced behavioral response of juvenile or adolescent and adult rodents (Olivier et al., 2011; Mitchell et al., 2013). In the current study all mice reached adulthood prior to being subjected to experiments (> 8 weeks). Although significant FST floating time differences were observed for mice between 18 and 20 weeks of age, no general age effect on FST floating time was detected. The relationship between body weight gain and chronic paroxetine treatment response was also investigated. Chronic paroxetine treatment induced a significant

increase of body weight. However, body weight gain and drug treatment response for each group did not correlate significantly.

My integrated metabolomics and proteomics data showed a differential effect of chronic paroxetine treatment on mouse hippocampal purine and pyrimidine metabolism. Purine and pyrimidine metabolites and their receptors have previously been shown to be associated with various neuropsychiatric disorders. The anti-purinerbic drug suramin was found to reverse autism-like behaviors and metabolism in mice (Naviaux et al., 2014). Polymorphisms of the P2RX7 gene, which encodes a purinerbic ion channel, have been associated with the development of MDD (Lucae et al., 2006). In addition, low brain purine levels were found in female depressed patients responding to treatment with the SSRI fluoxetine (Renshaw et al., 2001). Pyrimidines such as cytidine and uridine have been shown to have antidepressant-like activities in mice (Carlezon et al., 2002; 2005).

Hippocampal metabolome profiling also implicated other metabolites with elevated levels in PSF mice that have antidepressant-like activity. Folate has been shown to have an antidepressant-like effect in mice (Brocardo et al., 2008) and low folate levels were found to be associated with MDD (Gilbody et al., 2007). L-Methylfolate, the active metabolite of folate is used for patients with MDD who partially respond or do not respond to SSRIs (Papakostas et al., 2012). Myo-inositol has been identified as a potential biomarker of SSRI treatment response and innate anxiety disorder (Zhang et al., 2011a; Webhofer et al., 2013; Zhao et al., 2015) and has been shown to have anxiolytic and antidepressant-like effects in both animals and humans (Wurglics and Schubert-Zsilavecz, 2006; Herrera-Ruiz et al., 2011) Flavones are also known to have an antidepressant-like effect (Wurglics and Schubert-Zsilavecz, 2006; Herrera-Ruiz et al., 2011). The elevated levels of folate, myo-inositol and flavones that I found in the hippocampus of PSF mice might be of relevance for the favorable paroxetine response.

I also compared the plasma metabolome of the two mouse sub-groups, PLF and PSF, at baseline and after 28 days of treatment (T0 and T4) with the aim of identifying differentially affected pathways and potential biomarker candidates in the periphery. Plasma metabolome changes over time resulted in group-specific profiles. Major metabolite level alterations and elevation of purine and pyrimidine metabolites were observed in the PSF group. In contrast, the plasma metabolome was minimally affected by chronic paroxetine treatment in the PLF group. Despite the fact that the

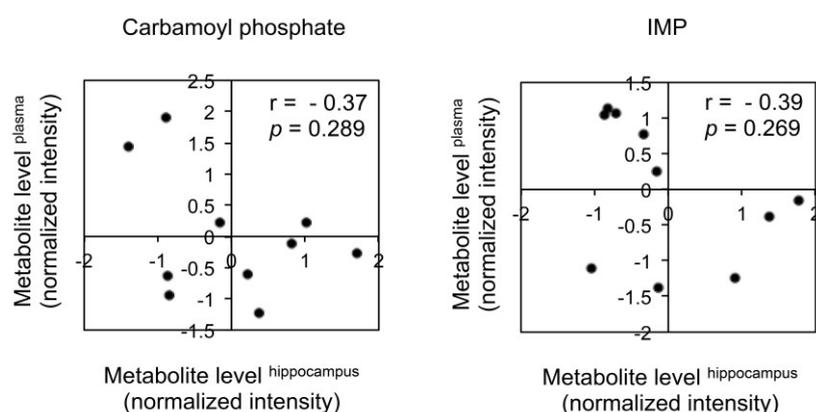
glycine, serine and threonine metabolism pathway was specifically enriched as a sub-pathway of PLF group, significant metabolite level changes were observed in both sub-groups. I also found plasma metabolite biosignatures commonly regulated between the PLF and PSF groups. In particular, levels of choline and threonine that are part of the glycine, serine and threonine metabolism pathway were significantly changed both in the PLF and PSF groups after chronic paroxetine treatment. Metabolites of glycine, serine and threonine metabolism may thus constitute confounding biosignatures.

In contrast to hippocampal purine/pyrimidine metabolites, plasma metabolite level changes were not correlated with FST floating time. Small plasma metabolite level differences between the PLF and PSF groups might be responsible for non-significant correlation. Despite non-significant correlation between plasma metabolite level changes and chronic paroxetine treatment response, group-specific analysis showed PSF-group specific pathway enrichment and significant metabolite changes.

Based on my integrated -omics data that implicate purine and pyrimidine metabolism pathways to be involved in paroxetine response I next wanted to corroborate these findings through the analysis of proteins that are part of these pathways. Based on the observed differences of carbamoyl phosphate and IMP levels between the PLF and PSF groups, ATIC, CPS2 and HPRT protein levels were analyzed. CPS2 catalyzes early steps of carbamoyl phosphate synthesis in the pyrimidine biosynthesis pathway. ATIC and HPRT play a central role in synthesis and conversion of inosine monophosphate (IMP), the end product of the purine biosynthesis pathway. For pathway validation I chose hippocampus, prefrontal cortex and erythrocytes and compared ATIC, CPS2 and HPRT protein levels between the PLF and PSF groups. Western blot analyses revealed that hippocampal and erythrocytic ATIC, CPS2 and HPRT proteins were differentially expressed between the two groups while prefrontal cortex protein expression showed no difference. Hippocampal and erythrocytic ATIC, CPS2 and HPRT protein levels were also highly correlated with FST floating time while no significant correlation was observed in the prefrontal cortex. This indicates that different purine and pyrimidine metabolism pathway activities between PLF and PSF groups might be specific for the hippocampus.

Interestingly, I observed an inverse relationship between hippocampal and erythrocytic protein expression for the three proteins. Inconsistent biosignature expression patterns in brain and peripheral tissues have been found in other cases

related to psychiatric disorders. Brain and blood BDNF levels were inversely correlated in a genetic rat model of depression (Elfving et al., 2010). Brain and white blood cell p11 protein levels also showed an inverse relationship (Svenningsson et al., 2014). Inverse myo-inositol levels between hippocampus and plasma were also reported previously in mice chronically treated with paroxetine (Webhofer et al., 2013). In the current study we found that carbamoyl phosphate and IMP levels have an inverse correlation between hippocampus and plasma. This might be caused by the observed inverse relationship of ATIC, CPS2 and HPRT enzyme expression levels between hippocampus and erythrocytes (Figure 53).



**Figure 53.** Correlation between hippocampus and plasma metabolite levels. Carbamoyl phosphate and IMP metabolite levels showed inverse relationships between hippocampus and plasma. Pearson correlation coefficients ( $r$ ) with  $P$  values are indicated in the correlation graphs.

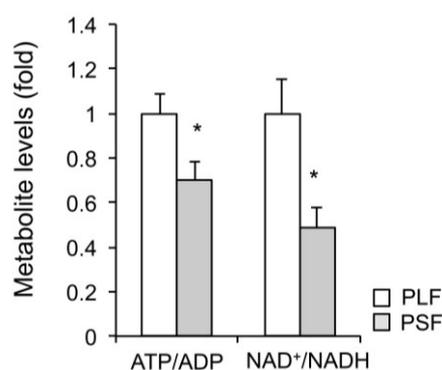
Lower expression of hippocampal ATIC, CPS2 and HPRT in the PSF group might be caused by negative feedback regulation in response to elevated pathway metabolite levels. Higher erythrocytic protein levels in the PSF mice might reflect PSF group-specific activation of purine and pyrimidine metabolisms.

To address the question whether the pathways for antidepressant response identified in the mouse are also relevant for patients' response, PBMCs from antidepressant responder and non-responder patients were analyzed for ATIC, CPS2 and HPRT protein expressions. PBMCs collected after 4-6 weeks of antidepressant treatment showed significant correlation between ATIC and CPS2 protein expression levels and HDRS score change between baseline and following chronic antidepressant treatment. Whereas PBMCs ATIC protein expression showed a similar pattern as the one

observed in mouse erythrocytes, PBMCs CPS2 protein expression resembled that of mouse hippocampus.

In *ex vivo* experiments, PBMCs ATIC protein levels were negatively correlated with clinical antidepressant response. The observed discrepancy between *in vivo* and *ex vivo* PBMCs ATIC protein expression could be due to different treatment conditions (chronic vs. subchronic) and exposure to multiple types of drugs. PBMCs' CPS2 protein expression consistently showed negative correlation with mouse hippocampal protein expression both in *in vivo* and *ex vivo*. ATIC and CPS2 proteins may thus represent candidate biomarkers to determine clinical antidepressant treatment response. For HPRT protein, I failed to determine a significant correlation between clinical antidepressant response and PBMCs protein levels both *in vivo* and *ex vivo*.

Previously obtained data by Webhofer et al. revealed altered energy metabolism upon chronic paroxetine treatment in DBA/2OlaHsd mice when compared to vehicle-treated control mice (Webhofer et al., 2011; 2013). The previous and current studies are not directly comparable since the studies were performed under different conditions (groups, dosage and route of paroxetine administration, mouse strain). In the current study, energy metabolism-related pathways including glycolysis, Krebs cycle and glycogen metabolism that came up in the previous study were not enriched when PLF and PSF groups were compared. The current -omics analyses were performed to compare PLF and PSF mouse groups and identified purine and pyrimidine metabolisms as the main distinguishing pathways. However, based on ATP/ADP and NAD<sup>+</sup>/NADH ratios found in the current study I can not exclude that energy metabolism might also distinguish the PLF and PSF groups (Figure 54).



**Figure 54.** Energy-related metabolite ratios in the hippocampus. ATP/ADP and NAD<sup>+</sup>/NADH ratios in the hippocampus in the PLF and PSF group ( $n = 5/\text{group}$ ). Bars represent mean  $\pm$  SEM. \* $p < 0.05$  (two-tailed  $t$ -test).

Hippocampal neurogenesis is known to be caused by the action of antidepressants (Malberg et al., 2000, Santarelli et al., 2003, Gundersen et al., 2013). Since purine and pyrimidine metabolites are critical for cell proliferation, increased purine/pyrimidine metabolism activity in PSF mice may also result in neurogenesis in these animals.

Based on evidence from the literature microRNAs (miRNAs) may be also relevant for the distinct regulation of purine/pyrimidine metabolisms in the PLF and PSF mouse groups. Feng et al have shown that miR-1 and miR-133a-3p regulate purine and pyrimidine metabolic pathways (Feng et al., 2015) and the purine metabolism gene *GART* is regulated by 16 miRNAs (Li et al., 2014). Since SSRIs impact miRNA levels (Hansen and Obrietan, 2013) this may explain the observed differences in the purine and pyrimidine metabolisms upon chronic paroxetine treatment.

Based on these data a pharmacological study with an inhibitor of the folate pathway that also regulates purine and pyrimidine metabolism could shed light on the functional relevance of the pathways in chronic antidepressant treatment response.

The current study used wild-type stress naïve DBA/2J mice to investigate the pharmacological heterogeneity of the antidepressant response. Wild-type stress naïve rodents have been used previously to evaluate antidepressant-like effects (Guzzetti et al., 2008; Gurbuz Ozgur et al., 2015; Taguchi et al., 2016). An extension of our studies using an animal model with a depression-like phenotype would further validate the identified pathways affected by the antidepressant treatment response and add relevant information for the antidepressant treatment of patients.

## 4.2. Glutamatergic pathway

My results also suggest that proteins and metabolites associated with the glutamatergic pathway are affected by chronic antidepressant treatment which became apparent with the categorization of a paroxetine-treated intermediate floating (PIF) group.

The glutamatergic pathway has previously been associated with MDD pathobiology and antidepressant response. In depressed patients, significantly elevated serum, plasma and cerebrospinal fluid glutamate levels were found (Kim et al., 1982; Altamura et al., 1993; Mauri et al., 1998; Levine et al., 2000; Mitani et al., 2006). A single nucleotide polymorphism (SNP) in metabotropic 7 glutamate receptor was shown to be involved in the onset of the clinical antidepressant effect (Fabbri et al.,

2013). Glutamate release decreases with chronic fluoxetine, desipramine, reboxetine, venlafaxine or agomelatine treatment (Bonanno et al., 2005; Musazzi et al., 2010). Numerous studies have shown that chronic antidepressant treatment regulates glutamatergic receptor expression in rodent hippocampus (Boyer et al., 1998; Skolnick, 1999; Martinez-Turrillas et al., 2002; Barbon et al., 2006; Pittaluga et al., 2007; Wieronska et al., 2007; Ryan et al., 2009; Calabrese et al., 2012; O' Connor et al., 2013). The current study also indicates that NRs and P-NRs expression levels were differentially affected in paroxetine-treated sub-groups.

We also observed different levels of proteins downstream of the glutamate receptor (CaMKII, GSK-3 $\beta$ , P-ERK), which might be a reflection of differential NMDA receptor activity. CaMKII has been linked to neurotransmitter release and synaptic plasticity (Lotrich and Pollock, 2005), which has been associated with neuropsychiatric disorders and antidepressant treatment effects (Pavlidis et al., 2002; Holderbach et al., 2007; Wang et al., 2008). Our observation of a lower CaMKII expression level in PSF mice suggests a regulatory mechanism that prevents synaptic connections becoming too strong as has been suggested by Robison et al (Robison et al., 2014).

Accumulating evidence has implicated GSK-3 in the pathogenesis of bipolar disorder and major depressive disorder (Gould et al., 2004; Lovestone et al., 2007) as well as the antidepressant treatment response. Tsai et al., reported that polymorphisms in GSK-3 $\beta$  gene were associated SSRI treatment response (Tsai et al., 2008). Paroxetine and lithium treatments were shown to regulate GSK-3 $\beta$  phosphorylation which also predicts clinical improvement of depressive patients (Gassen et al., 2016). Joaquim et al. showed that long-term treatment with sertraline induces increased expression and decreased phosphorylation of GSK-3 $\beta$  in MDD patient platelets (Joaquim et al., 2012). Our chronic paroxetine treatment did not induce significantly different inhibition of GSK-3 $\beta$  activity, which is evident from Ser9-P-GSK-3 $\beta$  protein levels. As GSK-3 protein suppresses Ca<sup>2+</sup>-current and neurotransmitter release by inhibiting calcium channels and soluble NSF attachment protein receptor (SNARE) complex interaction (Wildburger and Laezza, 2012), low levels of GSK-3 $\beta$  total protein in PSF mice might result in greater synaptic transmission and a more favourable antidepressant treatment outcome.

Several lines of evidence have also associated ERK with MDD and the antidepressant treatment response. *Post-mortem* brains of depressed suicide subjects showed reduced

ERK expression suggesting a role of the protein in MDD pathophysiology (Dwivedi et al., 2001). Antidepressant treatment increases ERK phosphorylation levels in rodent hippocampus (Gourley et al., 2008; Qi et al., 2008). Different P-ERK levels between PLF and PSF mice implicate a potential role in the heterogeneous antidepressant treatment response.

We also observed significantly different GDH1 and SYNJ1 protein levels between PLF and PSF groups. GDH1 has been associated with glutamatergic transmission and synaptic activity in the hippocampus (Bao et al., 2009; Michaelis et al., 2011). In addition, SYNJ1 gene expression, which is required for vesicle recycling and synaptic transmission (Cremona et al., 1999; Lüthi et al., 2001; Kim et al., 2002; Mani et al., 2007) was reported to be significantly altered by imipramine and St John's wort, an herbal product with antidepressant activities (Wong et al., 2004). GDH1 and SYNJ1 protein level alterations may indicate different synaptic transmission activity between PLF and PSF groups.

My results were further corroborated by metabolite profiling data. GDH1 catabolizes glutamate and its elevated levels may be caused by the low GDH1 protein expression I found in PSF mice. Alternatively, high glutamate levels might induce a compensatory feedback regulation of GDH1 protein expression to prevent pathway over-activation. Altered NR levels are consistent with the observed glutamate levels in the PLF and PSF mice. More glutamate and other NMDA receptor modulators could result in reduced NMDA receptor expression as previously reported in studies with L-trans-pyrrolidine-2,4-dicarboxylate, a high-affinity glutamate reuptake inhibitor (Cebers et al., 1999; 2001).

In particular PSD-95 and nNOS, which functionally interact with NMDA receptor (Bredt and Snyder, 1989; Vallebuona and Raiteri, 1994; Fedele et al., 2001) and produce NO, are of great interest with regard to the heterogeneous antidepressant response. Hippocampal nNOS was found to mediate glucocorticoid-induced depressive behavior in mice (Zhou et al., 2011) and the number of nNOS-immunoreactive neurons in *post-mortem* hippocampus samples was higher in MDD and bipolar disorder patients compared to the control group (Oliveira et al., 2008). Plasma NO metabolite levels were higher in MDD patients compared to healthy controls, which was reversed by 8 week paroxetine treatment (Chrapko et al., 2006). NO has also been implicated to play a role for the function of antidepressant-like agents tramadol, bupropion and lithium (Dhir and Kulkarni, 2007; Ghasemi et al.,

2008; Jesse et al., 2008). Serotonergic antidepressants including citalopram, imipramine, paroxetine and tianeptine have been shown to decrease hippocampal NOS activity *in vitro and in vivo* (Finkel et al., 1996; Wegener et al., 2003). Based on these findings targeting the NO system is a potential therapeutic strategy for major depressive disorders. Doucet et al., reported that a single administration of PSD-95/nNOS interface inhibitors, IC87201 and ZL006, produced an antidepressant-like effect in the FST and tail suspension test (TST) (Doucet et al., 2013). In addition, nNOS inhibitors like *N $\omega$ -Propyl-L-Arginine*, *7-nitroindazole* and *aminoguanidine* have been shown to produce antidepressant-like effects (Joca and Guimarães, 2006; Zhou et al., 2007; Hiroaki-Sato et al., 2014; Tomaz et al., 2014). I found that the hippocampal NMDA receptor/PSD-95/nNOS protein complex was expressed at significantly different levels between PLF and PSF mice, implying that it has an important role in the chronic antidepressant treatment response.

We also investigated CAPON and sGC- $\beta$ 1, proteins associated with the NO pathway, to further corroborate the differential activity of the NMDA receptor/PSD-95/nNOS protein complex between PLF and PSF groups. CAPON has been shown to disrupt the interaction between nNOS and postsynaptic proteins including PSD-95, which prevents NMDA receptor-mediated NO release (Xu et al., 2005). I found high levels of hippocampal CAPON protein in PSF mice which may contribute to a favorable antidepressant treatment response, possibly by suppressing nNOS activity.

sGC- $\beta$ 1 protein levels between PLF and PSF mice were not significantly different, which is in line with results from Reiersen et al. who reported that 8-weeks of SSRI fluoxetine treatment did not change sGC- $\beta$ 1mRNA levels in rat hippocampus (Reiersen et al., 2009). It has been also shown that sGC- $\beta$ 1mRNA levels were not affected in human schizophrenic prefrontal cortex *post-mortem* samples that had higher nNOS mRNA levels compared to controls (Baba et al., 2004). Although I did not find sGC- $\beta$ 1protein expression level differences, I cannot exclude the possibility that sGC enzymatic activity might be different between PLF and PSF groups.

Consistent with nNOS protein levels, citrulline levels between PLF and PSF mice were also altered after 28 days of paroxetine treatment. Since nNOS catalyzes the production of NO and citrulline from arginine, lower levels of citrulline in PSF mouse hippocampus may indicate lower enzymatic nNOS activity.

Hippocampal metabolome profiling also supported differential NMDA receptor activity between the two groups. Alanine and citrate are known to regulate NMDA

receptor activity. (Westergaard et al., 1995; Popescu et al., 2010) Glutathione is an NMDA receptor agonist (CHUEH, 2006; Rosa et al., 2013) and sarcosine is an NMDA receptor co-agonist (Zhang et al., 2009; Huang et al., 2013). The role of serine as a potent co-agonist of NMDA receptor has been demonstrated. (Mothet et al., 2000; Panatier et al., 2006) In addition, taurine is suggested to interact directly with NMDA receptor and regulates its function (Suárez and Solís, 2006).

Maes et al. found that low serum levels of asparagine, serine and taurine levels characterized non-responder patients after 5-weeks of antidepressants treatment (Maes et al., 1998). In addition, glutathione, sarcosine and taurine administration have antidepressant-like effects suggesting the here found elevated levels might be relevant for the favorable paroxetine response (Huang et al., 2013; Rosa et al., 2013; Toyoda and Iio, 2013).

The different glutamatergic pathway activity is also reflected in MDD patients' PBMCs by the observed sGC- $\beta$ 1 protein levels, which is downstream of the glutamatergic pathway.

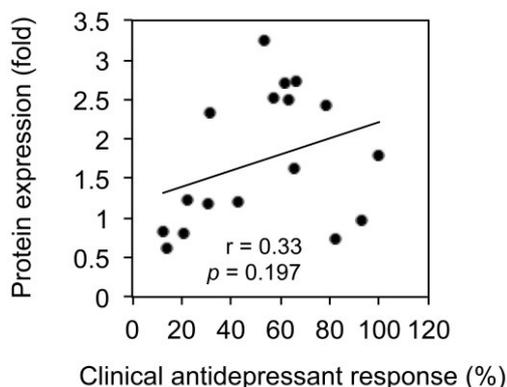
Taken together, my study suggests that monitoring glutamatergic pathway protein and metabolite levels can be used to determine whether paroxetine-treated patients with depressive disorders are responding to the drug.

### 4.3. Ubiquitin-proteasome system pathway

No significant mRNA level differences for NRs, PSD-95 and nNOS were found between PLF and PSF groups suggesting that post-translational modifications might be relevant instead. Supporting this notion I found proteomics signatures involved in the UPS pathway that significantly differed between the two groups. While my data indicate that ubiquitination is not associated with differential protein expression levels in paroxetine-treated mice, PM2A protein and total ubiquitination level differences suggest potential roles in the antidepressant treatment response.

Protein analyses of PBMCs showed that UBS pathway components including PM2A and ubiquitination can separate antidepressant treatment responder/non-responder patients. Especially PM2A levels were significantly lower at T6 only in antidepressant responder patients and could be used to monitor and determine the antidepressant treatment response. In addition, ubiquitination at baseline may be used

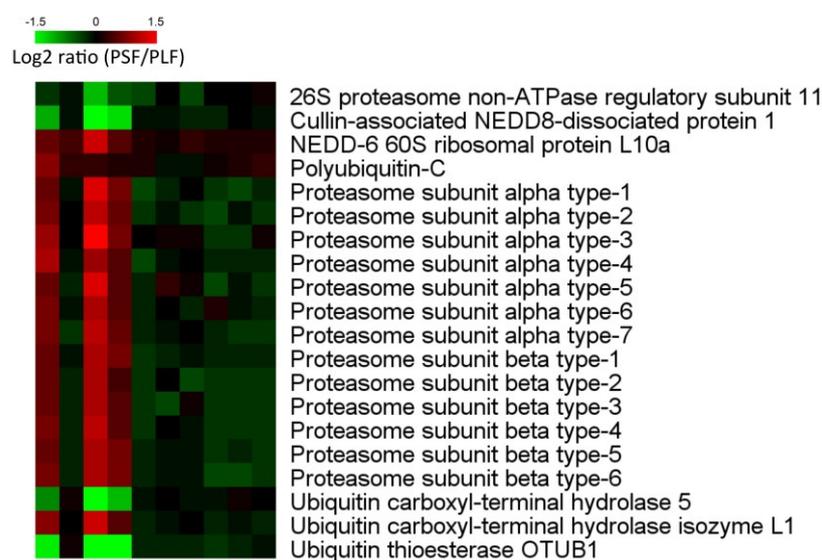
to predict the response to antidepressant treatment, as its levels stayed low in antidepressant responder patients at T0 and T6 compared to non-responder patients. Evidence from other studies suggests that the UPS may be linked to MDD treatment. In this regard, it has been shown that SNPs in proteasome subunit  $\alpha 7$  (PSMA7), proteasome 26S non-ATPase subunit 9 (PSMD9) and proteasome 26S non-ATPase subunit 13 (PSMD13) are associated with clinical antidepressant response (Wong et al., 2008; Gragnoli, 2014). Data from my thesis project suggest that UPS protein signatures such as PM2A and ubiquitination levels could be used to assess antidepressant treatment outcome. Especially ubiquitination levels at baseline may be able to predict the response to antidepressant treatment. As stressful events are known to alter the UPS pathway (Ryan et al., 2006; Karssen et al., 2007; Minelli et al., 2015), higher ubiquitination levels in antidepressant non-responder patients might be a reflection of that. Despite the fact that data from the present study failed to show a significant correlation between baseline ubiquitination levels and clinical antidepressant treatment response (Figure 55), the inclusion of larger sample size may increase predictability power of the analysis.



**Figure 55.** Correlation between baseline ubiquitination levels and clinical antidepressant response. Baseline ubiquitination levels did not show significant correlation with HDRS score changes after treatment. Pearson correlation coefficients ( $r$ ) with  $P$  values are indicated in the correlation graphs.

UPS was shown to be involved in the regulation of various neuronal pathways including synaptic formation and function (Yi and Ehlers, 2007). In light of this, significant differences of PM2A and ubiquitination levels between antidepressant responder and non-responder mice and humans implicate a potential involvement of systemic molecular pathways. For example, UPS has been shown to mediate NMDA

receptor degradation (Kato et al., 2005; Yi and Ehlers, 2007; Tsai, 2014). Tai *et al.* reported that NMDA treatment of cultured hippocampal neurons decreases UPS activity, suggesting an interaction between glutamatergic and proteasome pathways (Tai et al., 2010). This is in agreement with my data, which also point towards an involvement of both pathways in the different response of the PLF and PSF mouse groups. Although my results indicate that ubiquitination does not seem to be associated with the regulation of NMDA receptor/PSD-95 protein levels in the paroxetine-treated mice, my proteomics analysis significantly enriched the UPS pathway (Figure 56). The relationship of UPS and glutamatergic pathways is of interest and should be further explored in the future.



**Figure 56.** A heatmap of hippocampal UPS pathway proteins comparing PLF and PSF groups. In the heatmap, colors denote  $\log_2$  ratio. Proteins with adjusted  $p$  value  $< 0.05$  were considered significant,  $n=5/\text{group}$ .

Kaminsky and Kosenko showed that MK-801, an NMDA receptor blocker, and sodium nitroprusside, an NO donor, were able to modulate brain purine metabolism activity suggesting a functional involvement of NMDA receptor and nitric oxide in purine metabolism pathway (Kaminsky and Kosenko, 2009). GDA, which catalyzes conversion of guanine to xanthine in the purine metabolism pathway (Yuan et al., 1999; Paletzki, 2002), was found to be important for neuronal dendrite branching by regulating postsynaptic trafficking of PSD-95 protein, a major component of the glutamatergic synapse (Firestein et al., 1999). Antidepressant-like and neuroprotective effects of pyrimidines including cytidine and uridine have been linked to the

regulation of glutamatergic neurotransmission (Mir et al., 2003; Hurtado et al., 2005; Radad et al., 2007; Yoon et al., 2009). In this context, illuminating the crosstalk between purine/pyrimidine metabolism and glutamatergic pathways may aid in an improved understanding of their functions in the pathobiology of MDD and its treatment.

Whether the pathways I have identified in the mouse will qualify as predictive biosignatures for the antidepressant treatment response in patients and routine use in the clinic remains to be further investigated. Eventually biomarkers for the antidepressant treatment response will enable patient sub-group stratification and will render clinical decision making more objective to realize a personalized psychiatry approach.

#### 4.4. Outlook

This study aimed at the molecular delineation of the chronic antidepressant treatment response in mice, and validation in MDD patients' PBMCs. While my integrated -omics analyses of the hippocampus revealed novel pathways and biomarker candidates, additional analyses of other brain regions that are implicated in neuropsychiatric disorders may provide a more systemic understanding related to the antidepressant response heterogeneity.

As shown in previous reports several metabolites seem to play a role in the regulation of mood status and have potential to be used as supplements in treating psychiatric disorders (Papakostas et al., 2012; Foster and McVey Neufeld, 2013; Slyepchenko et al., 2014). Based on the dynamic changes of the metabolome in response to antidepressant treatment, the effect of nutritional supplements are also of great interest.

Conventional mental disorder diagnosis systems may benefit from Research Domain Criteria (RDoC), a new research framework initiated by the *U.S. National Institute of Mental Health*. Integration of multi-dimensional data from genomics to clinical reports can reduce the gap between molecular research and clinical practice, and therefore is expected to provide new evidence and perspectives on classification and treatment decision of neuropsychiatric disorders.

## Appendix

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## List of abbreviations

- ACTH: Adrenocorticotrophic hormone
- AdoHcyase: S-adenosyl-L-homocysteine hydrolase
- AdoHcyase 2: S-adenosyl-L-homocysteine hydrolase 2
- ALS: Amyotrophic lateral sclerosis
- AMPA:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- AVP: Arginine vasopressin
- ATIC: Aminoimidazole-4-carboxamide ribonucleotide transformylase/IMP  
cyclohydrolase
- BDNF: Brain-derived neurotrophic factor
- CaMK:  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase
- cAMP: cyclic adenosine monophosphate
- CAPON: Carboxy-terminal PDZ ligand of *nNOS*
- CPS2: Carbamoyl phosphate synthase 2
- CREB: cAMP response element binding protein
- CRH: Corticotrophin releasing hormone
- CRP: C-reactive protein
- DSM: Diagnostic and statistical manual of mental disorders
- DST: Dexamethasone suppression test
- ERK: Extracellular signal-regulated kinase
- FKBP51: FK506-binding protein 51
- FST: Forced swim test
- FUST: Female urine sniffing test
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- GDA: Guanine deaminase
- GDH1: Glutamate dehydrogenase 1
- GS: Glutamine synthetase
- GSK-3 $\beta$ : Glycogen synthase kinase-3 $\beta$
- HCA: Hierarchical clustering analysis
- HDRS: Hamilton Depression Rating Scale
- HPA: Human Protein Atlas / Hypothalamus-pituitary-adrenal

HPRT: Hypoxanthine-guanine phosphoribosyltransferase  
HRP: Horseradish peroxidase  
ICAT: Isotope-coded affinity tag  
ICD: International statistical classification of diseases and related health problems  
ICPL: Isotope-coded protein label  
IMP: Inosine monophosphate  
iPS: Induced pluripotent stem cell  
ITPase: Inosine triphosphate pyrophosphatase  
iTRAQ: Isobaric tags for relative and absolute quantification  
KEGG: Kyoto Encyclopedia of Genes and Genomes  
LTP: Long-term potentiation  
MAO: Monoamine oxidase  
MARS: Munich Antidepressant Response Signature  
mAST: Mitochondrial aspartate transaminase  
MDD: Major depressive disorder  
MEK: Mitogen-activated protein kinase kinase  
NAc: Nucleus accumbens  
NGF: Nerve growth factor  
NMDA: N-Methyl-D-aspartate  
nNOS: Neuronal nitric oxide synthase  
NO: Nitric oxide  
NR: N-Methyl-D-aspartate (NMDA) receptor subunit  
NT-3: Neurotrophin-3  
NT-4: Neurotrophin-4  
P-CaMKII : Phospho-CaMKII  
P-ERK: Phospho-ERK  
P-GSK-3 $\beta$ : Phospho-GSK-3 $\beta$   
P-MEK: Phospho-MEK  
P-NR1: Phospho-NR1  
P-NR2A: Phospho-NR2A  
P-NR2B: Phospho-NR2B  
PBMCs: Peripheral blood mononuclear cells  
PLF: Paroxetine-treated long-time floating  
PMSA2: Proteasome subunit  $\alpha$  type-2

PNP: Purine nucleoside phosphorylase  
PSD-95: Postsynaptic density protein 95  
PSMA7: Proteasome subunit  $\alpha$ 7  
PSF: Paroxetine-treated short-time floating  
PSMD9: Proteasome 26S non-ATPase subunit 9  
PSMD13: Proteasome 26S non-ATPase subunit 13  
qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction  
RDoC: Research domain of criteria  
SAM: Significant analysis of microarrays (and metabolites)  
sGC- $\beta$ 1: Soluble guanylate cyclase- $\beta$ 1  
SILAC: Stable isotope labeling of amino acids in cell culture  
SILAM: Stable isotope labeling in mammals  
SNPs: Single nucleotide polymorphisms  
SNRIs: Selective norepinephrine reuptake inhibitors  
SSRIs: Selective Serotonin Reuptake Inhibitors  
STXBP1: Syntaxin binding protein 1  
SV2A: Synaptic vesicle glycoprotein 2A  
SYNJ1: Synaptojanin 1  
TCA: Tricyclic antidepressant  
TMT: Tandem mass tag  
TRD: Treatment resistant depression  
Ub: Ubiquitin  
UMP/CMPK: UMP-CMP kinase  
UPS: Ubiquitin-proteasome system

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## Publications

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Shin, M.K., Kim, H.G., Baek, S.H., Jung, W.R., **Park, DI.**, Park, J.S., Jo, D.G., Kim, K.L. Neuropep-1 ameliorates learning and memory deficits in an Alzheimer's disease mouse model, increases brain-derived neurotrophic factor expression in the brain, and causes reduction of amyloid beta plaques. (2014). *Neurobiol Aging*. 35(5):990-1001.

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