Antidepressant activated biochemical pathways and biomarker candidates

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

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Abstract

Most of the commonly used antidepressants block monoamine reuptake transporters to enhance serotonergic or noradrenergic neurotransmission. Effects besides or downstream of increased monoaminergic neurotransmission are poorly understood and yet presumably important for the drugs' mode of action. In my PhD thesis I employed proteomics and metabolomics technologies combined with *in silico* analyses and identified cellular pathways affected by antidepressant drug treatment. DBA/2 mice were treated with paroxetine as a representative Selective Serotonin Reuptake Inhibitor (SSRI). Hippocampal protein levels were compared between chronic paroxetine- and vehicle-treated animals using in vivo ^{15}N metabolic labeling combined with mass spectrometry. I also studied chronic changes in the hippocampus using unbiased metabolite profiling and the time course of metabolic changes with the help of a targeted polar metabolomics profiling platform. I identified profound alterations related to hippocampal energy metabolism. Glycolytic metabolite levels acutely increased while Krebs cycle metabolite levels decreased upon chronic treatment. Changes in energy metabolism were influenced by altered glycogen metabolism rather than by altered glycolytic or Krebs cycle enzyme levels. Increased energy levels were reflected by an increased ATP/ADP ratio and by increased ratios of high-to-low energy purines and pyrimidines. Paralleling the shift towards aerobic glycolysis upon paroxetine treatment I identified decreased levels of Krebs cycle and oxidative phosphorylation enzyme levels upon the antidepressant-like ^{15}N isotope effect in high-anxiety behavior mice. In the course of my analyses I also identified GABA, galactose-6-phosphate and leucine as biomarker candidates for the assessment of chronic paroxetine treatment effects in the periphery and myo-inositol as biomarker candidate for an early assessment of chronic treatment effects. The identified antidepressant drug treatment affected molecular pathways and novel SSRI modes of action warrant consideration in antidepressant drug development efforts.

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Abbreviations

5-HT	serotonin
AC	adenylate cyclase
ADP	adenosine-5'-diphosphate
AGT	angiotensinogen
Ala	alanine
ALEX	automated liner exchange
AMP	adenosine-5'-monophosphate
ANXA4	annexin A4
API	atmospheric pressure ionization
ATP	adenosine-5'-triphosphate
BCAA	branched chain amino acid
BDNF	brain derived neurotrophic factor
cAMP	cyclic AMP
CDP	cytidine-5'-diphosphate
CID	collision induced dissociation
CMP	cytidine-5'-monophosphate
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	corticotropin-releasing hormone
CRYAB	alpha-crystallin B chain
CSF	cerebrospinal fluid
CTP	cytidine-5'-triphosphate
Cys	cysteine
Da	dalton
DAG	diacylglycerol
DALY	disability adjusted life year
dAMP	deoxy-AMP
DEX	dexamethasone
dGMP	deoxy-GMP
dGTP	deoxy-GTP
DPP4	dipeptidyl-peptidase 4
EDTA	ethylenediaminetetraacetic acid
EPM	elevated plus maze
ESI	electrospray ionization
ETD	electron transfer dissociation
FBP1	fructose biphosphatase 1
FDR	false discovery rate
FST	forced swim test
GAA	lysosomal alpha-glucosidase
GABA	gamma-aminobutyric acid
GC	gas chromatography
GCG	glucagon

GCK	glucokinase
GDP	guanosine-5'-diphosphate
GIT1	ARF GTPase-activating protein GIT1
Gln	glutamine
GLUL	glutamine syntethase
Glut	glutamate
Gly	glycine
GMP	guanosine-5'-monophosphate
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GTP	guanosine-5'-triphosphate
GYS2	glycogen synthase 2
HAB	high anxiety behavior
HCD	higher energy collision induced dissociation
HILIC	hydrophilic interaction liquid chromatography
HK1/2	hexokinase 1/2
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
ICAT	isotope coded affinity tags
ICPL	isotope coded protein label
Ile	isoleucine
IMP	inositol monophosphate
INS	insulin
IP	inositol phosphate
IQR	interquartile range
iTRAQ	isobaric tags for relative and absolute quantitation
K^+	potassium
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG orthologue
LAB	low anxiety behavior
LC	liquid chromatography
Leu	leucine
LTQ	linear trap quadrupole
m/z	mass-to-charge ratio
MALDI	matrix assisted laser desorption ionization
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
MeOH	methanol
mg	milligram
MGEA5	Mgea5 protein
min	minute
ml	milliliter
MPI	mannose phosphate isomerase
MRM	multiple reaction monitoring
mRNA	messenger RNA
MS	mass spectrometry

MSN	moesin
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
MYH9	myosin-9
Na^+	sodium
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NMR	nuclear magnetic resonance
NOS1	nitric oxide synthase 1
NSI	nanospray ionization
PCK2	phosphoenolpyruvate carboxykinase 2
PDE1	phosphodiesterase 1
PDE1A	phosphodiesterase 1A
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLS-DA	partial least square - discriminant analysis
PND	postnatal day
PPi	pyrophosphate
PPP1R1B	protein phosphatase 1 regulatory subunit 1B
Pro	proline
PTM	post-translational modification
PYGM	glycogen phosphorylase
Q	quadrupole
QqQ	triple quadrupole
R	Pearson´s correlation coefficient
RI	retention index
RP	reversed phase
S	second
S/N ratio	signal-to-noise ratio
SAM	significance analysis of microarrays
SDS	sodium dodecyl sulfate
Ser	serine
SILAC	stable isotope labeling by amino acids in cell culture
SILAM	stable isotope labeling of mammals
SNCA	alpha-synuclein
SNRI	selective noradrenaline reuptake inhibitor
SRM	selected reaction monitoring
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic agent, tricarboxylic acid cycle
Thr	threonine
TMP	thymidine monophosphate
TOF	time of flight
TPP	trans-proteomic pipeline
trkB	tyrosine-related kinase B
TST	tail suspension test

Tyr	tyrosine
UCHL1	ubiquitin carboxyl-terminal esterase L1
UDP	uridine-5'-diphosphate
UMP	uridine-5'-monophosphate
USV	ultrasonic vocalization
UTP	uridine-5'-triphosphate
Val	valine
VIP	variable importance in projection
w/v	weight/volume

1 INTRODUCTION

1.1 Major depressive disorder and the monoamine deficiency hypothesis of depression

1.1.1 Major depressive disorder

Major depressive disorder (MDD) is a severe and life-threatening disease (about 15% of depressed patients commit suicide (Manji et al., 2001)) with a population prevalence of 17% (Kessler et al., 2007; Burmeister et al., 2008). By the year 2030 major depression is projected to be the second leading contributor to the global burden of disease (disability adjusted life years, DALY) and the leading contributor in high-income countries (Mathers and Loncar, 2006).

MDD etiology is poorly understood and there are no objective diagnostic tests available. The diagnosis of depression is based on a highly subjective and variable set of symptoms such as depressed mood, loss of interest, diminished sense of pleasure, irritability, low self-esteem, weight loss or weight gain, insomnia or hypersomnia or recurrent thoughts of death and suicide that last for more than two weeks and disrupt normal social and occupational functioning (Nestler et al., 2002; Mann, 2005).

1.1.2 Current antidepressant treatment and the monoamine deficiency hypothesis of depression

More than 50 years ago it was observed serendipitously that the monoamine oxidase inhibitor (MAOI) iproniazid, initially developed for the treatment of tuberculosis (Bloch et al., 1954), and the tricyclic agent (TCA) imipramine, initially developed for the treatment of psychotic disorders (Kuhn, 1958), exerted antidepressant effects in man. Iproniazid increased monoamine levels by inhibiting their catabolism by monoamine oxidase and imipramine amplified monoamine signaling by inhibiting monoamine reuptake at the presynapse.

Based on these findings the monoamine deficiency hypothesis of depression proposed that increasing synaptic availability of monoamines elicits antidepressant effects and that MDD involved the decrease of monoamines in the brain (Schildkraut, 1965; Coppen, 1967; Wong and Licinio, 2004).

In the 1980s, based on the monoamine deficiency hypothesis of depression, antidepressant drug development efforts focused on the rational design of agents with selective monoamine-uptake inhibitory properties. Selective Serotonin Reuptake Inhibitors (SSRIs, e.g. paroxetine, fluoxetine) (Vaswani et al., 2003, see Figure 1) and, to a lesser extent, Selective Noradrenaline Reuptake Inhibitors (SNRIs, e.g. reboxetine) (Brunello et al., 2003) were developed that did not affect other neuroreceptor systems for increased safety and tolerability. According to meta-analyses, SSRIs and SNRIs exhibit favorable side effect profiles but equal treatment efficacy and onset of action compared to TCA treatment (Song et al., 1993; Montgomery et al., 1994; Anderson, 2000; Peretti et al., 2000). Shortcomings of current monoaminergic antidepressant treatment include a delayed onset of therapeutic action (up to 2-8 weeks), adverse side effects (e.g. sexual dysfunction or sleep disturbances) and a low response rate (about 50-80%) (Holsboer, 2001; Wong and Licinio, 2004; Berton and Nestler, 2006).

To face the challenges of increasing numbers of depressive patients and increasing economic burden in the coming decades it is mandatory to have better antidepressant drugs at hand with increased efficacy, less side effects and faster onset of therapeutic action.

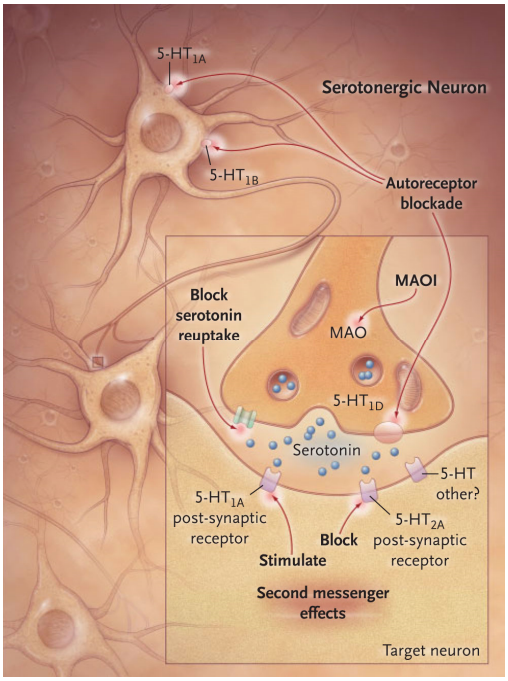

Figure 1. Mode of SSRI drug action.
Selective serotonin reuptake inhibitors increase serotonergic neurotransmission by blocking serotonin transporters in the presynapse.
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1.2 Antidepressant treatment effects beyond monoamine reuptake inhibition

Contrary to the monoamine deficiency hypothesis of depression, monoamine depletion does not induce depression in healthy humans (Ruhe et al., 2007). Moreover, although pharmacological modulation of monoamine receptors represents an acute antidepressant treatment effect therapeutic effects are delayed by several weeks (Gould and Manji, 2002). Additionally, a study by *Nickel et al*. (Nickel et al., 2003) found that serotonin reuptake inhibition (by paroxetine treatment) and serotonin reuptake enhancement (by tianeptine treatment) elicit antidepressant effects. Both paroxetine and tianeptine treatment normalized hypothalamic-pituitary-adrenal (HPA) axis reactivity, indicating that opposite effects on the serotonin system may lead to common downstream pathway alterations.

The most parsimonious explanation for these findings is that monoamine elevation *per se* is not the only source for therapeutic antidepressant activity but secondary long-term downstream effects may also be involved in the alleviation of depressive symptoms (Brady et al., 1992; Michelson et al., 1997; Holsboer, 2001). It is tempting to speculate that for improved antidepressant treatment it would be beneficial to pharmacologically interfere with these downstream mechanisms directly.

The following chapters (1.2.1-1.2.4) overview the major hypotheses of antidepressant treatment effects beyond monoamine reuptake inhibition. It is important to note that this overview is not exclusive, there is no consensus on which effects are most relevant for therapeutic action and molecular antidepressant treatment effects in general remain elusive.

1.2.1 Intracellular signaling events upon antidepressant treatment

Monoaminergic neurotransmission is mainly mediated via G-protein coupled receptor (GPCR) signaling and different monoamine receptor families are coupled to different G-proteins. Dependent on the receptor subtype two major ´prototypical´ intracellular signal transduction pathways are involved: (1) cAMP signaling upon $G_{i/o}$ or G_s activation and (2) phosphatidylinositol signaling upon G_q activation. $G_{i/o}$ inhibits adenylate cyclase (AC) activity leading to reduced second messenger cAMP generation and in turn reduced protein kinase A (PKA) activity whereas G_s activates AC activity leading to increased PKA activity. Gq activates phospholipase C (PLC) leading to increased generation of the second messengers inositol phosphate (IP) and diacylglycerol (DAG) and in turn increased protein kinase C (PKC) activity. PKA and PKC mediated protein phosphorylation eventually lead to gene expression alterations (Millan et al., 2008). PKA phosphorylates the cAMP response element binding protein (CREB) that in turn binds to the cAMP response element (CRE), a gene element found in the promoters of a few thousand genes (Gould and Manji, 2002; Impey et al., 2004).

Despite extensive cross-talk between PKA and PKC mediated systems, antidepressant treatment is believed to mainly influence cAMP/PKA signaling cascades and phosphatidylinositol/PKC signaling seems less relevant (Shelton, 2000; Perera et al., 2001). Specifically, chronic antidepressant treatment alters mRNA and protein levels of G protein alpha subunits in a brain region- and antidepressant drug-specific manner (Lesch and Manji, 1992; Li et al., 1996; Li et al., 1997; Raap et al., 1999). Antidepressant treatment also induces a translocation of PKA from the cytosol to the nucleus in rat frontal cortex cells (Nestler et al., 1989) and increases PKA dependent phosphorylation events (Perez et al., 1989; Perez et al., 1991).

1.2.2 Induced neurogenesis upon antidepressant treatment

Long-term antidepressant treatment increases cellular CREB mRNA, CREB protein and CREB/CRE binding in the hippocampus (Nibuya et al., 1995; Nibuya et al., 1996; Duman et al., 1997). The same studies found that the CREB regulated gene products BDNF and its receptor *trkB* were upregulated upon long-term treatment. Other studies indicate that antidepressant treatment regulates CREB in a brain region-specific manner and that CREB exerts opposing effects on depression-like features in different brain regions (Schwaninger et al., 1995; Nibuya et al., 1996; Manier et al., 2002; Newton et al., 2002).

CREB and the neurotrophin BDNF have been considered key regulators of neuronal survival and plasticity, including neurogenesis and synaptogenesis (Nakagawa et al., 2002; Castren, 2004; Malberg and Blendy, 2005; Gass and Riva, 2007). Antidepressant treatment appears to protect against hippocampal volume loss in depressed patients (Sheline et al., 2003), antidepressant drugs enhance neurogenesis in rodent hippocampi (Malberg et al., 2000) and *Santarelli et.al.* (Santarelli et al., 2003) have shown that hippocampal neurogenesis is required for antidepressant induced behavioral effects in mice. Based on these findings the neurotrophic hypothesis of depression postulates that hippocampal neuronal atrophy (due to stress and decreased expression of BDNF and other growth factors) contributes to depression and antidepressant treatment effects derive from an upregulation of BDNF in the hippocampus (Wong and Licinio, 2004; Duman and Monteggia, 2006).

1.2.3 Altered stress response upon antidepressant treatment

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is a putative risk factor predisposing to the development of depression. A significant percentage of depressed patients exhibit HPA axis hyperactivity due to, at least in part, impairment of corticosteroid receptor function which is also reflected by an inappropriate response in the dexamethasone/corticotropin-releasing hormone (DEX/CRH) test (Holsboer, 2001; Pariante and Lightman, 2008). Successful antidepressant treatment is associated with resolution of

the impairment in the negative feedback on the HPA axis by glucocorticoids (Pariante, 2006).

The CRH promotor region contains a CRE sequence (Spengler et al., 1992) and antidepressants may influence the stress system via PKA mediated signaling and regulation of CREB. Indeed, antidepressant treatment reduces CRH concentrations in the cerebrospinal fluid (CSF) (Nemeroff et al., 1991; De Bellis et al., 1993; Veith et al., 1993; Heuser et al., 1998). Additionally, antidepressant treatment decreases HPA axis stress reactivity associated with increased binding capacity of corticosteroid receptors in the hippocampus (Reul et al., 1994), alters glucocorticoid receptor (GR) expression, and improves GR function and GR-mediated feedback inhibition (Brady et al., 1991; Brady et al., 1992; Michelson et al., 1997; Holsboer, 2001; Pariante and Lightman, 2008).

1.2.4 Unifying link between distinct mechanisms of antidepressant drug action

A recent study identified a putative link between antidepressant treatment induced changes on intracellular PKA mediated signaling (see chapter 1.2.1), neurogenesis (see chapter 1.2.2) and the HPA axis (see chapter 1.2.3). Specifically, *in vitro* experiments indicated that induced neurogenesis upon SSRI (sertraline) treatment is dependent on GR function that is associated with GR phosphorylation via PKA activity (Anacker et al., 2011). This and other findings indicate that distinct hypotheses for the mode of antidepressant action do not compete and exclude each other but rather complement each other (Perera et al., 2001).

Taken together, despite tremendous efforts during the last decades, the precise mechanism of antidepressant action remains elusive and studies on current antidepressant treatment effects are in great demand for a better understanding of antidepressant elicited therapeutic as well as side effects.

1.3 Biomarker discovery

1.3.1 Biomarkers and surrogate endpoints

Biomarkers are used as diagnostic tools for the objective determination of disease state, disease progression, disease prognosis or treatment effects and response. According to the *National Institutes of Health*, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). A biomarker that is reasonably likely to predict clinical benefit may also be used as surrogate endpoint, i.e. be used as "biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence" (Biomarkers Definitions Working Group, 2001).

1.3.2 Biomarkers for antidepressant treatment effects

At present there are no biomarkers that are used in clinical practice for any psychiatric disorder. Biomarkers for antidepressant treatment response could accelerate drug discovery as surrogate endpoints and hence reduce production costs and pipeline development time (Schwarz and Bahn, 2008). Biomarkers could help to predict antidepressant treatment response early in the course of treatment in order to minimize protracted serial trial-and-error (Rush et al., 2009; Leuchter et al., 2010), inform on drug dosing and help minimize inter-individual variation in antidepressant treatment response (Biomarkers Definitions Working Group, 2001).

Because psychiatric diseases are considered complex disorders of the brain, major affected biochemical pathways in disease or upon treatment are expected to be primarily altered in the brain. Plasma, on the other hand, is the preferred specimen for a biomarker assay due to its accessibility, reproducible sampling methods and the fact that small brain molecules are excreted via plasma (Leuchter et al., 2010). The ideal biomarker for psychiatric disorders

would be measured in the periphery and represent a highly sensitive and specific indicator of disease pathways in the brain.

Due to the complex etiology and polygenic character of psychiatric disorders biomarker signatures rather than single biomarkers are expected to depict more accurately behavioral phenotype changes (Filiou et al., 2011a). A single biomarker will usually have small effect sizes and several biomarkers need to be combined for a robust prediction of disease state or treatment effects (Singh and Rose, 2009).

1.4 Mouse models in antidepressant treatment research

Although human material is the most relevant specimen for the analysis of psychiatric disease etiology and antidepressant treatment effects, exploratory studies frequently rely on the investigation of rodent brains (Nakatani et al., 2004; Wong and Licinio, 2004; Yamada and Higuchi, 2005; Conti et al., 2007; Sillaber et al., 2008) for the following reasons: (1) fresh human brain tissue, the most relevant organ for psychiatric disorders, is inaccessible due to ethical reasons (Sullivan et al., 2006); (2) human *post mortem* brain, cerebrospinal fluid (CSF) or plasma are of limited availability; (3) the inter-individual genetic and life style variability combined with the low sample quantity available pose serious challenges for analytical efforts (Filiou and Turck, 2011); (4) the presence of confounding factors like brain pH, *post mortem* interval, age, use of antidepressant or other drugs, smoking or substance abuse complicate statistical data analyses and thus (5) a great number of biological replicates is needed (Kim and Webster, 2009).

Therefore, for the exploratory phase of the antidepressant-affected pathways or biomarker signature discovery pipeline, animal model based studies tend to have higher success rates (Turck et al., 2005; Filiou and Turck, 2011). Animal models in combination with proteomic and metabolomic technologies promise to be useful tools for the hypothesis-free discovery of biomarker candidates (Turck et al., 2005; Reckow et al., 2008; Filiou and Turck, 2011).

1.4.1 Mouse models for psychiatric disorders

Major depression involves complex thought processes and emotions and it is not possible to model depression in mice in its entirety (Wong and Licinio, 2004). However, mouse models were successfully developed that model certain features, i.e. endophenotypes, of the disease by resembling selected characteristics of the human condition. Current animal models for depression-like behavior include uncontrollable stress models (e.g. forced swim test (FST), tail suspension test (TST)), reward models (e.g. sucrose prevalence), olfactory bulbectomy, chronic mild stress, drug withdrawal, maternal deprivation, neonatal clomipramine and genetic models (reviewed in (Wong and Licinio, 2004)). Important criteria for mouse models of depression are (1) face validity, i.e. whether the model phenomenologically resembles the human condition; (2) predictive validity, i.e. whether the performance of the model in a test is predictive for human performance and (3) construct/etiological validity, i.e. how accurately the test models the human condition and whether the model is based on known etiological factors. Predictive validity is sometimes used to indicate pharmacological isomorphism, i.e. whether the model predicts antidepressant treatment response in humans (Van Dam and De Deyn, 2006; Markou et al., 2009).

There are various models available to investigate the effects of currently used and novel antidepressants in mice. The following chapter (1.4.2) will focus on an animal model that was utilized in the present thesis for (1) the characterization of antidepressant treatment affected molecular pathways and (2) for the identification of biomarker candidates for antidepressant treatment effects. The subsequent chapter (1.4.3) will focus on an animal model of anxiety-related behavior and the recently discovered antidepressant-like ^{15}N isotope effect that was utilized for correlating antidepressant-like effects with molecular pathway alterations.

1.4.2 Paroxetine treatment of DBA/2 mice

Paroxetine is the most potent inhibitor of serotonin reuptake (Sanchez and Hyttel, 1999; Bourin et al., 2001) and a weak inhibitor of norepinephrine reuptake (Kelly and Leonnard, 1995; Sugimoto et al., 2011). The ratio of

inhibition of uptake of norepinephrine to serotonin (NE/5-HT) is second only to that of citalopram. There is a negligible affinity for other receptor systems, including catecholaminergic, dopaminergic, histaminergic and muscarinic cholinergic systems (Bourin et al., 2001). Next to depression the SSRI paroxetine is also used for treating panic disorder, obsessive compulsive disorder and social phobia (Wagstaff et al., 2002).

The inbred DBA/2 mouse strain displays a high level of innate anxiety-like behavior (Ohl et al., 2003) that is potentially linked to distinct neurotransmitter receptor densities (Yilmazer-Hanke et al., 2003), may exhibit aspects of hippocampal dysfunction (Thinus-Blanc et al., 1996) and displays a reduced HPA axis feedback inhibition compared to other inbred mouse strains (Thoeringer et al., 2007). DBA/2 mice are more sensitive towards paroxetine treatment compared to other mouse strains, as determined in the FST (Sugimoto et al., 2011). These studies suggest that DBA/2 mice reflect some psychiatric disorders endophenotypes and therefore might be suitable for the investigation of molecular antidepressant treatment effects (Sillaber et al., 2008). Paroxetine treatment of DBA/2 mice results in decreased passive stress coping behavior in the FST, an indication of antidepressant treatment efficacy in rodents (Porsolt et al., 1977).

Sillaber et al. investigated paroxetine induced alterations in DBA/2 mice at the mRNA level using transcriptomic analyses (Sillaber et al., 2008). In the present thesis the focus was expanded to the identification of molecular pathway alterations using proteomic and metabolomic analyses and the identification of biomarker candidates for antidepressant treatment effects.

1.4.3 The HAB/LAB mouse model and the antidepressant-like ^{15}N isotope effect in anxious HAB mice

The high anxiety-related behavior (HAB)/low anxiety-related behavior (LAB) mouse model is based on selective bidirectional breeding of mice for behavioral extremes according to their performance on the elevated plus-maze (EPM), a behavioral paradigm assessing anxiety-related behavior. HAB mice spend most of their time in the closed arms whereas LAB mice spend most of their time in the open arms (Pellow et al., 1985; Kromer et al., 2005).

Selective bidirectional inbreeding led to the enrichment of the genetic material associated with the anxiety phenotype (Landgraf et al., 2007; Plomin et al., 2009). The high comorbidity of anxiety with depression is reflected in HAB mice by an increased passive stress coping behavior in the FST and TST compared to LAB mice (Kromer et al., 2005; Landgraf et al., 2007).

In previous efforts for relative protein quantification ^{15}N metabolic labeling was applied to the HAB/LAB mouse model for the comparison of HAB and LAB proteomes (Filiou et al., 2011b; Zhang et al., 2011b). For that purpose mice were fed a diet enriched with the stable, heavy ^{15}N isotope (>97% ^{15}N) resulting in an enrichment of the ^{15}N isotope in the organism. Intriguingly, the introduction of the stable ^{15}N isotope resembled antidepressant treatment effects on the behavioral phenotype of HAB mice in the TST. We therefore carried out unbiased proteomic analyses of this antidepressant-like ^{15}N isotope effect in order to discover pathways that are potentially involved in antidepressant-like actions in the HAB mouse model (Frank et al., 2009).

1.5 Mass spectrometry based quantitative proteomics and metabolomics

1.5.1 Genomics and functional genomics in psychiatry research

Major depression is a complex, hereditary and polygenetic disease. The interplay of the genetic setup with environmental factors is believed to play a crucial role in psychiatric disease development (Taurines et al., 2011). There is a great demand for integrative analyses across a wide range of fields including omics-based systems biology strategies for a better understanding of disease related pathways (Kitano, 2002; Barabasi and Oltvai, 2004).

After the complete sequencing of the human genome about 10 years ago (Lander et al., 2001; Venter et al., 2001), psychiatry research has applied genomic approaches in human studies leading to valuable, though unfortunately limited, insights into disease pathways, treatment response and novel drug targets (Lucae et al., 2006; Burmeister et al., 2008; Sillaber et al., 2008; McQuillin et al., 2009).

Functional genomics (i.e. post-genomics) including transcriptomics, proteomics and metabolomics adds a very important new dimension to the study of psychiatric disorders: the analysis of spatial and temporal system alterations. Consequently, functional genomics facilitates an accurate and unbiased generation of hypotheses for pathophysiology and drug action mechanisms (Turck et al., 2005; Geschwind and Konopka, 2009).

Both transcriptomics and proteomics were successfully applied to the elucidation of antidepressant treatment affected pathways in rodents (Khawaja et al., 2004; Carboni et al., 2006; Cecconi et al., 2007; McHugh et al., 2008; Sillaber et al., 2008; McHugh et al., 2010). Compared to transcriptomics, proteomics exhibits several advantageous characteristics: (1) the level of mRNA does not always reflect protein expression indicating the need for complementary proteomics analyses; (2) proteomics allows for organelle specific analyses facilitating e.g. synapse specific alterations; (3) protein post-translational modifications (PTM) play an important role in protein function and activity, but this aspect is undetectable by transcriptomics and (4) proteins are the actual contributors to biochemical pathway activities and biological processes and therefore are more closely related to disease phenotype.

With the advance of novel instrumentation and data analysis strategies metabolomics represents the latest of functional genomics technology. Metabolomics complements transcriptomics and proteomics investigations especially in the area of systems biology and pathway analysis because metabolites are the final products of interactions between gene expression, protein expression and the cellular environment (Kaddurah-Daouk and Krishnan, 2009). At present only a limited number of studies have been performed investigating effects of antidepressant treatment at the metabolome level (Paige et al., 2007; Dai et al., 2010; Ji et al., 2011; Su et al., 2011).

The following chapters describe quantitative proteomic (1.5.2) and metabolomic (1.5.3) technologies with a focus on methods that were used in the current thesis.

1.5.2 Quantitative proteomics

Traditional proteomics strategies predominately exploited two-dimensional gel electrophoresis for high resolution protein separation and relative protein quantification. Complex protein mixtures (e.g. derived from brain tissue) are first separated according to their isoelectric point and in a second dimension by molecular weight. Staining of the proteins allows the identification of both quantitative (expression level) and qualitative (mainly post-translational modification) protein differences. Differentially expressed proteins between disease/treatment vs. control are then identified using mass spectrometry (O'Farrell, 1975). Limitations of two-dimensional gel electrophoresis include low throughput, limited dynamic range, low sensitivity, high technical variability and incompatibility for hydrophobic membrane proteins (Fey and Larsen, 2001; Lubec et al., 2003). Therefore quantitative proteomics has recently shifted towards shotgun proteomics approaches that aim at identifying and quantifying as many proteins as possible in a given sample by utilizing highly sensitive mass spectrometry (MS) in combination with multidimensional protein and peptide separation techniques.

1.5.2.1 The impact of tandem mass spectrometry in modern proteomics

Mass spectrometry is an analytical approach that measures the mass-to-charge (m/z) ratio of ions. In a typical bottom-up proteomics experiment MS is used to analyze peptides in complex protein digests (Steen and Mann, 2004). Peptide information is then conferred to corresponding proteins. For increased sensitivity sample complexity has to be reduced prior to mass spectrometry analysis at the protein level (e.g. by SDS gel electrophoresis or isoelectric focusing) and/or at the peptide level (e.g. by strong cation exchange or reversed phase high performance liquid chromatography).

The main components of a mass spectrometer are an ionization source, a mass analyzer and an ion detector. Current MS-based proteomics became possible only after the introduction of soft ionization methods like matrix-assisted-laser-desorption ionization (MALDI) (Karas and Hillenkamp, 1988), electrospray ionization (ESI) (Whitehouse et al., 1985) and, for increased sensitivity, nanospray ionization (NSI) (Shevchenko et al., 1996b; Shevchenko

et al., 1996a; Wilm and Mann, 1996; Wilm et al., 1996). There are different kinds of mass analyzers that, depending on the analytical question, exhibit distinct strengths and advantages. Mass analyzers include quadrupole (Q) mass filter, linear ion trap (LTQ), Orbitrap, time-of-flight (TOF), magnetic sector, ion cyclotron resonance (ICR), and combinations thereof like Triple-Quadrupole (QqQ), Q-TOF or LTQ-Orbitrap instruments.

LTQ-Orbitrap hybrid mass spectrometers are the instruments of choice for shotgun proteomics due to the high sensitivity, high scan rate, high dynamic range (>10^3), high mass accuracy (<5 ppm) and high mass resolution (up to 100.000 at m/z 400) (Yates et al., 2006). The final readout of all mass spectrometers is a mass spectrum (MS spectrum) which is a recording of the signal intensity of the ion at each value of the m/z scale (Steen and Mann, 2004).

Tandem mass spectrometry involves the fragmentation of precursor ions and m/z determination of product ions resulting in the generation of MS/MS spectra. By *in silico* comparison of precursor ion and fragment ion masses with a protein database it is then possible to identify peptides and corresponding proteins (Eng et al., 1994; Perkins et al., 1999). Fragmentation methods include collision-induced dissociation (CID), higher energy collision dissociation (HCD), or electron transfer dissociation (ETD).

1.5.2.2 Quantitative proteomics using stable isotope labeling

Mass spectrometry signal intensities are not a measure of protein amount because different peptides have different physico-chemical properties like solubility or ionization efficiency. However, because these factors are reproducible relative quantification is possible by using label free quantitation strategies (Bondarenko et al., 2002; Chelius and Bondarenko, 2002; Higgs et al., 2008) or strategies based on stable isotope labeling (Gygi and Aebersold, 2000; Steen and Mann, 2004).

According to the isotope dilution theory (De Leenheer, 1992), peptides that differ only in isotopic composition behave identically during an LC-MS experiment. Therefore, the ratio of unlabeled and labeled peptides within a mass spectrum or the ratio of extracted ion chromatograms represents an

accurate measure of relative protein concentration (Steen and Mann, 2004). Stable isotopes that are used for labeling of proteins or peptides include ^{2}H, ^{13}C, ^{15}N and ^{18}O, leading to a great variety of different methods for relative protein quantification like ICAT (Gygi et al., 1999), ICPL (Schmidt, 2005), iTRAQ (Ross et al., 2004), ^{18}O labeling (Reynolds et al., 2002), SILAC (Ong et al., 2002) and ^{15}N metabolic labeling (Oda et al., 1999). Unlabeled and labeled samples are combined at different experimental stages leading to different quantitation accuracies. If unlabeled and labeled samples are combined at the very beginning of sample preparation, as is the case for SILAC or ^{15}N metabolic labeling, quantitation accuracy is maximal because potential experimental biases affect both labeled and unlabeled sample in the same way and the ratio remains unchanged (Bantscheff et al., 2007; Bantscheff et al., 2012)(Figure 2). Therefore, SILAC and ^{15}N metabolic labeling represent the current gold standard for accurate relative protein quantification. ^{15}N metabolic labeling has been used in several model organisms including yeast (Oda et al., 1999), bacteria(Pan et al., 2008), *Drosophila melanogaster* (Krijgsveld et al., 2003), *Caenorhabditis elegans* (Dong et al., 2007), plants (Huttlin et al., 2007), mice (Huttlin et al., 2009; Filiou et al., 2011b; Zhang et al., 2011b) and rats (Wu et al., 2004). To avoid any biases due to the labeling itself the labeled sample is frequently used as internal standard for indirect comparison of relative protein levels (Pan et al., 2008).

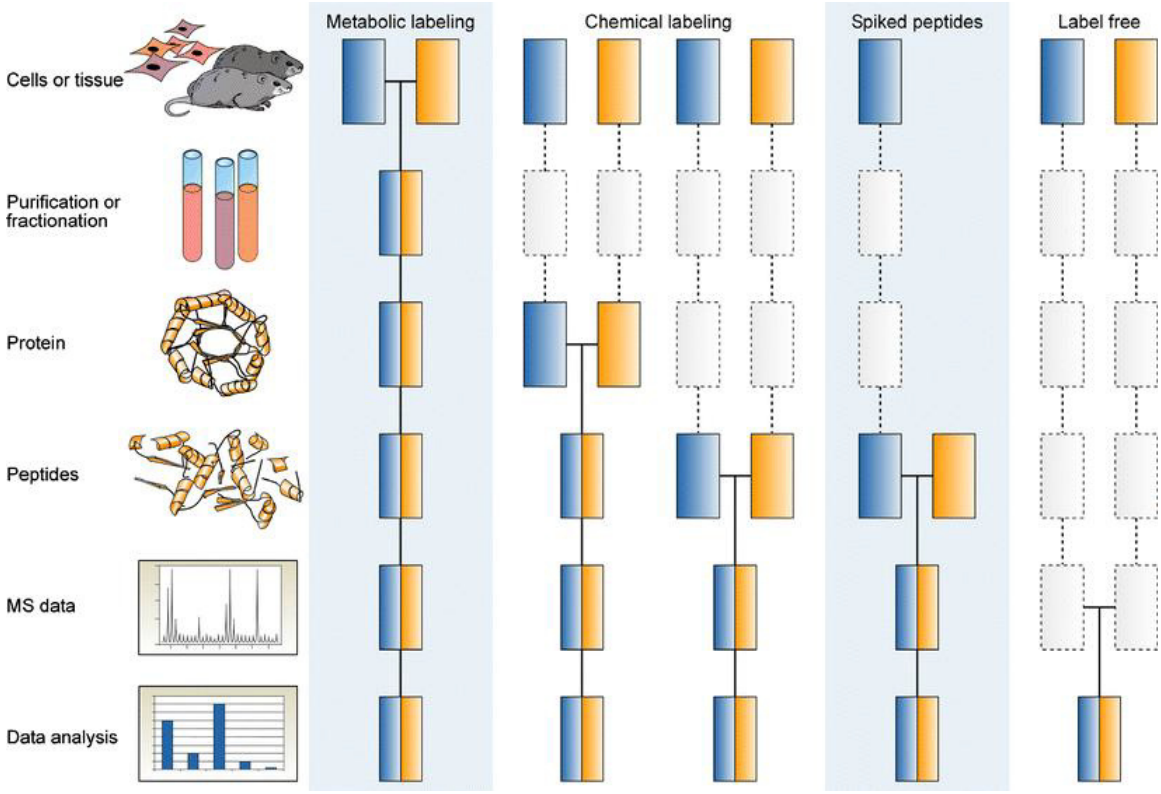

Figure 2. Quantitative proteomics workflows. If samples are combined prior to sample preparation, as seen in metabolic labeling approaches, quantitation accuracy is optimal. Blue and orange boxes represent two experimental conditions, horizontal lines indicate when samples are combined and dashed lines indicate possibilities for experimental variation. Reproduced with permission from (Bantscheff et al., 2007).

1.5.3 Quantitative metabolomics

Quantitative metabolomics complements previous -omics efforts due to its integrative capacity and was recently introduced into psychiatric research (Kaddurah-Daouk et al., 2007; Paige et al., 2007; Dai et al., 2010; Ji et al., 2011; Su et al., 2011).

A major challenge in metabolomic analyses is the large dynamic range (>10^6) and great chemical diversity of metabolites like amino acids, amino sugars, oligosaccharides, fatty acids or lipids. Another drawback is the absence of reliable and automated metabolite identification tools (Moco et al., 2007).

Due to the chemical variety diverse complementary analytical methods including sample preparation need to be applied for a comprehensive and

hypothesis-free holistic analysis. Depending on the biological question, however, the analysis of sub-metabolomes or targeted analyses may reveal significant insights into the system of interest. Most commonly used platforms are based on LC-MS (liquid chromatography-MS), GC-MS (gas chromatography-MS) and NMR (nuclear magnetic resonance) (Moco et al., 2007; Theodoridis et al., 2012).

Acquisition of NMR spectra of complex mixtures like biofluids, so called NMR fingerprinting, is used for the identification of biomarker patterns. NMR is a quantitative technique well suited for metabolomics analyses but lacks sensitivity compared to MS-based methods.

GC-MS was applied to metabolomics studies much earlier than LC-MS and offers high sensitivity, high resolution power, high throughput, excellent reproducibility, and highly reproducible fragmentation patterns. Disadvantages include tedious sample preparation because samples need to be derivatized before analysis and only volatile (or volatile-made) metabolites with molecular weights below 400-500Da can be analyzed (Tolstikov, 2009; Theodoridis et al., 2012).

LC-MS is the most widely used technique in metabolomics. Metabolites are first separated using reversed phase (RP) chromatography for hydrophobic compounds (e.g. phenolic acids, flavonoids) and hydrophilic chromatography (HILIC) for hydrophilic and neutral compounds (e.g. sugars, amino acids, nucleotides) (Tolstikov and Fiehn, 2002). Metabolite ions are generated by electrospray ionization (ESI, in positive or negative mode) or atmospheric pressure ionization (API). Ions are then analyzed mainly using quadrupole-time-of-flight (Q-TOF) instruments because of their high sensitivity, mass resolution (about 10.000) and mass accuracy with limitations in dynamic range. Metabolite ions may also be analyzed using FT-Orbitrap or FT-ICR-MS instruments for highest mass resolution (100.000-1.000.000) and mass accuracy (<2ppm) facilitating metabolite identification. Metabolites are identified using characteristic parameters like metabolite mass, isotopic distribution, fragmentation pattern and retention time (Moco et al., 2007).

Metabolomics analyses may also be performed in a targeted fashion. Targeted analyses mainly exploit Multiple-Reaction-Monitoring (MRM) on Triple-Quadrupole mass spectrometers that allow for accurate and

unambiguous quantification of metabolite precursor and product ions (Lu et al., 2008). Recently, a metabolomics platform was introduced targeting metabolites involved in cellular metabolism using LC-MRM-MS. The platform reliably quantifies more than 250 metabolites involved in major metabolic pathways, including glycolysis, Krebs cycle and metabolism of amino acids (Kelly et al., 2011; Yuan et al., 2012).

1.6 Aim and scope of thesis

The aim of the current thesis was to define antidepressant treatment effects at the molecular pathway level in mouse models using state of the art proteomic and metabolomic technologies. A further aim was to identify biomarker candidates for antidepressant treatment effects.

Specific aims of the individual manuscripts:

Chapter 2.1: Metabolite profiling of antidepressant drug action reveals novel drug targets beyond monoamine elevation

- Identification of paroxetine treatment affected molecular pathways beyond serotonin reuptake inhibition in hippocampus of chronically treated DBA/2 mice at the metabolome level.
- Identification of potential drug targets that may modulate these pathways.
- Identification of metabolite biomarker candidates in plasma.

Chapter 2.2: Proteomic and metabolomic profiling reveals time-dependent changes in hippocampal metabolism upon paroxetine treatment and biomarker candidates

- Identification of paroxetine treatment affected molecular pathways beyond serotonin reuptake inhibition in hippocampus at the metabolome and proteome levels.
- Identification of time course alterations.
- Identification of metabolite biomarker candidates in plasma.

Chapter 2.3: The ^{15}N isotope effect as a means for correlating phenotypic alterations and affected pathways in a trait anxiety mouse model

- Identification of molecular correlates of the behavioral antidepressant-like ^{15}N isotope effect in various brain regions at the proteome level.

2 RESEARCH ARTICLES

2.1 Metabolite profiling of antidepressant drug action reveals novel drug targets beyond monoamine elevation

Webhofer C, Gormanns P, Tolstikov V, Zieglgänsberger W, Sillaber I, Holsboer F, Turck CW (2011). Metabolite Profiling of Antidepressant Drug Action Reveals Novel Drug Targets Beyond Monoamine Elevation. Transl Psychiatry 1:e58.

Declaration of contribution: Christian Webhofer designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with co-authors (for detailed information on author contributions see chapter ´Eidesstattliche Versicherung/Affidavit´).

Supplemental material is available on enclosed CD and upon request.

Citation: Transl Psychiatry (2011) 1, e58, doi:10.1038/tp.2011.56

www.nature.com/tp

Metabolite profiling of antidepressant drug action reveals novel drug targets beyond monoamine elevation

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Currently used antidepressants elevate monoamine levels in the synaptic cleft. There is good reason to assume that this is not the only source for antidepressant therapeutic activities and that secondary downstream effects may be relevant for alleviating symptoms of depression. We attempted to elucidate affected biochemical pathways downstream of monoamine reuptake inhibition by interrogating metabolomic profiles in DBA/2Ola mice after chronic paroxetine treatment. Metabolomic changes were investigated using gas chromatography-mass spectrometry profiling and group differences were analyzed by univariate and multivariate statistics. Pathways affected by antidepressant treatment were related to energy metabolism, amino acid metabolism and hormone signaling. The identified pathways reveal further antidepressant therapeutic action and represent targets for drug development efforts. A comparison of the central nervous system with blood plasma metabolite alterations identified GABA, galactose-6-phosphate and leucine as biomarker candidates for assessment of antidepressant treatment effects in the periphery.
Translational Psychiatry (2011) **1,** e58; doi:10.1038/tp.2011.56; published online 13 December 2011

Introduction

Major depression is a severe and life-threatening disease with a population prevalence of 17%[1] and is projected to be the second leading contributor to the global burden of disease (DALYs) by the year 2020.[2] At present, selective serotonin reuptake inhibitors, selective noradrenaline reuptake inhibitors and monoamine oxidase inhibitors are the most commonly prescribed antidepressants.[3,4] Shortcomings of current antidepressant treatments include a delayed onset of therapeutic action, adverse side effects and response in only a subset of patients.[4,5]

The elevation of serotonin or noradrenaline levels by tricyclic and currently used antidepressants had led to the monoamine deficiency hypothesis of depression[6,7] with clinical research over the past 40 years suggesting that increased monoaminergic neurotransmission is essential for antidepressant efficiency.[6] However, contrary to this hypothesis, monoamine depletion does not induce depression in healthy humans.[8] Moreover, a study by Nickel *et al.*[9] found that paroxetine, a serotonin reuptake inhibitor and tianeptine, a serotonin reuptake enhancer are both effective antidepressants. A common downstream effect of both drugs was to normalize hypothalamic-pituitary-adrenal (HPA) axis reactivity. These findings indicate that opposite effects on the serotonin system may lead to common downstream pathway alterations. The most parsimonious explanation is that monoamine elevation *per se* is not the only source for antidepressant activity but secondary downstream effects may also be involved in the alleviation of depressive symptoms.[10–12] Therefore, it is tempting to speculate that for improved antidepressant treatment it would be beneficial to pharmacologically interfere with these downstream mechanisms directly. Proposed antidepressant downstream effects include neurogenesis,[13,14] strengthened neuronal plasticity[15] and attenuation of HPA axis reactivity.[10,16] Despite several suggested modes for antidepressant action beyond monoamine elevation, the precise mechanisms at the cellular metabolism and pathway levels remain elusive. Activation of postsynaptic monoamine receptors in response to antidepressant treatment triggers intracellular signaling cascades relayed by G proteins that are coupled to several effector systems including adenylate cyclase, phospholipase C, phospholipase A_2 and ion channels.[17,18] Second messengers like cyclic AMP and diacylglycerol induce intracellular protein phosphorylation events mediated by protein kinase A and protein kinase C, respectively. Phosphorylation events cause gene expression alterations through transcription factors like cyclic AMP response element-binding protein resulting in further downstream alterations.[19–22] Several studies have investigated the effects of antidepressants in unbiased transcriptomic or proteomic studies. Sillaber *et al.*[23] identified a number of paroxetine-induced changes on the transcriptome of DBA/2 mice, including alterations in glial fibrillary acidic protein and brain-derived neurotrophic factor (BDNF) mRNA expression. Proteomic studies include paroxetine treatment effects on embryonic stem cell-derived neural cells[24] and fluoxetine and venlafaxine effects on the rat hippocampus and frontal cortex[25,26] protein expression.

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Metabolites reflect the ultimate response of an organism to any biological effect[27] as they are the final products of interactions between gene expression, protein expression and the cellular environment.[28] Therefore, metabolite profiling holds great promise for the identification of affected pathways in depression, and for the study of antidepressant drug treatment.

Recent studies have used metabolomic methods to interrogate psychiatric drug treatment effects. Atypical antipsychotic treatment influenced metabolism of specific lipid classes in patients with schizophrenia.[29] Metabolomic studies on treatment effects of traditional Chinese medicine in rats identified potential biomarker candidates.[30,31] Ji *et al*.[32] applied a pharmacometabolomic approach to guide targeted pharmacogenomic analyses in antidepressant responders versus non-responders. A pilot study in depressed patients of old age revealed alterations in plasma metabolite levels of GABA, glycerol and several fatty acids compared with controls.[33] Most importantly, many of these alterations normalized after remission. Unfortunately, in this study, metabolomic changes could not be unambiguously attributed to depression or antidepressant treatment-related alterations.

In this study, we have for the first time analyzed metabolomic changes after chronic paroxetine treatment in DBA/2Ola mice. Using multivariate and univariate statistics, we have identified affected biochemical pathways downstream of serotonin reuptake inhibition and potential antidepressant drug targets in the hippocampus. Alterations in the plasma as the preferred specimen in a clinical setting represent a starting point for the implementation of a clinical biomarker assay for an early assessment of antidepressant treatment response.

Materials and methods

Antidepressant treatment of DBA/2Ola mice and organ sampling. Eight-week-old DBA/2OlaHsd mice were purchased from Harlan Laboratories (Harlan Winkelmann, Borchen, Germany). Upon arrival, mice were housed singly in standard cages and habituated for 2 weeks under standard laboratory conditions (food and water *ad libitum*, 12 h dark–light cycle: lights on 0700–1900 hours, 45–55% humidity, $21 \pm 2\,^{\circ}C$). After 2 weeks, mice received either paroxetine at $10\,mg\,kg^{-1}$ (1 mg paroxetine–hydrochloride–hemihydrate (Sigma-Aldrich, St Louis, MO, USA) in 1 ml tap water, thoroughly mixed before each application) or vehicle (tap water) twice per day (between 0800 and 0900 hours and 1800 and 1900 hours) by gavaging for 28 days. In the morning of day 29, mice received a final treatment, and 60 min later, the behavior in the forced swim test (FST), water temperature 25–26 °C, was observed during a 5-min test period. Immediately after the FST, mice were killed by an overdose of Forene isoflurane (Abbott, Wiesbaden, Germany), and blood was drawn by heart puncture and collected in EDTA tubes (Kabe Labortechnik, Nuembrecht-Elsenroth, Germany). Plasma was separated from serum by centrifugation (1300 *g*, 10 min) and immediately frozen in liquid nitrogen. Mice were perfused with 0.9% ice-cold saline (Merck, Darmstadt, Germany) and their brains sampled. Hippocampi were dissected, weighed, individually shock frozen in liquid nitrogen and stored at −80 °C until further analysis.

Metabolite analysis. Metabolite analysis was performed by the UC Davis Metabolomics Core Facility (http://www.metabolomics-core.ucdavis.edu/) using validated protocols and standard operation procedures (see Supplementary Figure S1). For quality control purposes, 30 standard metabolites were included in the samples in each randomized batch. FAMEs C08–C30 were used as internal references for retention index (RI) calibration, the Leco proprietary deconvolution protocol for peak finding (Leco, St Joseph, MI, USA) and the BinBase database (http://fiehnlab.ucdavis.edu/db/) for metabolite annotation.

Metabolite extraction. Per 10 mg of frozen hippocampus, 500 µl pre-chilled extraction solution (acetonitrile, isopropanole, water, 3:3:2 (v/v/v)) was added and the mixture was homogenized for 45 s using Tissue Master 125 (Omni International, Kennesaw, GA, USA). After centrifugation (13,000 *g*, 5 min), supernatants were dried and resuspended with 500 µl acetonitrile:water (1:1, v/v). After centrifugation (14 000 *g*, 2 min), supernatants were completely dried and stored in darkness under argon for further analysis. Plasma metabolite extraction was performed as described previously by Urayama *et al*.[34]

Sample derivatization and GC-time of flight-mass spectrometry metabolite profiling. Sample derivatization with methoxylamine hydrochloride in pyridine and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was performed as described previously.[34] Gas chromatography (GC)-time of flight-mass spectrometry analysis was performed using an Agilent 6890N gas chromatograph (Agilent, Palo Alto, CA, USA) interfaced to a time-of-flight Pegasus III mass spectrometer (Leco). Automated injections were performed using an MPS2 programmable robotic multipurpose sampler (Gerstel, Mühlheim an der Ruhr, Germany). The GC system was fitted with both an Agilent injector and a Gerstel temperature-programmed injector, cooled injection system (model CIS 4), with a Peltier cooling source. An automated liner exchange (ALEX) designed by Gerstel was used to eliminate cross-contamination from sample matrix occurring between sample runs. Multiple baffled liners for the GC inlet were deactivated with 1-µl injections of MSTFA. The Agilent injector temperature was held constant at 250 °C, whereas the Gerstel injector was programmed (initial temperature 50 °C, hold 0.1 min and increased at a rate of $10\,^{\circ}C\,s^{-1}$ to a final temperature of 330 °C, hold time 10 min). Injections of 1 µl were made in the split (1:5) mode (purge time 120 s, purge flow $40\,ml\,min^{-1}$).

Chromatography was performed on an Rtx-5Sil MS column (30 m × 0.25 mm i.d., 0.25-µm film thickness) with an Integra-Guard column (Restek, Bellefonte, PA, USA). Helium carrier gas was used at a constant flow of $1\,ml\,min^{-1}$. The GC oven temperature program was 50 °C initial temperature, with 1-min hold time and ramping at 208 °C/min to a final temperature of 330 °C with 5-min hold time. Both the transfer line and the source temperatures were 250 °C. The Pegasus III time of

flight-mass spectrometer ion source operated at −70 kV filament voltage. After a solvent delay of 350 s, mass spectra were acquired at 20 scans per second with a mass range of 50–500 *m*/*z*.

Data analysis and statistics. 2-Monostearin, fructose, glucose, glucose-6-phosphate, glycine and phenylalanine were identified twice during chromatographic separation in different chemical derivatization states and their signal intensities were summed up for further analysis. Metabolite signal intensities were normalized by the total sum of all metabolite intensities. Pareto scaled metabolite signal intensities were then analyzed using MetaboAnalyst (http://www.metaboanalyst.ca).[35]

Downstream pathway analysis in the hippocampus and antidepressant drug targets. Identification of altered metabolites for subsequent pathway analysis in the hippocampus was performed by a combined univariate and multivariate data analysis strategy. Metabolites with altered concentrations were identified by SAM (significance analysis of microarrays)[36] applying a *q*-value threshold of 15%. Multivariate data structure was revealed by partial least square—discriminant analysis considering metabolites with a variable importance in projection >1, using just one component (accuracy and $R^2>80\%$, $Q2=0.57$ and $P<0.18$ in permutation tests).[37] By exclusively considering the overlap between the two statistically different methods, we improved robustness of data analysis and increased confidence in significantly altered metabolites.

Pathway analysis was performed using the Pathway Studio software v7.1 (Ariadne Genomics, Rockville, MD, USA), which contains literature-based relations between proteins, functional classes, small molecules and cellular processes. For an increased confidence, relations were only considered if there were at least two reported literature references.

For identification of affected molecular pathways, a search for common molecular upstream regulators was performed including proteins and functional classes and for downstream targets including proteins, functional classes and cellular processes of altered metabolites. A conservative hypergeometric test[38] was performed to detect significant overrepresented ($P<0.05$) common regulators and targets. The background for each common regulator/target was set to the number of metabolites it relates to (minimum two references) in the Ariadne database. Owing to the conservative test, common regulators/targets with only a few interactions are highly penalized.[38] Finally, metabolites along with their regulators and targets were grouped according to biological function into pathway clusters. In addition to the identified pathway clusters, potential antidepressant drug targets were identified. These comprise common regulators and targets (proteins and functional classes) that modulate these pathways.

Biomarker candidates in the hippocampus and plasma. To detect biomarker candidates for antidepressant treatment effects in the hippocampus, SAM was performed by applying a *q*-value threshold of $q<0.05$. Each metabolite is presented by a scatter plot indicating inter- and intra-group biological variabilities. Metabolite/behavior correlation was assessed using Pearson's correlation of metabolite intensities versus floating behavior in the FST. In addition, mice were grouped according to floating time independent of treatment group and corresponding average metabolite intensities are shown.

To detect biomarker candidates in the plasma, we correlated hippocampal and plasma metabolite intensities ($SAM_{hippocampus}$ $q<0.05$, Pearson's correlation $P<0.10$).

Results

We performed a comparative metabolomic study in chronic paroxetine- versus vehicle-treated DBA/2 mice. As expected, paroxetine treatment significantly reduced depression-like behavior in the FST, indicated by a reduced time of floating ($P<0.0001$, Figure 1a). Six biological replicates per group were selected for metabolite profiling based on immobility behavior in the FST (Figure 1b).

A total of 270 metabolites were quantified in the hippocampus and plasma, of which 110 were of known identity including 25 sugars, 24 amino acids, 17 fatty acids, 12 lipids, 12 organic acids and 20 of other categories (Supplementary Tables S1 and S2).

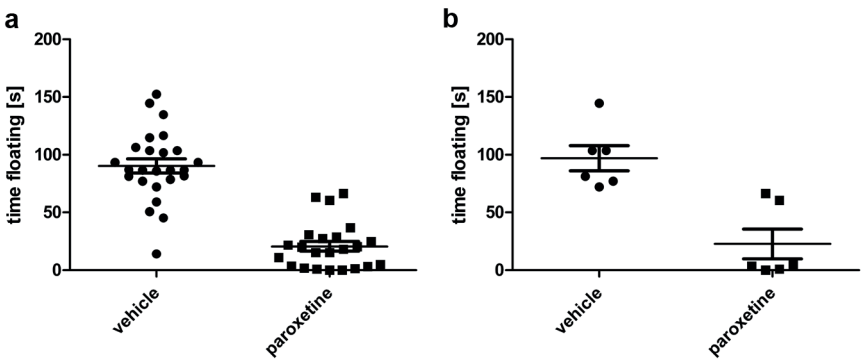

Figure 1 Behavioral analysis of paroxetine-treated mice. (**a**) Chronic paroxetine treatment reduced passive behavior in the forced swim test determined by time of floating, $P<0.0001$, $t_{floating}$ (vehicle) = 90.3 ± 6.1 s (mean ± s.e.m.), $t_{floating}$ (paroxetine) = 20.6 ± 4.2 s. (**b**) Six biological replicates were selected for metabolomics analysis based on the time of floating, $P=0.0014$, $t_{floating}$ (vehicle) = 97.0 ± 11.0 s (mean ± s.e.m.), $t_{floating}$ (paroxetine) = 22.7 ± 12.9 s.

Downstream pathway analysis and antidepressant drug target candidates in the hippocampus. For the investigation of affected hippocampal downstream pathways, we analyzed differences in metabolomic profiles using a combined univariate and multivariate data analysis strategy as described in the 'Materials and methods' section. In total, 28 metabolites including 9 amino acids, 5 sugars, 2 organic acids, 1 fatty acid, 4 of other categories and 7 of unknown identity were considered for pathway analysis (Table 1a). Twenty four significantly altered metabolites in the hippocampus were present at higher levels upon paroxetine compared with vehicle treatment. A log2 abundance factor distribution of all metabolites for the hippocampus and plasma is shown in Supplementary Figure S2, indicating slightly higher metabolite levels in paroxetine-treated hippocampi.

Identified pathway clusters included energy metabolism, amino-acid metabolism and hormone signaling (Figure 2). Regulators of these pathways, as illustrated in Figure 2 and summarized in Table 2, represent antidepressant drug targets that have the potential to modulate these pathways.

Energy metabolism. Most profound alterations at the pathway level were found to be related to energy metabolism. Glucose, glucose-6-phosphate and fructose-6-phosphate, metabolites involved in glycolysis, were present at 2–4-fold higher levels upon paroxetine treatment. Levels of lactate, a metabolite linked to glycolysis, were reduced by 30%. Potential tricarboxylic acid (TCA) cycle alterations were reflected by a 2.5-fold increase in fumarate levels. Regulators of energy metabolism were identified as hexokinase 1 and 2, glucokinase, glycogen synthase 2, glucose transporter, fructose 1,6 diphosphatase, mannose phosphate isomerase and fructose bisphosphatase 1.

Amino acid metabolism. Eight proteinogenic amino acids were considered significant for pathway analysis (Ala, Ile, Leu, Pro, Ser, Thr, Tyr, Val), all being upregulated by paroxetine treatment (23–72% increase). All but two (Ser, Thr) amino acids are metabolically linked to TCA cycle intermediates linking amino-acid changes to energy metabolism alterations. Potential regulators are represented by 3-methyl-2-oxobutanoate dehydrogenase, an important enzyme in branched-chain amino acid (BCAA), Val, Leu, Ile, degradation and glutamate dehydrogenase, converting glutamate to alphaketoglutarate, a TCA cycle intermediate.

Hormone signaling. Hormone signaling was dominated by glucagon and insulin. Both control blood glucose levels and the pre-hormone angiotensinogen that is involved in blood pressure regulation.

Biomarker candidates in the hippocampus and plasma. Biomarker candidates were identified by univariate data analysis, as described in the 'Materials and methods' section. A total of 34 metabolites were found in the hippocampus (7 amino acids, 4 sugars, 2 fatty acids, 1 lipid, 2 organic acids, 1 of other categories and 17 of unknown identity), of which 24 were present at higher levels upon paroxetine treatment (Table 1b). Galactose-6-phosphate (4.88), fumaric acid (2.47), fructose (2.19) and xylulose (2.08) were among the metabolites with the greatest fold change and the most significant changes were found for threonic acid ($q=0.009$), xylulose ($q=0.024$) and valine ($q=0.024$). Supplementary Figure S3 provides detailed information on significant metabolites (scatter plots and correlation analysis). For many metabolites there was a high correlation between metabolite intensities and depression-like behavior in the FST. This represents a first hint that paroxetine-induced metabolite alterations may be linked to a depression-like phenotype and do not represent a mere side effect (Supplementary Figure S3).

For identification of plasma biomarker candidates, a correlation analysis of hippocampus versus plasma metabolite intensities was performed. Out of 17 hippocampus biomarker candidates ($SAM_{hippocampus}$ $q<0.05$) of known identity, 3 metabolites show high correlation values (two-tailed $P<0.10$) upon paroxetine treatment. Calculated Pearson's coefficient values were $r=0.77$ for GABA, $r=0.75$ for galactose-6-phosphate and $r=-0.73$ for leucine (Figure 3).

Discussion

Metabolomic studies hold great promise for the identification of molecular alterations upon drug treatment.[29–32,39]

To exclude any metabolite level alterations that are caused by environmental factors such as nutritional effects, animals with homogeneous genetic backgrounds and housed under controlled conditions are the preferred study objects. This way, inter-individual variability that is commonplace in patient studies can be avoided resulting in a better signal-to-noise ratio of the drug-elicited metabolite level changes. To our knowledge, this is the first study identifying metabolite signatures in chronically paroxetine-treated DBA/2 mice. We aimed at revealing treatment effects beyond elevation of serotonin levels in the synaptic cleft that are involved in therapeutic antidepressant effects. Understanding the cross-talk between altered metabolomic pathways will greatly enhance our understanding of the drug's mode of action and adverse side effects.

Affected downstream pathways in the hippocampus. The selective serotonin reuptake inhibitor paroxetine inhibits presynaptic serotonin transporters leading to enhanced serotonergic synaptic transmission, which was previously shown to be essential for therapeutic efficiency.[6] Our results indicate that enhanced serotonin availability results in diverse downstream pathway alterations.

Most significantly, paroxetine treatment altered hippocampal energy metabolism (Figure 2, Tables 1a and 2), particularly the initial steps of glycolysis. Recent studies have linked antidepressant treatment to energy metabolism alterations. Scaini *et al.*[40] demonstrated increased mitochondrial respiratory chain activity in selected brain regions after chronic antidepressant treatment. Prefrontal cortex, hippocampal and striatal, but not cerebellar citrate synthase and succinate dehydrogenase activities were increased after paroxetine treatment.[41] Santos *et al.*[42] found increased brain creatine kinase activity after chronic paroxetine treatment. All these findings indicate that in order to normalize metabolic

Table 1 (a) Hippocampal metabolite level alterations after paroxetine treatment reveal affected pathways (PLS-DA VIP>1 and SAM $q<0.15$, see Figure 2). (b) Hippocampal biomarker candidates for monitoring antidepressant treatment response (SAM $q<0.05$)

Metabolite	*Pubchem CID*	*Chemical class*	*Fold change*	*PLS-DA, VIP score*	*SAM, P-value*	*SAM, q-value*
(a)						
Alanine	5950	Amino acid	1.48	4.6	0.006	0.031
GABA	119	Amino acid	0.87	5.3	0.005	0.026
Isoleucine	6306	Amino acid	1.58	2.0	0.002	0.026
Leucine	6106	Amino acid	1.66	2.8	0.003	0.026
Proline	145742	Amino acid	1.65	2.2	0.039	0.075
Serine	5951	Amino acid	1.18	2.0	0.082	0.103
Threonine	6288	Amino acid	1.25	1.2	0.056	0.087
Tyrosine	6057	Amino acid	1.23	1.1	0.051	0.087
Valine	6287	Amino acid	1.72	2.8	0.001	0.024
Threonic acid	439535	Fatty acid	0.59	1.1	<0.001	0.009
Fumaric acid	444972	Organic acid	2.47	1.8	0.004	0.026
Lactic acid	612	Organic acid	0.70	3.3	0.018	0.054
Fructose	5984	Sugar	2.19	1.9	0.003	0.026
Fructose-6-phosphate	69507	Sugar	1.99	1.2	0.029	0.062
Glucose	5793	Sugar	3.75	2.9	0.033	0.067
Glucose-6-phosphate	5958	Sugar	2.55	1.6	0.027	0.061
Inositol myo-	892	Sugar	0.75	3.7	0.021	0.057
Ethanolamine	700	Other	1.69	3.3	0.003	0.026
Hypoxanthine	790	Other	1.09	1.2	0.108	0.125
Taurine	1123	Other	1.34	3.1	0.071	0.094
Uracil	1174	Other	1.27	1.4	0.022	0.057
199786	NA	Unknown	1.32	1.1	0.026	0.061
202571	NA	Unknown	3.08	1.0	0.017	0.054
203259	NA	Unknown	1.50	1.1	0.025	0.060
223535	NA	Unknown	1.69	1.2	0.013	0.046
227964	NA	Unknown	4.14	1.1	0.031	0.064
295226	NA	Unknown	1.61	1.8	0.016	0.052
301745	NA	Unknown	1.75	1.6	0.049	0.087
(b)						
Alanine	5950	Amino acid	1.48	4.6	0.006	0.031
β-Alanine	239	Amino acid	1.40	<1	0.002	0.026
GABA	119	Amino acid	0.87	5.3	0.005	0.026
Isoleucine	6306	Amino acid	1.58	2.0	0.002	0.026
Leucine	6106	Amino acid	1.66	2.8	0.003	0.026
Ornithine	6262	Amino acid	1.25	<1	0.008	0.034
Valine	6287	Amino acid	1.72	2.8	0.001	0.024
Lignoceric acid	11197	Fatty acid	0.65	<1	0.013	0.046
Threonic acid	439535	Fatty acid	0.59	1.1	<0.001	0.009
2-Monopalmitin	123409	Lipid	1.52	<1	0.011	0.044
Fumaric acid	444972	Organic acid	2.47	1.8	0.004	0.026
Ribonic acid	5460677	Organic acid	1.33	<1	0.005	0.026
Fructose	5984	Sugar	2.19	1.9	0.003	0.026
Galactose-6-phosphate	99058	Sugar	4.88	<1	0.002	0.026
Xylose	6027	Sugar	1.84	<1	0.004	0.026
Xylulose	5289590	Sugar	2.08	<1	<0.001	0.024
Ethanolamine	700	Other	1.69	3.3	0.003	0.026
199239	NA	Unknown	0.77	<1	0.014	0.048
199553	NA	Unknown	1.23	<1	0.004	0.026
202572	NA	Unknown	3.45	<1	0.005	0.026
202573	NA	Unknown	2.54	<1	0.001	0.026
214201	NA	Unknown	2.29	<1	0.002	0.026
214537	NA	Unknown	0.63	<1	0.007	0.032
216860	NA	Unknown	1.31	<1	0.005	0.026
217797	NA	Unknown	8.00	<1	0.004	0.026
219021	NA	Unknown	1.93	<1	0.012	0.046
219169	NA	Unknown	0.40	<1	0.012	0.045
223535	NA	Unknown	1.69	1.2	0.013	0.046
231659	NA	Unknown	0.62	<1	0.010	0.043
234563	NA	Unknown	0.32	<1	0.005	0.027
236605	NA	Unknown	2.93	<1	0.003	0.026
239332	NA	Unknown	0.43	<1	0.001	0.026
241111	NA	Unknown	2.79	<1	0.003	0.026
270407	NA	Unknown	0.67	<1	0.008	0.034

Abbreviations: PLS-DA, partial least square—discriminant analysis; NA, not applicable; SAM, significance analysis of microarrays; VIP, variable importance in projection.

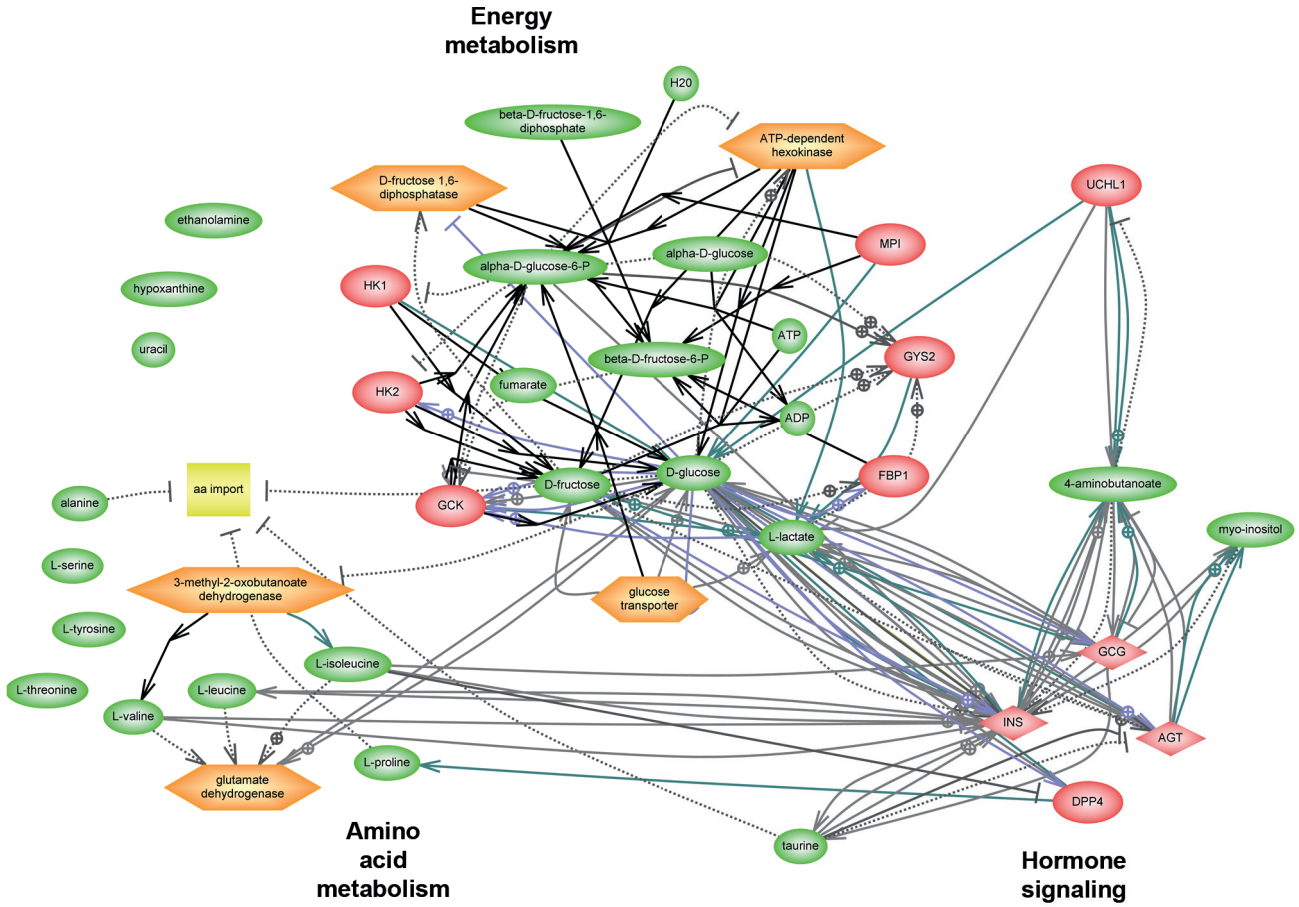

Figure 2 Pathway analysis of altered metabolites upon paroxetine treatment. Common targets and regulators of significant metabolites were identified by a conservative hypergeometric test expression analysis systematic explorer (EASE), score < 0.05. Metabolites and targets/regulators were clustered into energy metabolism, amino acid metabolism and hormone signaling pathways. Green: metabolites, red: proteins, yellow: cellular processes, orange: functional classes.

Table 2 Antidepressant drug target candidates that are regulators and targets of altered metabolites, expression analysis systematic explorer (EASE), score < 0.05

Pathway cluster	*Antidepressant drug target candidate*	*Category*	P-*value*	*EASE score*
Carbohydrate metabolism	ATP-dependent hexokinase	Functional class	0.005	0.033
	D-fructose 1,6-diphosphatase	Functional class	4.1E-04	0.012
	FBP1 (fructose bisphosphatase 1)	Protein	0.004	0.042
	GCK (glucokinase)	Protein	8.8E-05	0.001
	glucose transporter	Functional class	0.002	0.021
	GYS2 (glycogen synthase 2)	Protein	0.001	0.008
	HK1 (hexokinase 1)	Protein	1.5E-04	0.006
	HK2 (hexokinase 2)	Protein	7.5E-05	0.004
	MPI (mannose phosphate isomerase)	Protein	1.5E-04	0.006
Amino acid metabolism	3-Methyl-2-oxobutanoate dehydrogenase	Functional class	0.002	0.033
	aa (amino acid) import	Cellular process	0.005	0.048
	Glutamate dehydrogenase	Functional class	0.004	0.030
Hormone signaling	AGT (angiotensinogen)	Protein	0.012	0.047
	DPP4 (dipeptidyl-peptidase 4)	Protein	1.5E-04	0.006
	GCG (glucagon)	Protein	0.004	0.021
	INS (insulin)	Protein	0.003	0.013
Other	Uchl1 (ubiquitin carboxyl-terminal esterase L1)	Protein	0.002	0.032

hypoactivity, which is frequently observed in depressed patients, energy pathways are a pharmacological target worth pursuing. Increased ATP levels could also facilitate energy-dependent antidepressant-treatment-associated cellular processes like G-protein activity, protein kinase activities (protein kinase A, protein kinase C), synaptic activity

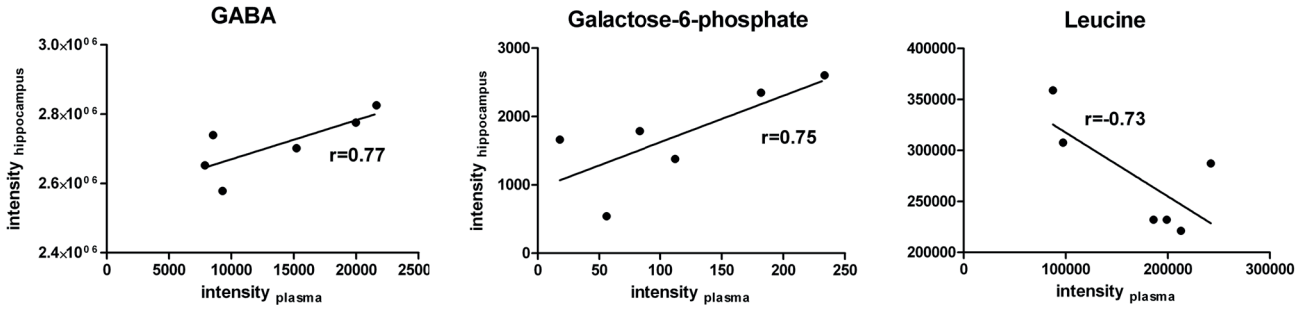

Figure 3 Plasma metabolite biomarker candidates for monitoring antidepressant treatment response. Hippocampal metabolite intensities were correlated with plasma metabolite intensities ($P<0.10$).

(transport of ions and neurotransmitter uptake) and protein synthesis (BDNF).[43]

We further identified significant alterations in amino acid metabolism, which is closely linked to energy metabolism through TCA cycle intermediates. However, from our data it is not clear whether increased amino acid concentrations contribute to or are a consequence of altered energy metabolism and further studies are required to answer this question.

We also identified significantly altered neuroactive amino acid level including β-alanine, a glycine receptor agonist, and decreased levels of GABA, the most prominent inhibitory neurotransmitter, indicating a direct link of amino acid metabolism to neurotransmission. Furthermore, significantly increased levels of tyrosine, the amino acid precursor of epinephrine, could contribute to elevated noradrenergic neurotransmission, a target of selective noradrenaline reuptake inhibitors.

Biomarker candidates for antidepressant treatment effects in the hippocampus. Biomarkers for antidepressant treatment response that indicate expected treatment efficiency at an early time point are in great demand. The metabolites identified in this study can provide a basis for future *in vivo* studies using either nuclear magnetic resonance spectroscopy, or cerebrospinal fluid and blood samples, the latter being the preferred specimen for implementation of a clinical biomarker assay.

Branched chain amino acids. Concentrations of the essential amino acids valine, leucine and isoleucine increased by 50–70% upon paroxetine treatment. Elevated levels of these amino acids, especially leucine, have been shown to increase protein synthesis through increased mammalian target of rapamycin signaling in liver, skeletal muscle, kidney and adipose tissues.[44–46] Until now, however, there is no evidence that BCAAs also induce protein synthesis in the brain. Mammalian target of rapamycin signaling, which is closely linked to synaptic plasticity,[47] was shown to be inhibited by the selective serotonin reuptake inhibitor sertraline leading to decreased, not elevated, protein synthesis,[48] seen in other tissues. Rapamycin, an inhibitor of mammalian target of rapamycin signaling exhibited antidepressant-like effects in mice and rats,[49] whereas antidepressant effects of ketamine in treatment-resistant depressed patients were attributed to activated mammalian target of rapamycin signaling.[50]

Different pathways could be affected by increased BCAA levels in the hippocampus since BCAA metabolism is directly connected to energy metabolism. Specifically, oxidative BCAA degradation leads to Krebs cycle intermediates.[51,52] Thus, alterations in energy metabolism upon paroxetine treatment could, at least in part, be influenced by altered BCAA concentrations. Furthermore, alterations in Krebs cycle intermediates were shown to result in altered neurotransmitter synthesis,[53] suggesting that increased BCAA levels could also influence synaptic transmission.

Biomarker candidates for antidepressant treatment effects in the plasma. Metabolite alterations in the plasma were less pronounced than those in the hippocampus. This finding is not unexpected as paroxetine primarily targets cerebral neurotransmission. To see whether central nervous system alterations are also reflected in the plasma, we performed a brain/plasma metabolite correlation analysis. We identified GABA, galactose-6-phosphate and leucine as biomarker candidates for antidepressant treatment effects. All three plasma biomarker candidates represent alterations also observed in the brain. Further studies need to validate these findings in humans by a targeted analysis in specimens from antidepressant treatment responders versus non-responders.

Future studies. Although we have identified a significant intensity level/phenotype correlation for a number of metabolites (Supplementary Figure S3), we cannot unambiguously associate identified pathway alterations or biomarker candidates with antidepressant therapeutic effects. To address this issue further, one line of investigation could include a pharmacological analysis of different mouse models of depression including chronic mild stress,[54] social defeat[55,56] or early-life stress through maternal separation,[57] wherein antidepressant treatment effects could be more directly associated with decreased depression-like behavior. Directly targeting the identified pathways in mouse models of depression may also help in this endeavor. In this pilot study, we have identified molecular changes upon antidepressant treatment at the metabolome level in DBA/2 mice that reflect

pathway alterations beyond monoamine reuptake inhibition. On the basis of pathway information, we revealed putative antidepressant drug targets and biomarker candidates for the assessment of antidepressant treatment effects elicited through novel modes of action.

Conflict of interest

The authors declare no conflict of interest.

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2.2 Proteomic and metabolomic profiling reveals time-dependent changes in hippocampal metabolism upon paroxetine treatment and biomarker candidates

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Declaration of contribution: Christian Webhofer designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with co-authors (for detailed information on author contributions see chapter ´Eidesstattliche Versicherung/Affidavit´).

Supplemental material is available on enclosed CD and upon request.

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Proteomic and metabolomic profiling reveals time-dependent changes in hippocampal metabolism upon paroxetine treatment and biomarker candidates

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ABSTRACT

Most of the commonly used antidepressants block monoamine reuptake transporters to enhance serotonergic or noradrenergic neurotransmission. Effects besides or downstream of monoamine reuptake inhibition are poorly understood and yet presumably important for the drugs' mode of action. In the present study we aimed at identifying hippocampal cellular pathway alterations in DBA/2 mice using paroxetine as a representative *Selective Serotonin Reuptake Inhibitor* (SSRI). Furthermore we identified biomarker candidates for the assessment of antidepressant treatment effects in plasma. Hippocampal protein levels were compared between chronic paroxetine- and vehicle-treated animals using *in vivo* ^{15}N metabolic labeling combined with mass spectrometry. We also studied the time course of metabolite level changes in hippocampus and plasma using a targeted polar metabolomics profiling platform. *In silico* pathway analyses revealed profound alterations related to hippocampal energy metabolism. Glycolytic metabolite levels acutely increased while Krebs cycle metabolite levels decreased upon chronic treatment. Changes in energy metabolism were influenced by altered glycogen metabolism rather than by altered glycolytic or Krebs cycle enzyme levels. Increased energy levels were reflected by an increased ATP/ADP ratio and by increased ratios of high-to-low energy purines and pyrimidines. In the course of our analyses we also identified myo-inositol as a biomarker candidate for the assessment of antidepressant treatment effects in the periphery. This study defines the cellular response to paroxetine treatment at the proteome and metabolome levels in the hippocampus of DBA/2 mice and suggests novel SSRI modes of action that warrant consideration in antidepressant development efforts.

1. Introduction

Major depression is one of the leading causes of morbidity and mortality worldwide. Antidepressant treatment which selectively inhibits monoamine reuptake alleviates symptoms of depression only after several weeks of medication in only a subset of patients (Berton and Nestler, 2006). Novel antidepressants with rapid onset and high treatment efficacy are in great demand. Biomarkers that facilitate prediction of treatment outcome are required in this endeavor.

Besides monoamine reuptake inhibition other downstream effects have been implicated in alleviating symptoms of depression (Moretti et al., 2003). An in-depth analysis of currently used antidepressants at the cellular and molecular level may reveal novel targets for drug development. Direct pharmacological targeting of relevant cellular pathways represents a promising strategy for the development of novel antidepressants. Non-hypothesis driven approaches – like transcriptomics, proteomics or metabolomics – can identify such cellular pathway alterations (Filiou et al., 2011) and represent suitable tools to investigate antidepressant treatment effects (Sillaber et al., 2008).

Abbreviations: FDR, False Discovery Rate; GABA, γ-Aminobutyric acid; MeOH, methanol; mg, milligram; min, minute; ml, milliliter; *m/z*, mass-to-charge ratio; PLS-DA, Partial Least Squares Discriminat Analysis; R, Pearson's correlation coefficient; RP, Reversed Phase; s, second; SAM, Significance Analysis of Microarrays; SRM, Selected Reaction Monitoring; SSRI, Selective Serotonin Reuptake Inhibitor; VIP, Variable Importance in Projection; w/v, weight/volume.

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With the help of unbiased metabolite profiling we have recently shown that chronic paroxetine treatment targets hippocampal glucose metabolism and identified antidepressant drug target candidates (Webhofer et al., 2011).

In the present study we have extended our efforts to identify cellular alterations at the proteome level after chronic paroxetine treatment using ^{15}N metabolic labeling combined with mass spectrometry. To corroborate our findings we investigated cellular metabolism by using a targeted polar metabolomics profiling platform (Yuan et al., 2012). In order to shed light on the delayed onset of therapeutic SSRI action in patients we performed time course experiments and studied acute versus chronic treatment effects on metabolic pathways. Furthermore, we have identified a biomarker candidate that can be used for monitoring hippocampal antidepressant treatment effects in the periphery.

2. Materials and methods

2.1. Paroxetine treatment and organ sampling

Male DBA/2OlaHsd mice (8 weeks old at arrival, Harlan Winkelmann, Borchen, Germany) were singly housed upon arrival for two weeks under standard laboratory conditions (food and water *ad libitum*, 12 h dark–light cycle with lights on at 7 am, 45–55% humidity, 21 ± 2 °C).

After habituation mice were treated twice per day (between 8 and 9 am and 6–7 pm) with paroxetine (10 mg/kg, paroxetine-hemihydrate, Sigma–Aldrich, St. Louis, MO, USA) or vehicle (tap water) by gavaging. Paroxetine was prepared freshly before each treatment by dissolving it in tap water (1 mg/ml). One hour after the last treatment mice were euthanized by an overdose of isoflurane (Forene®, Abbott, Wiesbaden, Germany). Blood was drawn by heart puncture and collected in EDTA tubes (Kabe Labortechnik, Nuembrecht-Elsenroth, Germany). Plasma was separated from serum by centrifugation (1300g, 10 min, 4 °C). Organs were perfused with 0.9% ice-cold saline solution (Merck, Darmstadt, Germany). Mice were decapitated, brains harvested and dissected. Plasma and hippocampi were shock frozen in liquid nitrogen and stored at −80 °C until further analysis.

The experiments were performed in accordance with European Communities Council Directive 86/609/EEC. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

2.2. Quantitative proteomics analysis

2.2.1. Protein sample preparation

Hippocampal soluble proteins were extracted according to Emili and Cox (Cox and Emili, 2006). To all biological replicates the same ^{15}N-labeled internal standard was mixed at equal protein amounts. *In vivo* ^{15}N-labeled hippocampal proteins were derived from DBA/2 mice that were raised with a ^{15}N mouse diet (Silantes GmbH, Munich, Germany) for 12 weeks (Filiou et al., 2011). One hundred μg of the $^{14}N/^{15}N$ protein mixture were separated by one-dimensional SDS gel electrophoresis. Separated proteins were fixed and stained with Coomassie Brilliant Blue R-250 (Biorad, Hercules, CA, USA). The gel was destained and each gel lane was cut into 2.5 mm slices (22–23 slices per biological replicate) and tryptic in-gel digestion and peptide extraction were performed as described previously (Filiou et al., 2011).

2.2.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Tryptic peptides were dissolved in 0.1% formic acid and analyzed with a nanoflow HPLC-2D system (Eksigent, Dublin, CA, USA) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were on-line desalted for 10 min with 0.1% formic acid at 3 μl/min (Zorbax-C18 (5 μm) guard column, 300 μm × 5 mm, Agilent Technologies, Santa Clara, CA, USA) and separated via RP-C18 (3 μm) chromatography (in-house packed Pico-frit column, 75 μm × 15 cm, New Objective, Woburn, MA, USA). Peptides were eluted with a gradient of 95% acetonitrile/0.1% HCOOH from 10% to 45% over 93 min at a flow rate of 200 nl/min. Column effluents were directly infused into the mass spectrometer via a nanoelectrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in positive mode applying a data-dependent scan switch between MS and MS/MS acquisition. Full scans were recorded in the Orbitrap mass analyzer (profile mode, m/z 380–1600, resolution $R = 60000$ at m/z 400). The MS/MS analyses of the five most intense peptide ions for each scan were recorded in the LTQ mass analyzer in centroid mode. Other MS parameters were set as described previously (Filiou et al., 2011).

2.2.3. Protein identification and quantitation

Peptides were identified by ^{14}N and ^{15}N database searches against a decoy Uniprot mouse protein database (release 2010_02) containing 119,128 entries (including forward and reverse sequences) using Sequest (implemented in Bioworks v3.3, Thermo Fisher). Enzyme specificity was set to trypsin. Mass accuracy settings were 10 ppm and 1 Da for MS and MS/MS, respectively. Two missed cleavages were allowed, carboxyamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modifications. ^{15}N peptide identification was facilitated by a variable modification of −0.9970 Da for lysine and arginine to account for the frequent shift from ^{15}N monoisotopic to most intense ^{15}N isotopomer (at 90% ^{15}N incorporation) as described previously (Zhang et al., 2009). Peptide hits were filtered at a 'False Discovery Rate (FDR)' of 1% using PeptideProphet, ^{14}N and ^{15}N database searches were combined using iProphet and protein groups were detected using ProteinProphet using default parameters (FDR < 1%)(Keller and Shteynberg, 2011).

Relative protein quantitation was performed with the ProRata software (v1.0) using default parameters and excluding ambiguous peptides (Pan et al., 2006). Log_2 ($^{14}N/^{15}N$) ratios of protein groups were then compared between biological replicates. Briefly, protein identifications were assigned to the protein group's log_2 ($^{14}N/^{15}N$) ratio. As protein identifications from different biological replicates could be assigned to distinct protein groups (due to the existence of different peptide sets identified by shotgun proteomics) we initially considered each protein identification individually for inter-experimental comparison. To avoid redundancies in final protein quantification, protein identifications with identical log_2 ($^{14}N/^{15}N$) ratios assigned to the same protein group were combined again.

2.3. Targeted metabolomics analysis

2.3.1. Polar metabolite extraction

Hippocampal tissue was ground (2 min × 1200 min^{-1}, homogenizer PotterS, Sartorius, Göttingen, Germany) in 30-fold (w/v) ice-cold 80% MeOH. Samples were centrifuged (14,000 g, 10 min, 4 °C) and supernatants incubated on dry ice. Tissue pellets were further disrupted in 6-fold (w/v) ice-cold 80% MeOH (Branson Sonifier, Branson Ultrasonics, Danbury, CT, USA) and combined with previous supernatants. Plasma metabolites were extracted twice in 80% ice-cold MeOH. Metabolite extracts were vortexed and centrifuged (14,000 g, 10 min, 4 °C). Supernatants were evaporated and stored at −80 °C until further analysis.

2.3.2. Targeted LC-MS/MS

Samples were re-suspended using 20 μl LC-MS grade water for mass spectrometry. Ten μl were injected and analyzed using

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a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX, Framingham, MA, USA) coupled to a Prominence UFLC HPLC system (Shimadzu, Columbia, MD, USA) via selected reaction monitoring (SRM) of a total of 254 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 mm i.d × 10 cm Amide Xbridge HILIC column (Waters Corp., Milford, MA, USA) at 350 μl/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0 to 5 min; 42% 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 was comprised of 20 mM ammonium hydroxide/20 mM ammonium acetate (pH = 9.0) in 95:5 water:acetonitrile. Some metabolites were targeted in both positive and negative ion modes for a total of 285 SRM transitions using positive/negative polarity switching. ESI voltage was +4900 V in positive ion mode and −4500 V in negative ion mode. The dwell time was 4 ms per SRM transition and the total cycle time was 1.89 s. Approximately 9–12 data points were acquired per detected metabolite. Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/Sciex).

2.4. Statistics and data analysis

2.4.1. Significant protein alterations

Median normalized protein group $\log_2$ (paroxetine/vehicle) was used for statistical analysis. Protein groups were considered for further analysis if they were quantified in all biological replicates. Significance Analysis of Microarrays (SAM) was used to identify significantly altered protein groups (FDR < 0.05) using MetaboAnalyst (Xia et al., 2009).

2.4.2. Significant metabolite alterations

Metabolite intensities were median normalized and Pareto scaled for statistical analysis. Missing values (10% allowed for individual metabolites) were replaced by the half of the minimum values in all replicates. Significant metabolite level changes after 24 days of treatment were identified by SAM ($p < 0.05$, FDR < 0.05). Time course analysis of median $\log_2$ (paroxetine/vehicle) was performed by hierarchical cluster analysis for metabolites that were significantly altered after chronic (24 days) treatment.

2.4.3. Protein and metabolite pathway analysis in the hippocampus

Pathway analysis was performed using the Pathway Studio software v8.0 (Ariadne Genomics, Rockville, MD, USA), which contains literature-based relations between proteins, metabolites, functional classes and cellular processes. Subnetwork detection in Pathway Studio was used to detect affected molecular pathways and chemical reactions. Cellular processes were considered affected if they were significantly enriched ($p_{\text{Benjamini–Hochberg corrected}} < 0.05$) by significantly altered proteins and at the same time were not significantly enriched by all quantified hippocampal proteins. Subnetworks were considered significantly enriched at p-value thresholds after Benjamini–Hochberg correction (Benjamini and Hochberg, 1995) as indicated.

2.4.4. Biomarker discovery in the hippocampus and plasma

To detect metabolite biomarker candidates for antidepressant treatment effects, multivariate PLS-DA analyses were performed for hippocampal metabolite intensities at individual time points (3, 7, 14 and 24 days). At 14 days of treatment one biological replicate was missing and data was imputed by average intensities of the other biological replicates. This was necessary to calculate sophisticated quality criteria for the PLS-DA model by Cross Validation. The qualities of the models were assessed in terms of accuracy, R^2 and Q^2. Biomarker candidates in hippocampus were detected by an increasing importance in the contribution to the model over time by regression analysis ($\text{VIP}_{\text{hippocampus}} > 1$ for all time points, $R > 0.6$). Univariate changes of metabolite levels were identified using Student's t-test. To detect biomarker candidates in plasma, hippocampal and plasma metabolite intensities were correlated ($p < 0.05$) and manually validated.

3. Results

3.1. Chronic paroxetine treatment alterations at the proteome level

DBA/2 mice were chronically treated with the SSRI paroxetine (2 × 10 mg/kg*day) and vehicle for 28 days. In order to quantify paroxetine treatment induced proteome changes in the hippocampus we used a quantitative proteomics platform based on ^{15}N metabolic labeling and LC-MS/MS analysis (Filiou et al., 2011; McClatchy et al., 2007; Wu et al., 2004; Zhang et al., 2011). ^{15}N-labeled proteins were used as internal standard for the indirect comparison of paroxetine- versus vehicle-treated mice. *In vivo* metabolic labeling of DBA/2 mice resulted in 90% ^{15}N incorporation at postnatal day 94 allowing for accurate protein quantitation. Using five biological replicates per group, 1366 non-redundant protein groups were quantified in at least three biological replicates per group, 1124 in at least four replicates per group and 800 protein groups were quantified in every biological replicate (Supplemental Table S1). We found increased protein quantitation accuracy for proteins that were quantified in every biological replicate that correlated with increased quantified peptide numbers per protein (Supplemental Table S2, Figure S1). For increased robustness of subsequent data analysis we only considered protein groups quantified in every biological replicate because overall protein concentration changes were relatively small (Supplemental Figure S2). Using *Significance Analysis of Microarrays* (SAM) we identified 54 significant protein group alterations (FDR < 0.05, 24 increased and 30 decreased after paroxetine treatment) (Supplemental Table S3, Figure S3).

Significantly enriched cellular processes were identified using the *Pathway Studio* software as described in 'Materials and Methods'. Significant pathway alterations ($p_{\text{Benjamini–Hochberg corrected}} < 0.05$) included *glycogen metabolism* (NOS1, GAA, PYGM), *gametogenesis* (NOS1, GLUL, UCHL1), *membrane fluidity* (GLUL, ANXA4, SNCA) and *contraction* (CRYAB, NOS1, MYH9, PDE1A, GIT1, MSN, MGEA5, SNCA, PYGM). In a previous study applying identical treatment conditions we have identified profound increases in early glycolytic metabolites (Webhofer et al., 2011). Alterations in glycolysis, however, were not reflected by altered glycolytic enzyme levels. We therefore decided to further interrogate paroxetine treatment induced downstream pathways by complementary metabolomics analyses targeted at cellular metabolism.

3.2. Time course paroxetine treatment effects on cellular metabolism

We performed an independent paroxetine treatment time course analysis. Mice were treated with paroxetine (2 × 10 mg/kg*day) and vehicle for 3, 7, 14 and 24 days, respectively to track acute, sub-chronic and chronic pathway alterations. A metabolomics platform specific for the analysis of cellular metabolism was applied to five biological replicates per time point. Two hundred thirty metabolites were relatively quantified of which 209 were KEGG annotated (Supplemental Table S4, Figure S4).

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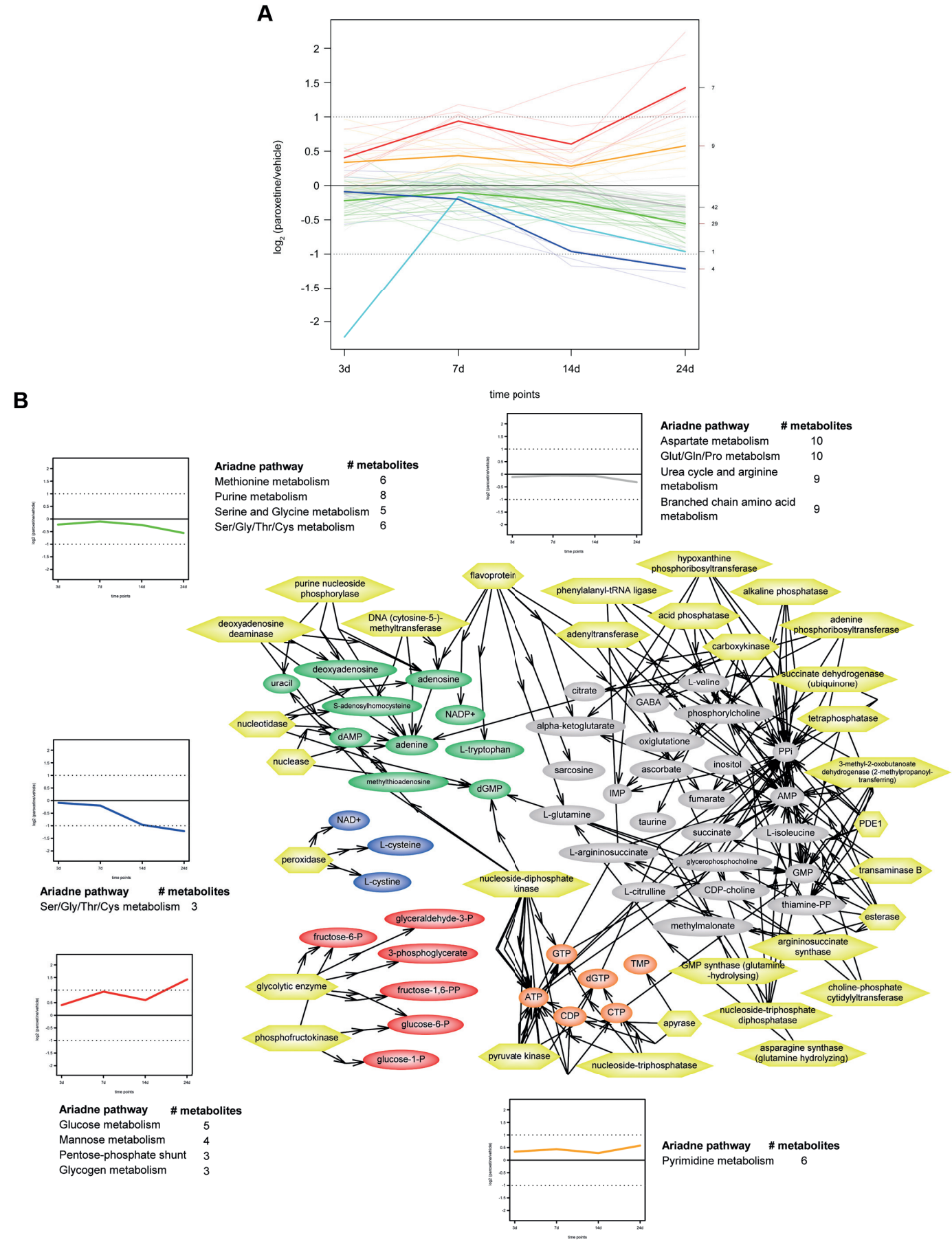

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Significantly altered metabolites after chronic (24 days) paroxetine treatment were identified using SAM. Ninety two metabolites were considered significant ($p < 0.05$, FDR < 0.05, 17 increased and 75 decreased after paroxetine treatment) (Supplemental Table S5, Figure S5). Hierarchical clustering of the time course data of chronically altered metabolites revealed six metabolite clusters with distinct temporal patterns (Fig. 1A). As expected, correlating metabolites were linked to common pathways after performing individual pathway analyses for metabolite time course clusters (Fig. 1B).

3.2.1. Increased aerobic glycolysis

After chronic treatment, levels of all metabolites resulting from the initial glycolytic steps were significantly increased up to 5-fold (glucose-1P: 2.4-fold, glucose-6P: 2.1-fold, fructose-6P: 2-fold, fructose-1,6PP: 3.8-fold, glyceraldehyde-3P: 4.7-fold and 3-P-glycerate: 2.6-fold). These represent the most severe alterations in the present dataset. Time course analyses indicated that these metabolites cluster together and alterations were detectable within several days of treatment (Fig. 2A).

3.2.2. Decreased Krebs cycle

Krebs cycle intermediates clustered together and paroxetine treatment effects were delayed. Interestingly, all Krebs cycle intermediates were decreased after 24 days of treatment contrasting increased glycolytic metabolite levels (Fig. 2B).

3.2.3. Altered glycogen metabolism

Increased glycolytic and decreased Krebs cycle metabolite levels were not reflected by altered glycolytic and Krebs cycle enzyme levels, respectively (Fig. 2). This phenomenon can be explained by increased glucose uptake or increased glycogen degradation which can both result in changes in glycolysis without affecting enzyme levels. Indeed, our results indicate that the cellular process "glycogen metabolism" was affected upon paroxetine treatment ($p < 0.05$). "Glycogen phosphorylase" was identified as significant regulator of metabolite alterations ($p < 0.05$) and glycogen itself tended to be affected by altered proteins ($p = 0.08$). Five altered proteins were significantly linked to altered metabolites (glutamine synthetase, glycogen phosphorylase, nitric oxide synthase 1, phosphoenolpyruvate carboxykinase 2, protein phosphatase 1 regulatory subunit 1B, $p < 0.05$). Remarkably, four of the five proteins were involved in glycogen metabolism. This suggests that altered glycogen metabolism constitutes a potential link between the observed protein and metabolite alterations (Fig. 3).

3.2.4. Increased energy levels and altered redox state

We further investigated the ATP/ADP ratio, a measure of energy state, and the NAD^+/NADH ratio, a measure of redox potential. The ATP/ADP ratio was increased ($p < 0.05$) whereas the NAD^+/NADH ratio tended to decrease ($p < 0.10$) after chronic treatment. NAD^+ levels negatively correlated with ATP and NADH levels (Fig. 4). In concordance with increased energy levels the ratios of high-to-low energy adenosine phosphates were elevated after chronic paroxetine treatment (ATP/ADP/AMP). Adenosine phosphate levels highly correlated with guanosine, uridine and cytidine phosphate levels (Supplemental Figure S6) and the ratios GTP/GDP/GMP, UTP/UDP/UMP and CTP/CDP/CMP exhibited a similar pattern compared to ratios of ATP/ADP/AMP (Supplemental Figure S7).

3.3. Biomarker candidates in the periphery

For the identification of predictive biomarker candidates for paroxetine treatment response we performed multivariate data analysis based on hippocampal metabolite intensities for all time points (3, 7, 14 and 24 days). Quality criteria of the PLS-DA models improved over time indicating that paroxetine treatment exerts its effect on hippocampal metabolism chronically (Fig. 5A). Metabolites with VIP > 1 at all time points were considered important contributors to multivariate group separations. Importantly, these metabolites indicated chronic changes already after 3 days of treatment. Moreover, a specific cluster of these metabolites showed increasing VIP scores over time (2-isopropylmalic acid, acetylcarnitine, AMP, betaine, carbamoyl phosphate, glutamine, glutathione, glycerophosphocholine, IMP, myo-inositol, phosphorylcholine, taurine; Pearson's correlation coefficient $R > 0.6$, Fig. 5B). Metabolites with increasing significance in PLS-DA models and prediction accuracy were considered important effectors of paroxetine treatment induced alterations. This is further supported by the fact that these metabolites exhibited significant univariate level differences after chronic paroxetine treatment (eleven of twelve metabolites $p < 0.05$, gluathione $p < 0.10$) (Fig. 5B). Comparison of hippocampal and plasma metabolite levels revealed a significant correlation for myo-inositol ($p < 0.05$) (Fig. 5C).

4. Discussion

We have for the first time combined proteomic and metabolomic analyses to unravel the *in vivo* effects of antidepressant treatment at the cellular systems level. *In silico* pathway analyses revealed altered energy metabolism as a key element of paroxetine treatment effects. Importantly, we identified an energy metabolism shift toward aerobic glycolysis. In support of increased aerobic glycolysis we found (1) acutely increased glycolytic metabolite levels, (2) chronically decreased Krebs cycle metabolite levels, (3) increased ATP/ADP ratios, and (4) decreased NAD^+/NADH ratios. Altered glycogen metabolism represents a putative link between protein and metabolite changes. Increased energy levels correlated with elevated purine- and pyrimidine triphosphate levels that in turn may be involved in modulating depression-like behavior as has been reported previously (Moretti et al., 2003; Renshaw et al., 2001).

For unraveling molecular antidepressant treatment effects we focused on paroxetine as a representative SSRI in the inbred DBA/2 mouse strain. We aimed at elucidating downstream effects besides serotonin reuptake inhibition that are involved in antidepressant action (Berton et al., 2006). By using inbred mice under standard laboratory and treatment conditions we significantly reduced biological variability for a better signal-to-noise ratio of paroxetine-induced changes. Using a standardized proteomics platform based on ^{15}N metabolic labeling and mass spectrometry we accurately quantified paroxetine treatment effects for 800 proteins. Proteomics data were complemented by targeted profiling of hippocampal metabolism. We investigated the time course of metabolic alterations to mimic long-term treatment effects in patients.

We observed a profound increase in early glycolysis metabolite levels within several days of treatment. These data extend our previous findings that chronic paroxetine treatment increases early

Fig. 1. Paroxetine treatment metabolite pathway analysis. DBA/2 mice were treated with paroxetine (2 × 10 mg/kg*day) or vehicle for 3, 7, 14 and 24 days, respectively. (A) Significantly altered metabolites after 24 days were identified by 'Significance Analysis of Microarrays' ($p < 0.05$, FDR<0.05) and hierarchical clustering of the time course data performed. The size of each metabolite cluster is shown. (B) Chemical reaction analysis for individual metabolite clusters was performed using subnetwork detection with Pathway Studio v8.0 ($p < 0.01$, Benjamini–Hochberg adjusted). Time course and number of metabolites in Ariadne pathways are presented.

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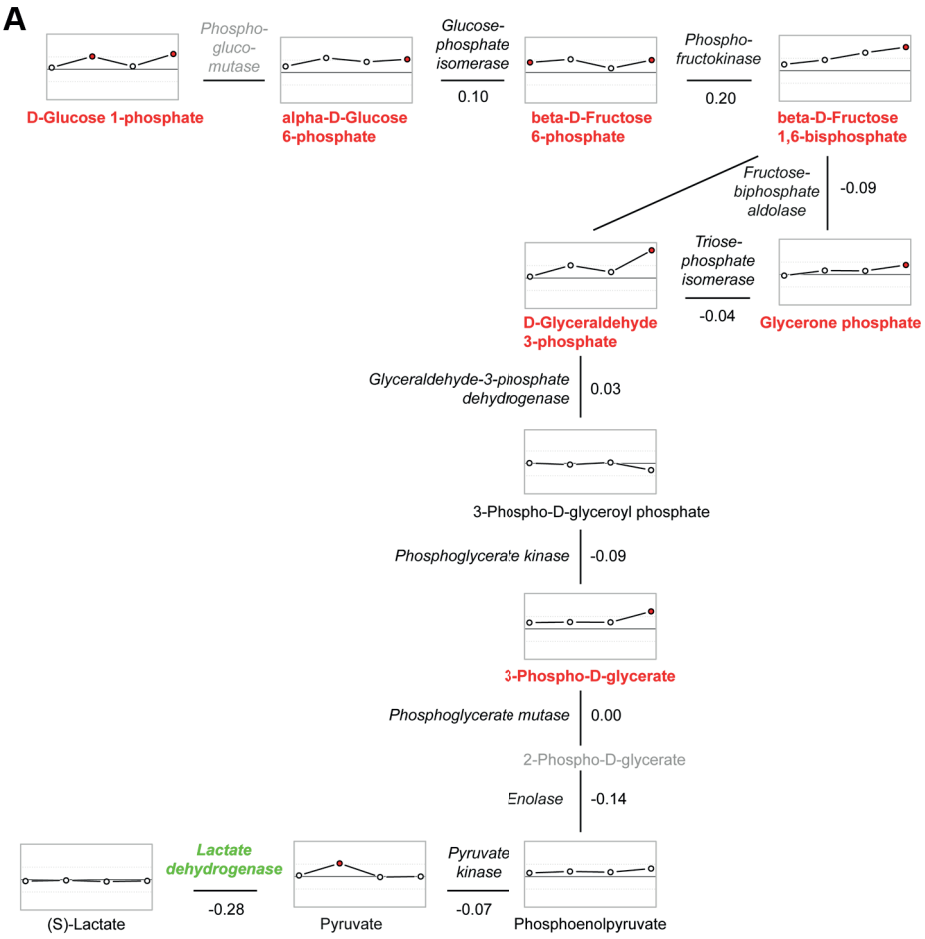

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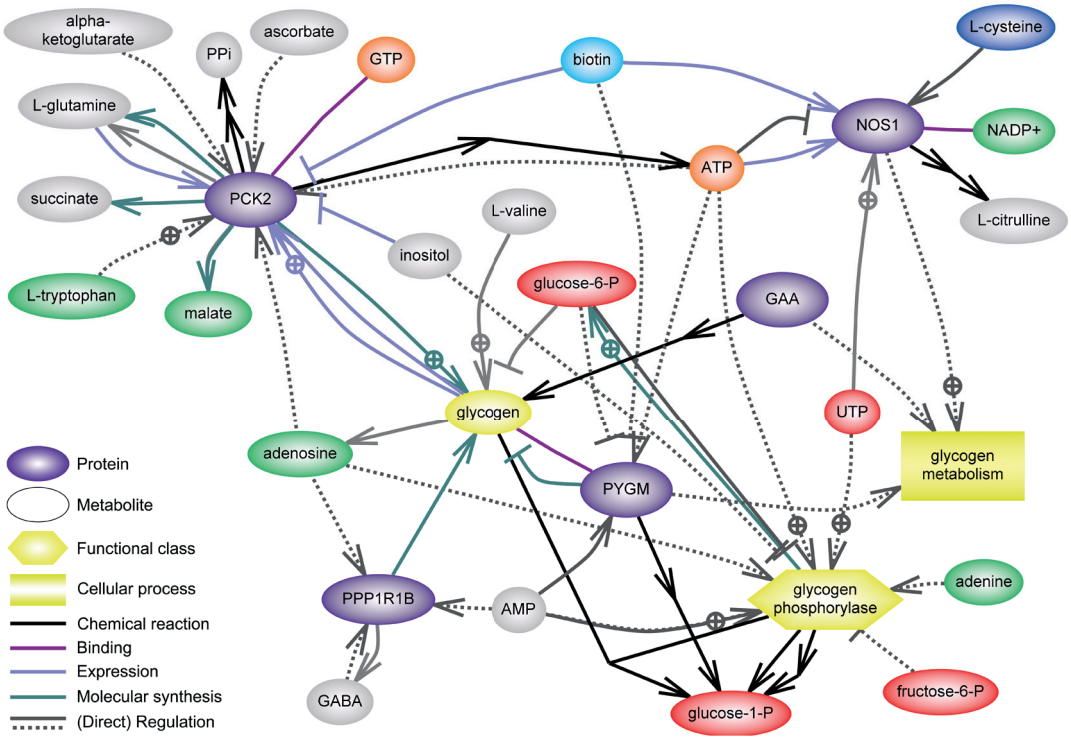

Fig. 3. Glycogen metabolism. Altered glycogen metabolism was identified as link between significant protein and metabolite alterations (FDR<5%) upon paroxetine treatment. Significantly enriched subnetworks were identified using Pathway Studio v8.0. Metabolite subnetworks: nitric oxide synthase 1 (NOS1), phosphoenolpyruvate carboxykinase 2 (PCK2), protein phosphatase 1 regulatory subunit 1B (PPP1R1B), glycogen phosphorylase (PYGM) ($p < 0.05$); Protein subnetworks: glycogen metabolism ($p < 0.05$) and glycogen ($p = 0.08$). p-Values are corrected for multiple testing using the Benjamini–Hochberg procedure.

glycolytic metabolite levels (Webhofer et al., 2011). At the same time we found decreased Krebs cycle intermediate levels after chronic paroxetine treatment. An energy metabolism shift toward aerobic glycolysis has been described in cancer cells by Otto Warburg (Warburg et al., 1924). The "Warburg effect" describes excess utilization of glucose under aerobic conditions to produce lactate (Koppenol et al., 2011). In our case lactate dehydrogenase B levels were reduced upon paroxetine treatment which could provide an explanation for the unaltered lactate levels in paroxetine-treated mice despite an increased aerobic glycolysis (Fig. 2A).

Proliferating cells utilize aerobic glycolysis for the generation of nucleotides, amino acids or lipids needed to produce new cells (Vander Heiden, Cantley, & Thompson, 2009). Neural cells also extensively utilize glucose for aerobic glycolysis to reduce apoptosis and increase long-term neuronal survival (Vaughn and Deshmukh, 2008). Aerobic glycolysis facilitates catabolic processes via the "Pentose Phosphate Shunt" that was shown to be affected in our study (Fig. 1B). Elevated aerobic glycolysis in SSRI-treated mice may be responsible for facilitating neurogenesis which has been repeatedly shown to be required for antidepressant effects (Santarelli et al., 2003). Cells with increased aerobic glycolysis have been reported to exhibit high ATP/ADP and low NAD^+/NADH ratios, both also found in the present study (Fig. 4) (DeBerardinis et al., 2008). The fact that ATP/ADP and NAD^+/NADH ratios were altered only after chronic treatment could explain the delayed onset of therapeutic action in a clinical setting.

Reduced levels of the inhibitory neurotransmitter GABA point toward increased neuronal activity after paroxetine treatment. Neuronal activity induces glucose uptake and astrocytic glycolysis, but not oxidative phosphorylation (Fox et al., 1988). Glycolysis predominantly takes place in astrocytes to fuel energy demands of astrocytic Na^+/K^+ ATPase activity, the main energy consuming process in neural cells (Hertz et al., 2007). Two recent studies revealed differential regulation of aerobic glycolysis in distinct brain areas in healthy human brain (Vaishnavi et al., 2010) and specific alterations in dementia of the Alzheimer type (Vlassenko et al., 2010). The present study indicates for the first time that shifting energy metabolism toward aerobic glycolysis might provide a venue for an effective and fast acting antidepressive treatment.

We have found elevated glucose 1-phosphate levels suggesting that increased glycolytic metabolite levels derive from elevated glycogenolysis and identified altered glycogen metabolism as potential link between protein and metabolite changes (Fig. 3). Glycogenolysis was shown to be induced at high energy demand (Magistretti et al., 2000). Although brain glycogen levels are comparably small, a significant amount is found in astrocytes (Gruetter, 2003) further indicating an important role of astrocytes in antidepressant action (Iwata et al., 2011). Strikingly, Allaman et al. (Allaman et al., 2011) showed that fluoxetine and paroxetine treatment reduced glycogen levels, increased glucose utilization and lactate release by astrocytes. There is increasing evidence that

Fig. 2. Energy metabolism alterations. (A) Glycolysis (B) Krebs cycle. DBA/2 mice were treated with paroxetine (2 × 10 mg/kg*day) or vehicle. Metabolite $\log_2$ (paroxetine/vehicle) ratios (3, 7, 14 and 24 days) are depicted. Solid lines indicate $\log_2 = 0$, dashed lines indicate $|\log_2| = 1$. Protein $\log_2$ (paroxetine/vehicle) ratios (28 days) are shown. Red: Significant upregulation, green: significant downregulation (FDR < 0.05) after chronic treatment.

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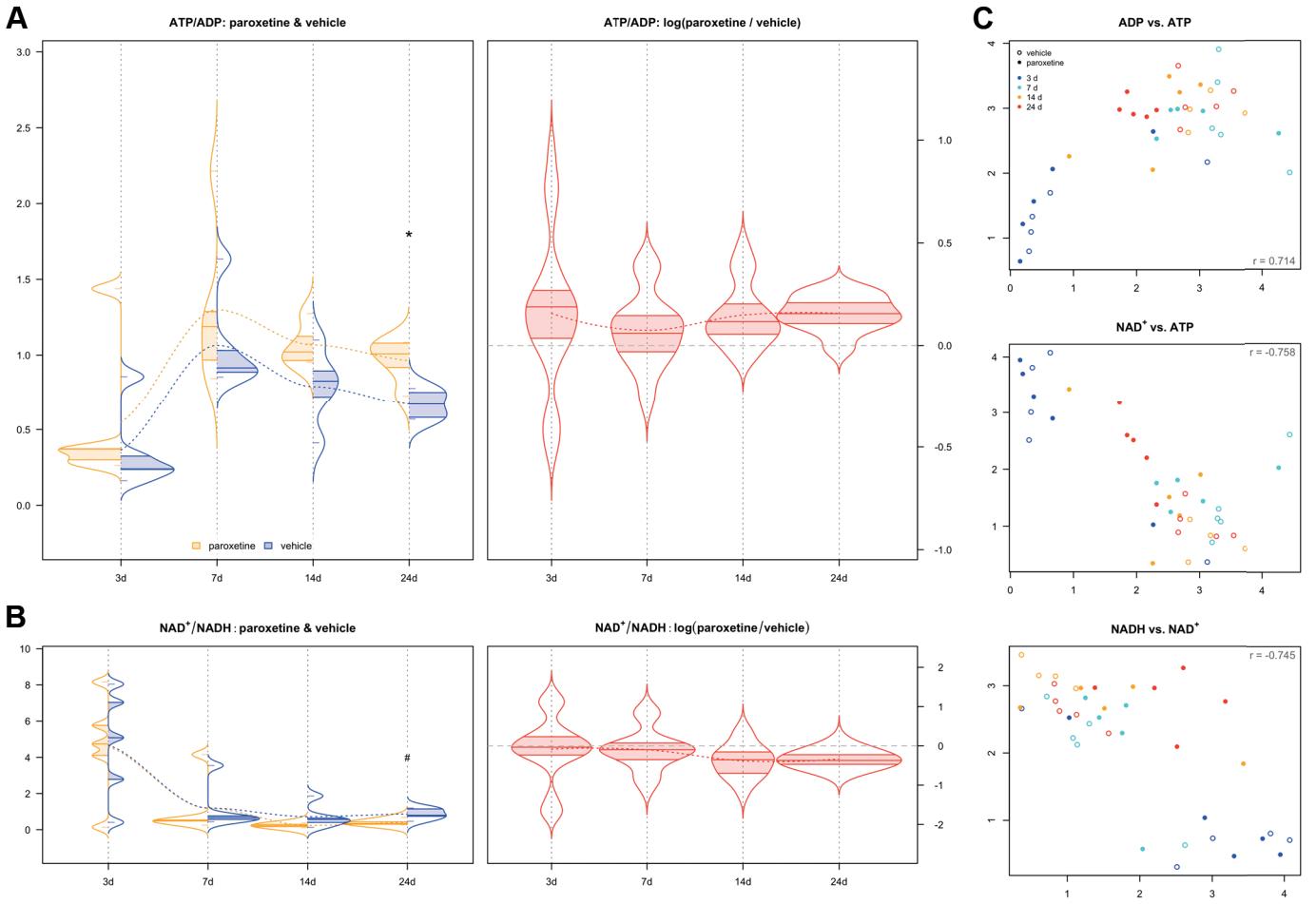

Fig. 4. Energy levels and redox state upon paroxetine treatment. Violine plots illustrating ratios (paroxetine/vehicle) of (A) ATP levels, ADP levels and ATP/ADP ratios (B) NAD^+ levels, NADH levels and NAD^+/NADH ratios after 3, 7, 14 and 24 days treatment; asterisks denote significant differences versus vehicle treatment as determined by two-way ANOVA followed by Student's *t*-test: $^{\#}p < 0.1$; $^{*}p < 0.05$, Benjamini–Hochberg adjusted. (C) Correlation analysis for ADP vs. ATP, NAD^+ vs. ATP and NADH vs. NAD^+; Pearson's correlation (*r*) values are indicated within each graph.

astrocytes influence synapse development, synaptic plasticity and long-term potentiation (Paixao and Klein, 2010) and are involved in the pathophysiology of major depression (Banasr and Duman, 2008). Thus, astrocytic energy metabolism represents a potential antidepressant drug target that warrants further investigation.

We identified several alterations that are closely related to mitochondrial function including the Krebs cycle and increased energy levels. Several studies identified direct effects of antidepressive treatment on mitochondrial functionality apart from serotonergic neurotransmission. Activities of mitochondrial electron transfer chain complexes were inhibited in isolated mitochondria upon clomipramine, desipramine and norfluoxetine treatment (Abdel-Razaq et al., 2011). Citrate synthase and succinate dehydrogenase enzyme activities were increased upon antidepressant treatment in diverse model systems (Scaini et al., 2011). Fluoxetine treatment of isolated rat brain mitochondria interfered with ATPase activity and decreased mitochondrial ATP production (Curti et al., 1999). In line with reduced oxidative energy production, oxygen consumption was reduced upon imipramine and clorgyline treatments (Nag and Nandi, 1991). Taken together, mitochondria appear to be targets for a wide range of antidepressants. Future studies need to investigate whether these mitochondrial alterations are related to therapeutic actions or side effect profiles of distinct antidepressant drugs.

We observed profound alterations in purine and pyrimidine metabolism. Ratios of high-to-low energy purines and pyrimidines increased after chronic treatment suggesting an involvement of a delayed therapeutic treatment effect. Several purines and pyrimidines act as neuroactive substances and therefore may influence neurotransmission besides the monoaminergic system. Extracellular ATP acts on ligand-gated P2X and G protein-coupled P2Y receptors. UTP, ADP and UDP-glucose act on P2Y receptors whereas adenosine acts on P1 receptors (Khakh and North, 2006). Physiological effects of P2 receptor signaling involve changes in proliferation and apoptosis (Ciccarelli et al., 2001), neuronal maturation, neural outgrowth (Swanson et al., 1998) and the expression of transmitter receptors on target cells (Choi et al., 2003). ATP released from astrocytes may modulate neuronal and synaptic activity (Khakh, 2001) and purinergic signaling modulates neuron–glia interactions (Fields and Burnstock, 2006). Purinergic signaling has been associated with panic disorder (Lam et al., 2005), anxiety disorders (Erhardt et al., 2007) and fear behavior (Corodimas and Tomita, 2001). P2X receptors modulate the release of neurotransmitters like noradrenaline, GABA and glutamate (Gu and MacDermott, 1997; Hugel and Schlichter, 2000) that play important roles in antidepressant action (Krystal et al., 2002). The P2X7 receptor gene was shown to be involved in major depression (Lucae et al., 2006). Moreover, increased ATP levels are present in antidepressant treatment responders compared to non-responders

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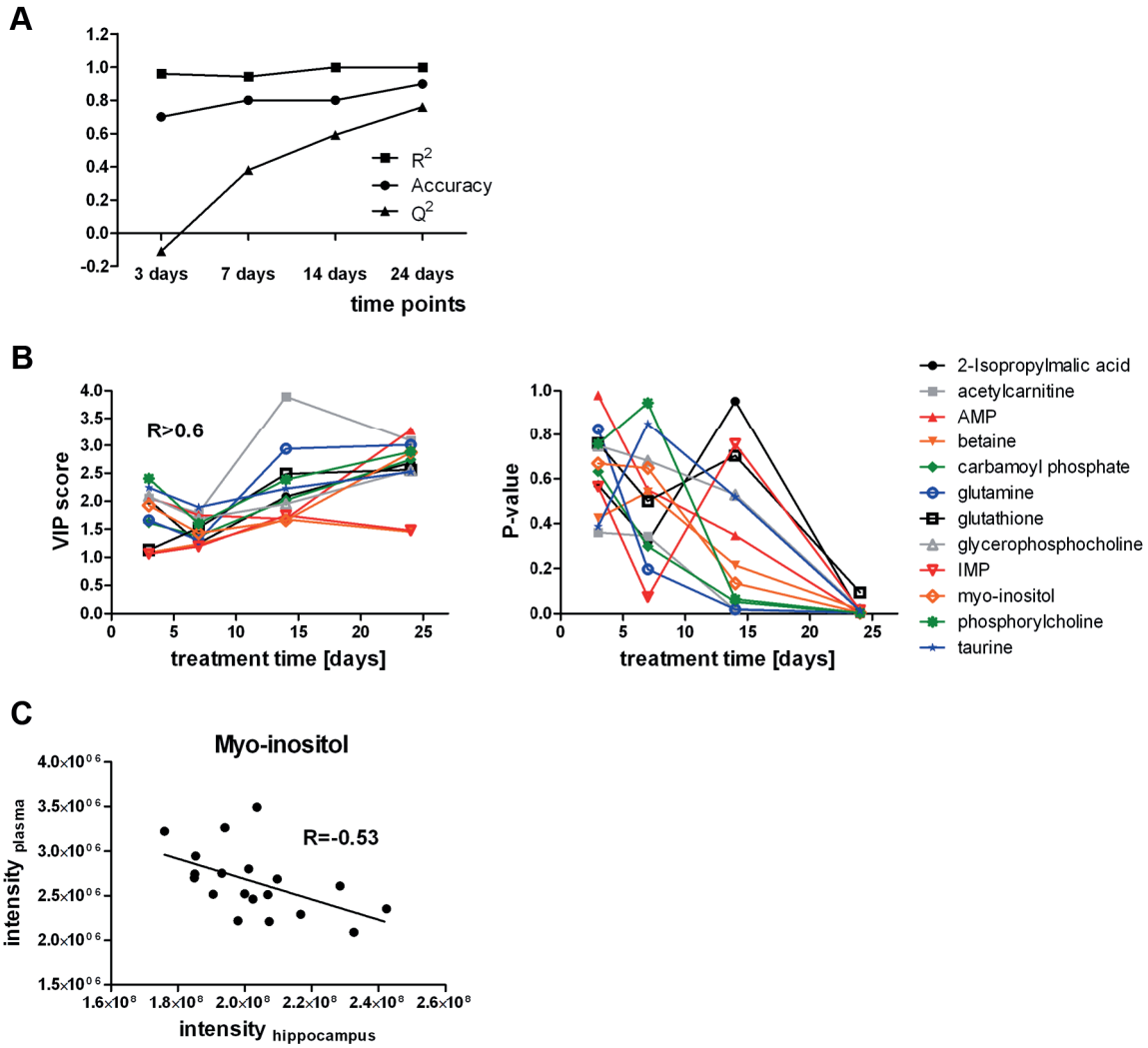

Fig. 5. Biomarker discovery. (A) Multivariate data analysis was performed using Partial Least Squares Discriminant Analysis (PLS-DA). Quality criteria (accuracy, R^2, Q^2) of the PLS-DA models improved over the time course of treatment. (B) VIP score and p-value time courses are shown for hippocampal biomarker candidates (VIP>1 for all time points; increasing VIP scores, Pearson's correlation coefficient $R > 0.6$). (C) Plasma metabolite intensities are plotted versus hippocampal metabolite intensities for biomarker candidates with significant hippocampus/plasma correlation ($p < 0.05$).

(Iosifescu et al., 2008) and it has been suggested that increased purine levels may exert antidepressant effects (Renshaw et al., 2001).

In addition to studying hippocampal antidepressant treatment effects we identified a metabolite that reflects cerebral alterations in the periphery. We focused on the identification of potential markers that allow prediction of chronic hippocampal treatment effects and identified myo-inositol as a predictive biomarker candidate in plasma. Noteworthy, myo-inositol is also found in urine which can be used as a source for future biomarker studies in patients. Based on its biology myo-inositol represents a very interesting biomarker candidate. On one hand it is involved in second messenger signaling upon serotonergic neurotransmission, the primary target of paroxetine treatment. At the same time myo-inositol is closely linked to glucose and glycogen metabolism, pathways we found affected upon paroxetine treatment in the hippocampus.

In the present study we have identified paroxetine treatment effects in DBA/2 mice providing evidence for an involvement of energy metabolism as a key mechanism at the cellular pathway level. For a more detailed understanding of paroxetine treatment effects future studies need to investigate which alterations are induced by serotonin-dependent or serotonin-independent mechanisms. Also, other antidepressants including monoamine oxidase inhibitors, selective noradrenaline reuptake inhibitors and tricyclic agents as well as different mouse models such as chronic mild stress (Willner et al., 2005), social defeat (Kudryavtseva et al., 1991) and early-life stress via maternal separation (Millstein et al., 2007) ought to be considered for this purpose. Eventually, a better understanding of current antidepressive treatment effects will allow the development of tailored antidepressants with novel modes of action.

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Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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2.3 The ^{15}N isotope effect as a means for correlating phenotypic alterations and affected pathways in a trait anxiety mouse model

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* equal contribution

Declaration of contribution: Christian Webhofer designed the study, performed research, analyzed the data and wrote the manuscript in collaboration with co-authors (for detailed information on author contributions see chapter ´Eidesstattliche Versicherung/Affidavit´).

Supplemental material is available on enclosed CD and upon request.

RESEARCH ARTICLE

The ^{15}N isotope effect as a means for correlating phenotypic alterations and affected pathways in a trait anxiety mouse model

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Stable isotope labeling techniques hold great potential for accurate quantitative proteomics comparisons by MS. To investigate the effect of stable isotopes in vivo, we metabolically labeled high anxiety-related behavior (HAB) mice with the heavy nitrogen isotope ^{15}N. ^{15}N-labeled HAB mice exhibited behavioral alterations compared to unlabeled (^{14}N) HAB mice in their depression-like phenotype. To correlate behavioral alterations with changes on the molecular level, we explored the ^{15}N isotope effect on the brain proteome by comparing protein expression levels between ^{15}N-labeled and ^{14}N HAB mouse brains using quantitative MS. By implementing two complementary in silico pathway analysis approaches, we were able to identify altered networks in ^{15}N-labeled HAB mice, including major metabolic pathways such as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Here, we discuss the affected pathways with regard to their relevance for the behavioral phenotype and critically assess the utility of exploiting the ^{15}N isotope effect for correlating phenotypic and molecular alterations.

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1 Introduction

Stable isotope labeling techniques have provided quantitative proteomics with a methodological toolbox that ensures high accuracy and precision. In vivo metabolic labeling approaches such as stable isotope labeling in cell culture (SILAC) [1] and stable isotope labeling of mammals (SILAM) [2] enable proteome comparisons at a systemic or organismal level. ^{15}N metabolic labeling has been applied to a wide variety of organisms [3, 4] and is a robust proteomics-based platform for biomarker discovery [5].

We have previously applied ^{15}N metabolic labeling to an animal model of trait anxiety in order to compare the proteomes of high anxiety-related behavior (HAB) versus low anxiety-related behavior (LAB) mice [6, 7]. The HAB/LAB mouse model is based on selective bidirectional inbreeding of mice for behavioral extremes according to their performance

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Abbreviations: EPM, elevated plus-maze; **HAB**, high anxiety-related behavior; **IQR**, interquartile range; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **KO**, KEGG orthologue; **LAB**, low anxiety related behavior; **PND**, postnatal day; **SILAM**, stable isotope labeling of mammals; **TCA**, tricarboxylic acid cycle; **TPP**, trans-proteomic pipeline; **TST**, tail suspension test

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on the elevated plus-maze (EPM), a behavioral paradigm assessing anxiety-related behavior. Mice that spent most of their time in the closed arms gave rise to the HAB line whereas mice that spent most of the time in the open arms gave rise to the LAB line [8, 9]. Selective bidirectional inbreeding led to an enrichment of the genetic material related to the anxiety phenotype, while retaining similarity in nonselected traits [10, 11].

Anxiety disorders are the most common psychiatric disorders [12] and are highly comorbid with depression [13]. Intriguingly, we observed that the introduction of the ^{15}N isotope had an antidepressant-like effect on the behavioral phenotype of HAB mice [14].

Although the ^{15}N isotope effect has no clinical relevance for depression treatment, the analysis of molecular correlates of this effect may shed light on biological mechanisms involved in depression-like behavior. We have investigated the ^{15}N isotope effect by comparing the brain proteomes of ^{15}N-labeled and ^{14}N HAB mice. For an in-depth analysis of the ^{15}N isotope effect, we generated ^{15}N metabolically labeled HAB mouse populations that were fed with two different ^{15}N-enriched diets. Several subproteomes from different brain regions were then subjected to quantitative mass spectrometry (MS) analysis.

Since it is widely believed that affected networks rather than individual proteins are responsible for complex brain disorder phenotypes, we followed up our proteomics analyses with an extensive in silico pathway investigation. Our results show that the introduction of the ^{15}N isotope results in expression level changes of proteins involved in molecular pathways that are critical for key organismal functions such as energy metabolism, presumably leading to the behavioral changes observed in the ^{15}N-labeled HAB mice.

2 Materials and methods

2.1 Animals

HAB mice were housed under standard conditions (12 h light/dark cycle, lights on at 6 am, room temperature 23 ± 2°C, humidity 60%, tap water, and food ad libitum) in the animal facility of the Max Planck Institute of Psychiatry. Only male mice were studied. The animal experiments were approved by local authorities and conducted according to current regulations for animal experimentation in Germany and the European Union (European Communities Council Directive 86/609/EEC).

2.2 ^{15}N Metabolic labeling of HAB mice

^{15}N metabolic labeling, breeding, behavioral testing, and tissue acquisition were performed as described previously [14]. In brief, HAB mice were labeled with ^{15}N starting in utero upon pregnancy detection and for 8 weeks post partum by receiving an ^{15}N-labeled diet either based on *Spirulina* (Harlan Laboratories, Andover, MA, USA) or *Ralstonia eutropha* (U-15N-SILAM-Mouse, Silantes, Munich, Germany) [14]. To avoid any diet-specific effects on the proteome, the unlabeled HAB mice used for the proteomics comparisons, referred to as ^{14}N HAB mice, received an unlabeled (^{14}N) *Spirulina*-based (Harlan Laboratories) or *Ralstonia eutropha* based (U-14N-SILAM-Mouse, Silantes) diet, respectively, with the same composition as the corresponding ^{15}N diet.

Both ^{14}N and ^{15}N-labeled HAB mice were bred simultaneously and subjected to the same feeding protocol. To assess anxiety-related behavior, ultrasonic vocalization (USV) and EPM were performed on postnatal day (PND) 5 and 49 [14], respectively. To assess depression-like behavior, the tail suspension test (TST) was performed on PND 51. Brain dissection for cingulate cortex, cerebrum, cerebellum, and hippocampus was performed according to the mouse brain atlas [15]. ^{15}N-labeling efficiency at different developmental time points was estimated in the cerebellum with the *QuantiSpec* software [16].

2.3 Experimental set up

From *Spirulina*-fed HAB animals, cerebrum cytosol was analyzed. From *Ralstonia eutropha* fed HAB animals, cingulate cortex synaptosomes and hippocampus cytosol were analyzed. An overview of the specimens used for MS analyses is provided in Table 1. The cerebrum cytosol was used to assess protein expression level alterations in brain tissue. In addition, we analyzed brain areas related to anxiety and fear processing (i.e. hippocampus and cingulate cortex). These analyses included hippocampus cytosol and cingulate cortex synaptosomes. The latter was chosen due to its central role for neurotransmission. To ensure that the ^{15}N isotope effect is independent of the ^{15}N protein source, two different ^{15}N-enriched diets were used. For each data set, three ^{14}N HAB/^{15}N HAB animal pairs were compared by quantitative MS. The study design details are shown in Fig. 1.

2.4 Proteomics sample preparation

Cytosol from cerebrum and hippocampus was obtained according to [17]. Synaptosomes from cingulate cortex were enriched as described previously [18]. For each animal pair, 100 μg subproteome fraction from the corresponding ^{14}N/^{15}N mixture was resolved by SDS gel electrophoresis. In-gel digestion and peptide extraction were performed as described elsewhere [14]. Peptides were lyophilized, and each fraction was dissolved in 10 μL 1% formic acid.

2.5 MS

Five microliters per SDS gel fraction were analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) using a nanoflow HPLC-2D system (Eksigent, Dublin, CA, USA) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific,

Table 1. Proteomics data sets used for the ^{15}N isotope effect analysis

HAB specimen	Subproteome	^{15}N-enriched diet	Biological replicates (No. of $^{14}N/^{15}N$ pairs analyzed)
Cingulate cortex	Synaptosomes	*Ralstonia eutropha*	3
Hippocampus	Cytosol	*Ralstonia eutropha*	3
Cerebrum	Cytosol	*Spirulina*	3

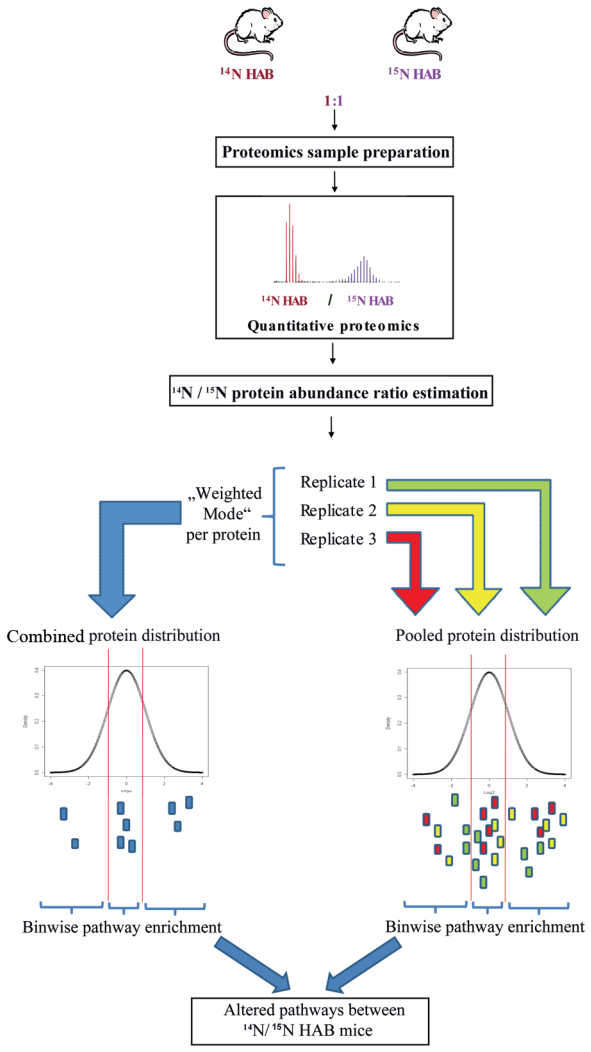

Figure 1. Experimental study design. Experimental workflow for each data set analyzed. ^{14}N- and ^{15}N-labeled homogenates were mixed at a 1:1 ratio based on protein content. The protein population of interest was enriched and separated by SDS gel electrophoresis. After in-gel digestion and peptide extraction, the $^{14}N/^{15}N$ peptide extracts were subjected to LC-ESI-MS/MS and in silico pathway analyses. The proteomics data were analyzed by two in silico pathway-based methods. Only significantly altered pathways found with both methods after multiple testing correction were considered affected.

Bremen, Germany). The mass spectrometer was operated in the positive ion mode using a data-dependent automatic scan switch between MS and MS/MS acquisition. Full scans were recorded in the Orbitrap mass analyzer at a mass range of 380–1600 m/z and a resolution of R = 60 000 (m/z 400) in profile mode. The MS/MS analysis of the five most intense peptide ions for each scan (top 5) was recorded in the LTQ mass analyzer in centroid mode. All other parameters were as described previously [18].

2.6 Quantitative proteomics data analysis

MS raw files were searched twice against an ^{14}N and an ^{15}N decoy Uniprot mouse protein database (release 2010_02) utilizing BioWorks (version 3.3.1) (Thermo Fischer Scientific, San Jose, CA) and SEQUEST (version 28) (Thermo Fischer Scientific). Peptide and fragment ion mass tolerance were set to 20 ppm and 1 Da, respectively. Trypsin was chosen as enzyme and up to two missed cleavage sites and only fully tryptic peptides were allowed. Cysteine carboxyamidomethylation was used as a static and methionine oxidation as a variable modification. To facilitate ^{15}N peptide identification, a variable modification of –1 Da for lysine and arginine was introduced to account for the frequent shift from the monoisotopic ^{15}N to the most intense ^{15}N isotopomer triggered for MS/MS [19]. Peptide hits were filtered at a false discovery rate of 1% using PeptideProphet [20]. ^{14}N and ^{15}N database searches were combined using iProphet [21] in the trans-proteomic pipeline (TPP) [22]. Relative peptide quantification was performed with the *ProRata* software (version 1.0) using default parameters [23]. Protein quantification was done by a density estimation approach, which allows weighing replicates by the number of identified peptides and the quality of the protein-specific peptide distribution. The S/N ratio of each peptide was used to estimate a density distribution per peptide. The peptide density distributions per protein were mixed and the protein abundance ratio was determined using the point with the highest probability content. This approach has a similar success as the *ProRata* point estimation (Supporting Information Table S1). The biological replicates of $^{14}N/^{15}N$ $\log_2$ ratios of a protein were then combined by weighing each protein abundance ratio using its probability content. The probability content for each replicate is a result of the protein-specific peptide distribution, the number of peptides and their corresponding S/N ratios (Supporting Information Fig. S1). For details see Supporting Information.

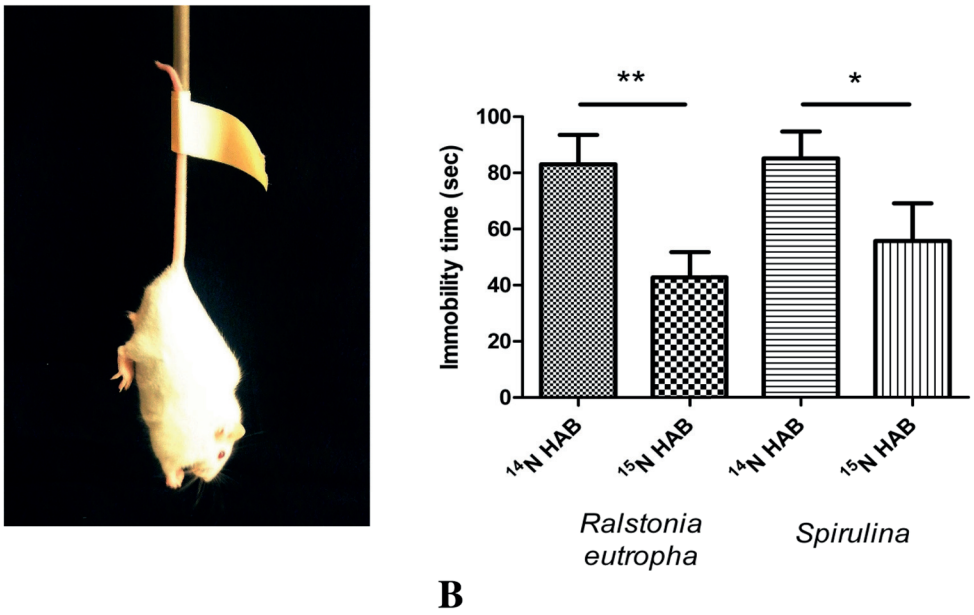

Figure 2. Behavioral alterations in ^{15}N-labeled HAB mice. (A) The tail suspension test (TST) behavioral paradigm. Mice are hung by their tails for 6 min and their immobility time, which is indicative of depression-like behavior, is measured (kindly provided by Markus Nussbaumer, Max Planck Institute of Psychiatry). (B) Reduced depression-like behavior as indicated by the immobility time in TST in ^{15}N-labeled compared to ^{14}N HAB mice fed either *Spirulina* or *Ralstonia eutropha* diets (adapted from [14]).

2.7 In silico pathway analysis

To identify enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways [24], Uniprot IDs were first mapped to Gene IDs and then to KEGG orthologue (KO) IDs. *p*-values were calculated by the hypergeometric test implemented in the statistical package R (www.R-project.org). To account for multiple testing and to penalize pathways with only a small number of entries, the input size of each test was reduced by 1 [25]. The background was set to all mouse KO IDs (3319). We used two different approaches (combined and pooled) to address pathway alterations related to ^{15}N metabolic labeling. In the combined approach, the distribution of averaged protein $\log_2$ (^{14}N/^{15}N) values was divided into three bins: $(-\infty, -0.4)$, $[-0.4, 0.4]$, $(0.4, +\infty)$, and enriched KEGG pathways were calculated for each bin. The pooled approach focused on the general variation between the biological replicates for each data set. We calculated a pooled protein distribution for each data set and each data set distribution was again divided into three bins $(-\infty, -0.4)$, $[-0.4, 0.4]$, $(0.4, +\infty)$.

In the combined approach, pathways of interest are statistically significant ($p < 0.001$) in the first or third bin and have a better *p*-value compared to the second bin. This implied a tendency of these pathways toward a regulation direction (up or down in ^{14}N compared to ^{15}N-labeled HAB mice). Ideally, this was also reflected in the second approach when biological variation was taken into account, allowing an assessment of the robustness and regulation tendency of enriched pathways found in the first approach.

3 Results

3.1 ^{15}N metabolic labeling of HAB mice

HAB mice were in vivo metabolically labeled using two different ^{15}N-enriched diets based on *Spirulina* and *Ralstonia eutropha* proteins. ^{15}N-labeling efficiency in the brain (cerebellum) was monitored at different developmental time points and found to be >91% for both diets on PND 56 [14]. The high ^{15}N incorporation enabled an accurate relative quantification of ^{14}N/^{15}N peptide pairs using MS analysis.

3.2 Behavioral alterations in ^{15}N-labeled HAB mice

Anxiety-related and depression-like behaviors were assessed. No differences were observed in anxiety-related behavior in EPM or USV tests between ^{15}N-labeled and ^{14}N HAB mice for both diets [14]. However, in TST (Fig. 2A), a significant decrease in immobility time, which is indicative of a decreased depression-like behavior, was observed for ^{15}N-labeled compared to ^{14}N HAB mice for both diets (Fig. 2B) [14].

3.3 Relative protein quantification between ^{14}N- and ^{15}N-labeled HAB mice

We focused our analyses on proteins that were quantified in all three biological replicates per data set. This resulted in

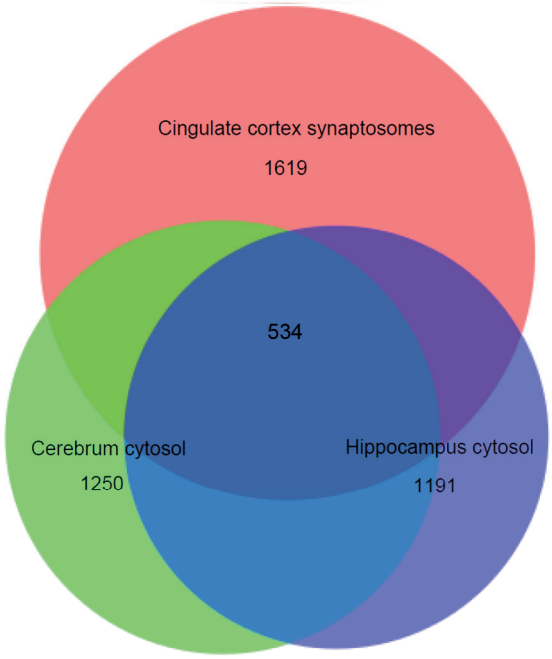

Figure 3. Venn diagram of quantified proteins. Only proteins present in all three biological replicates of an analyzed data set were used for the in silico pathway analysis. An overlap of 534 proteins was found for all three data sets.

1619, 1250, and 1191 quantified proteins in cingulate cortex synaptosomes, cerebrum cytosol, and hippocampus cytosol, respectively (Fig. 3). An overlap of 534 proteins was found for all three data sets.

3.4 Pathway alterations in ^{15}N-labeled HAB mice

To identify pathways with a high level of significance altered by the ^{15}N isotope, we submitted the proteomics data to two complementary in silico pathway analysis methods. We considered as affected only those pathways that were significantly altered in both analyses after correction for multiple testing (Supporting Information Tables S2 and S3). Alterations were found in major energy metabolism pathways, including the tricarboxylic acid cycle (TCA) cycle, oxidative phosphorylation, and glyoxylate and dicarboxylate metabolism. The TCA cycle showed a predominant downregulation in ^{15}N-labeled HAB mice in the cingulate cortex synaptosomes. In the pooled analysis, no significant ($p = 0.204$) enrichment of the TCA cycle was found in the second bin, indicating a clear trend toward downregulation ($p = 2.49 \times 10^{-11}$). A similar trend was confirmed in the cerebrum cytosol and hippocampus cytosol with *p*-values in the pooled analysis indicating a downregulation in ^{15}N-labeled HAB mice (cerebrum, $p = 3.87 \times 10^{-15}$; hippocampus, $p = 8.21 \times 10^{-12}$). The combined analysis also showed downregulation for hippocampus cytosol ($p = 2.99 \times 10^{-6}$) but not for the cerebrum cytosol data ($p = 0.0013$) when compared to second bin *p*-values (hippocampus, $p = 0.00014$; cerebrum, $p = 3.10 \times 10^{-6}$).

Oxidative phosphorylation was only altered in the cingulate cortex synaptosomes data set. Both in the combined ($p = 2.85 \times 10^{-42}$) and pooled ($p = 7.88 \times 10^{-46}$) analyses a tendency was found for a downregulation in ^{15}N-labeled HAB mice. This trend was not seen in cerebrum cytosol or hippocampus cytosol. Even in the pooled analysis, no clear direction of regulation could be identified. Finally, the glyoxylate and bicarboxylate metabolism pathway was only found to be significantly altered in the cerebrum cytosol (combined *p*-value $= 4.29 \times 10^{-6}$; pooled *p*-value $= 3.61 \times 10^{-6}$). Comparing the *p*-values of the third and second bins showed a trend toward downregulation. No pathways exclusively affected in the hippocampus cytosol were identified.

4 Discussion

In the present study, we report for the first time an ^{15}N isotope effect on the mouse brain proteome. When comparing ^{15}N-labeled and ^{14}N HAB mice, we found altered protein expression levels in all brain regions and subproteomes that were analyzed. Employing an in silico pathway-based analysis, we were able to pinpoint alterations in major metabolic pathways such as the TCA cycle, oxidative phosphorylation, and glyoxylate and bicarboxylate metabolism.

Although the ^{15}N isotope effect has been considered insignificant due to the small relative mass difference between the ^{14}N and ^{15}N isotopes, there are several lines of evidence that support an ^{15}N isotope effect on the molecular level. Alterations in growth rates have been observed in *Escherichia coli* bacteria and fungi growing in ^{15}N-labeled compared to ^{14}N media (M. D. Filiou et al., submitted for publication) [3, 26]. Others have reported changes in spectroscopic properties between ^{15}N- or ^{13}C-labeled and unlabeled Cu-thiolate clusters in yeast, suggesting that the ^{15}N incorporation in an organism can affect protein architecture [27]. As a consequence, enzymatic reactions and pathways could be altered in an unpredictable manner.

In the current study, ^{15}N-derived protein expression alterations in energy metabolism pathways were accompanied by alterations in depression-like behavior in HAB mice. Interestingly, several studies have already found a dysregulation of these pathways in depression and other psychiatric conditions. Energy metabolism imbalance has been repeatedly reported in a series of rat models of depression [28–31]. In a rat model of social defeat, alterations in TCA cycle and electron transport chain proteins were found [28]. Expression-level changes of proteins involved in TCA cycle and glycolysis were also observed in rats subjected to chronic mild stress [29]. Maternal separation and/or escitalopram treatment also resulted in alterations of TCA cycle and subunits of the electron transport chain [30] whereas similar energy metabolism alterations were reported for a gene-environment rat model of depression [31]. Altered brain levels of energy metabolism

enzymes were found in normal rats under chronic antidepressant treatment [32]. In human cohorts, enzymes of the TCA cycle were also found differentially expressed between patients suffering from major depression and controls [33].

Oxidative phosphorylation in the cingulate cortex synaptosomes was the most significantly altered pathway found in the current study. Oxidative phosphorylation is not only the major mechanism for ATP production in the cell but also the main source of reactive oxygen species that cause oxidative stress. There is a well-established link between oxidative stress and major psychiatric disorders including depression [30], schizophrenia [34], anxiety [6], and bipolar disorder [35, 36]. The fact that different pathways were found affected in the different data sets analyzed suggests a proteome- and area-specific effect of the ^{15}N isotope, in line with the spatially-dependent functional organization of the brain [37].

Taken together, the molecular changes observed in ^{15}N-labeled compared to ^{14}N HAB mice are related to the decreased depression-like behavior observed in these animals. Our findings do not imply that the ^{15}N incorporation has antidepressant properties and care should be taken not to overinterpret such isotope effects. In clinical practice, there is no evidence that the introduction of the ^{15}N isotope has a therapeutic potential. However, in the trait anxiety mouse model investigated in the present study, the introduction of the ^{15}N isotope results in behavioral changes similar to the ones caused by antidepressant agents. The ^{15}N isotope effect can be exploited to learn about molecular correlates associated with the behavioral phenotype in an animal model. In quantitative proteomics studies using stable isotopes, the ^{15}N isotope effect can be circumvented by using appropriate labeling controls (i.e. ^{15}N-labeled standard or reverse ^{15}N labeling). By implementing such controls in a comparative proteomics study design, the ^{15}N isotope effect will not influence the quantification results.

Our exploratory study demonstrates that the in vivo introduction of the ^{15}N isotope can have unpredictable effects on molecular pathways and behavioral characteristics. We therefore submit that the ^{15}N metabolic labeling approach, apart from a method for accurate protein quantification, has the potential to reveal molecular pathways involved in phenotypic alterations. The investigation of the ^{15}N isotope can be extended to behavioral phenotypes other than anxiety and even to mice with different genetic backgrounds. To this end, ^{15}N metabolic labeling may provide us with a powerful tool for unraveling the molecular underpinnings of dysfunctional circuits relevant for neuropsychiatric disorders.

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3 DISCUSSION AND FUTURE PERSPECTIVES

The implementation of functional genomics in psychiatry research - in particular high throughput proteomics and metabolomics - is only at its very beginning and its great potential is just being realized. In a disease with unknown etiology like major depression exploratory and unbiased -omics studies in well-established mouse models are of major importance to generate novel hypotheses of disease etiology and mode of drug action. Before reaching clinical significance, generated hypotheses have to be further addressed, validated or modified in targeted studies in other mouse models and clinical settings.

In the present study we employed proteomics and metabolomics technologies in combination with *in silico* pathway analyses in order to define antidepressant treatment effects in mouse models. DBA/2 mice were treated with paroxetine and molecular pathway alterations identified upon treatment with a representative SSRI. The antidepressant-like ^{15}N isotope effect on the HAB proteome was investigated and antidepressant-like behavioral and molecular alterations correlated. In addition, metabolite biomarker candidates for paroxetine treatment effects that reflect hippocampal alterations were identified in plasma.

Most importantly, we have shown that antidepressant treatment affects a great variety of biochemical pathways and is not restricted to alterations in monoaminergic neurotransmission. Novel hypotheses for the mode of antidepressant drug action were generated that lay the ground for future investigations in the quest for improved antidepressant treatment strategies.

3.1 Antidepressant treatment influences key cellular processes

Psychiatric disorders have traditionally been viewed as neurochemical diseases (Manji et al., 2001) and currently used antidepressants increase neurotransmitter availability. The present study has shown that apart from neurotransmission antidepressant treatment alters key cellular processes like energy metabolism supporting findings from previous functional genomic

studies (Khawaja et al., 2004; Carboni et al., 2006; McHugh et al., 2008). It is tempting to speculate that these altered key cellular processes are directly or indirectly involved in antidepressant therapeutic effects.

3.1.1 Antidepressant treatment shifts energy metabolism towards aerobic glycolysis

We identified significant alterations in energy metabolism upon paroxetine treatment (Webhofer et al., 2011). Most importantly, there was a fast and strong increase of early glycolytic intermediates in the hippocampus of DBA/2 mice upon paroxetine treatment (Webhofer et al., 2013). Supporting the shift towards aerobic glycolysis upon antidepressant treatment, the antidepressant-like ^{15}N isotope effect in HAB mice correlated with decreases in Krebs cycle and oxidative phosphorylation enzyme levels (Filiou et al., 2012). In agreement with these results, Filiou et al. (Filiou et al., 2011b) found decreased Krebs cycle and oxidative phosphorylation enzyme levels in low anxiety LAB mice compared to anxious HAB mice. It is important to note that aerobic glycolysis not only provides ATP for neuronal activity but also influences other important cellular parameters like the redox state and pathways like neurogenesis (Vaishnavi et al., 2010) – a known target of antidepressant treatment (Santarelli et al., 2003).

Future studies need to address the question whether directly targeting energy metabolism and shifting it towards aerobic glycolysis can have antidepressant-like effects in mouse models. In addition to assessing behavioral effects the emphasis of such studies should be on a critical evaluation of undesired side effects when directly interfering with a ubiquitous pathway such as energy metabolism. It may be beneficial to not directly target energy metabolism but related pathways that may be more relevant for antidepressant therapeutic effects. Future systems-based studies will eventually lead to an advanced understanding of neuronal and glial energy metabolism and its cross-talk with other pathways facilitating targeted therapeutic interventions.

3.1.2 Role of astrocytes in paroxetine treatment

Combined proteomic and metabolomic analyses indicated that increased glycolytic intermediate levels may, at least in part, be a consequence of an altered glycogen metabolism (Webhofer et al., 2013). The involvement of glycogen metabolism points towards an important role of astrocytes, the primary source of glycogen in the brain (Magistretti et al., 2000), in antidepressant action as has been postulated before (Czeh and Di Benedetto, 2012). It is therefore important that future studies elucidate specific roles of distinct cell types in antidepressant treatment. This notion is underscored by recent findings indicating that single cell types play distinct roles in antidepressant therapy (Schmidt et al., 2012) and that distinct cell types in different brain regions may elicit bidirectional effects on psychiatric phenotypes (Refojo et al., 2012). At present, however, functional genomic studies on single cell systems are analytically very challenging due to low tissue amounts and advances in analytical platforms are necessary to enable these studies.

3.1.3 Paroxetine treatment influences amino acid metabolism

Interestingly, there were opposing findings regarding proteinogenic amino acids that were significantly altered upon paroxetine treatment as described in chapters 2.1 and 2.2. Seven proteinogenic amino acids that were significantly increased in one study (chapter 2.1) were significantly decreased in the other study (chapter 2.2, alanine, leucine/isoleucine, serine, threonine, tyrosine and valine). GABA, a non-proteinogenic amino acid, was significantly decreased upon paroxetine treatment in both studies. Similar treatment paradigms but distinct sample preparation protocols and metabolomic platforms were employed in the two studies (Webhofer et al., 2011; Webhofer et al., 2013). Major differences between the studies included (1) duration of paroxetine treatment, i.e. 28 days versus 24 days, (2) metabolite extraction buffers and extraction protocol, i.e. acetonitrile/isopropanol/water 3:3:2 versus methanol/water 4:1 and (3) metabolomics platform, i.e. GC-TOF-MS versus LC-SRM-MS/MS based metabolite quantitation.

Although the duration of chronic paroxetine treatment was similar (24 days versus 28 days) differences could still result from different treatment duration. However, both studies show increased glycolytic metabolites. The observed differences of proteinogenic amino acids may be due to distinct sample preparation strategies rather than random or systematic technical or treatment variability. We speculate that different sub-metabolomes may have been analyzed in the described studies. Future studies need to systematically investigate metabolic alterations in different subcellular compartments in order to address this question. A first line of investigation may include mitochondrial versus cytosol metabolite alterations upon antidepressant treatment with respect to the current findings related to energy metabolism.

3.2 Future developments in functional genomics

The present study has demonstrated the importance and benefits of integrated pathway analyses at both proteome and metabolome levels. Previous studies have also shown that there is a weak correlation between mRNA and protein levels (Nie et al., 2006; Gry et al., 2009) further supporting the need for integrated complementary -omics analyses. However, for successful data integration from different analytical platforms and realizing truly holistic answers, advances in post genomic studies have to be made.

Post-genomic analyses are expensive and instrument-time consuming and it therefore takes a considerable amount of time to describe alterations at mRNA, protein and metabolite levels. Modern instrumentation that allows for reduced analysis time and increased throughput may help in this endeavor. In addition, instruments need to be sensitive for increased information output and robust in every day performance. There is also a great demand for novel computational tools integrating heterogeneous datasets from diverse analytical platforms to achieve a holistic, quantitative and predictive understanding of the system under investigation (Sauer and Zamboni, 2008). Finally, the current psychiatric pathway discovery pipeline should be expanded to shed light on disease etiology and mode of drug action from different perspectives. Important future paths may include studies on biological parameters such as mRNA turnover (Mitchell and Tollervey, 2001;

Munchel et al., 2011), protein turnover (Price et al., 2010; Zhang et al., 2011a), protein post-translational modifications like phosphorylation (Christensen et al., 2010) and using diverse platforms such as single-cell analyses (Shi et al., 2012) or imaging studies like MALDI imaging (Schuerenberg et al., 2007) or multi-isotope imaging mass spectrometry (Steinhauser et al., 2012; Zhang et al., 2012).

The integration of diverse technologies in combination with well characterized mouse models and clinical samples will lay the ground for an improved understanding of psychiatric diseases and treatment pathways and eventually lead to better diagnosis and improved treatment strategies.

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List of publications

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these publications are part of the dissertation

Book Chapters

Webhofer C and Schrader M. Bioinformatical Tools for LC-MS/MS Analysis of Proteins and Peptides. Experimental Strategies for LC-MS Analysis of Proteins and Peptides. Letzel (eds). RSC publishing, 2011.

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation ´Antidepressant activated biochemical pathways and biomarker candidates´ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum oder annähernd übernommen worden sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Zu den Manuskripten habe ich wie folgt beigetragen:

Webhofer C, Gormanns P, Tolstikov V, Zieglgänsberger W, Sillaber I, Holsboer F, Turck CW (2011). Metabolite Profiling of Antidepressant Drug Action Reveals Novel Drug Targets Beyond Monoamine Elevation. Transl Psychiatry 1:e58.

- Studiendesign und –planung: in Zusammenarbeit mit IS, FH, CT
- Durchführung der Experimente: in Zusammenarbeit mit VT
- Datenanalyse: in Zusammenarbeit mit PG
- Verfassen des Manuskriptes: in Zusammenarbeit mit WZ, IS, CT

Webhofer C, Gormanns P, Reckow S, Lebar M, Maccarrone G, Ludwig T, Pütz B, Asara JM, Holsboer F, Sillaber I, Zieglgänsberger W, Turck CW (2013). Proteomic and Metabolomic Profiling Reveals Time-Dependent Changes in Hippocampal Metabolism Upon Paroxetine Treatment and Biomarker Candidates. J Psychiatric Res 47:289-298.

- Studiendesign und –planung: in Zusammenarbeit mit GM, FH, IS, WZ, CT
- Durchführung der Experimente: in Zusammenarbeit mit ML, JA, IS
- Datenanalyse: in Zusammenarbeit mit PG, SR, TL, BP
- Verfassen des Manuskriptes: in Zusammenarbeit mit PG, WZ, CT

Filiou MD*, Webhofer C*, Gormanns P*, Zhang Y, Reckow S, Bisle B, Teplytska L, Frank E, Kessler MS, Maccarrone G, Landgraf R, Turck CW (2012). The ^{15}N Isotope Effect as a Means for Correlating Phenotypic Alterations and Affected Pathways in a Trait Anxiety Mouse Model. Proteomics 12:2421-2427. *equal contribution

- Studiendesign und –planung: in Zusammenarbeit mit MF, PG, YZ, BB, GM, RL, CT
- Durchführung der Experimente: in Zusammenarbeit mit MF, PG, YZ, BB, LT, EF, MK
- Datenanalyse: in Zusammenarbeit mit MF, PG, SR
- Verfassen des Manuskriptes: in Zusammenarbeit mit MF, PG, CT

Eidesstattliche Versicherung/Affidavit

I hereby confirm that the dissertation ´Antidepressant activated biochemical pathways and biomarker candidates´ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

Munich, March 2013

Christian Webhofer

Hiermit bestätigen die Mitautoren die von Herrn Webhofer angegebenen Beiträge zu den einzelnen Publikationen

München, März 2013

Walter Zieglgänsberger	Chris W. Turck	Michaela D. Filiou	Philipp Gormanns