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GENE EXPRESSION STUDIES FOR THE EVALUATION OF MOLECULAR INTERACTIONS BETWEEN ECSTASY AND ANTIDEPRESSANTS

PhD thesis

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5-HT	serotonin, 5-hydroxytryptamine
Ace	angiotensin-converting enzyme
Alpl1	tissue-nonspecific alkaline phosphatase
ANOVA	analysis of variance
Ascl1	Achaete-scute complex like 1
Bcl2	B-cell CLL/lymphoma 2
BDNF	brain-derived neurotrophic factor
Ca2	carbonic anhydrase 2
Camk1g	calcium/calmodulin-dependent protein kinase I gamma
Camk2b	calcium/calmodulin-dependent protein kinase II beta
Camk2g	calcium/calmodulin-dependent protein kinase II gamma
Camk2n2	calcium/calmodulin-dependent protein kinase II inhibitor 2
cAMP	cyclic adenosine monophosphate
Cd47	CD 47 antigen
Cdh22	cadherin 22
Cdh7	cadherin 7, type 2
Clstn2	calsyntenin 2
Cnr1	cannabinoid receptor, type 1
Cntn2	contactin 2
Col27a1	procollagen, type XXVII, alpha 1
Col4a2	procollagen, type IV, alpha 2
Col4a3bp	procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein
Col5a1	collagen, type V, alpha 1
Cox17	copper chaperone
Cox4i1	cytochrome c oxidase subunit IV isoform 1
DA	Dark Agouti
Dao1	D-amino acid oxidase
Dpp4	dipeptidyl-peptidase 4
DR	dorsal raphe nucleus
Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1
Enpp1	ectonucleotide pyrophosphatase/phosphodiesterase1
Epha5a	ephrin receptor 5a
Erp29	endoplasmic retuclum protein 29
Faah	fatty acid amid hydrolase
FC	frontal cortex
FDR	false discovery rate
FLX	fluoxetine
fMRI	functional magnetic resonance imaging
FST	forced swimming test
Gabrb2	gamma-aminobutyric acid receptor, subunit beta 2
Gabrb3	gamma-aminobutyric acid receptor, subunit beta 3

Gad2	glutamic acid decarboxilase 2
Galp	galanin-like peptide
GalR1	galanin receptor 1
GalR2	galanin receptor 2
GalR3	galanin receptor 3
Gas2	growth arrest-specific protein 2
GEO	gene expression omnibus
Gfap	glial fibrillary acidic protein
Glp1r2	glucagone-like peptide 1 receptor
Gnao	guanine nucleotide binding protein, alpha o
Gnaq	guanine nucleotide binding protein, q polypeptide
GO	gene ontology
Gpx1	glutathione peroxidase 1
Gria3	glutamate receptor, AMPA 3
Grin2a	NMDA-type glutamate receptor, type 2A
Grin2b	NMDA-type glutamate receptor, type 2B
GSEA	gene set enrichment analysis
Hcn1	hyperpolarisation-activated cyclic nucleotide gated potassium channel 1
HPA	hypothalamus-pituitary-adrenal axis
Hsd11b	hydroxysteroid 11-beta dehydrogenase 2
Hsf2	heat shock factor 2
HSP	heat-shock protein
Hspca	heat-shock protein 1
Igf2	insulin like growth factor 2
Il1rapl1	interleukin 1 receptor accessory protein-like 1
Kcnc2	potassium voltage gated channel, Shaw-related subfamily, member 2
Kcnd2	potassium voltage gated channel, Shal-related family, member 2
Kif1b	Kinesin family member 1b
Kif2b	Kinesin family member 2b
Kif5a	Kinesin family member 5a
Lphn1	latrophilin 1
MDD	major depressive disorder
MDMA	±3,4-methylenedioxy-methamphetamine
MinPplr	minimum probability of positive log ratio
Miz1	Msx-interacting-zinc finger
Mmp9	matrix metallopeptidase 9
Mrpl42	mitochondrial ribosomal protein L42
Mrpl48	mitochondrial ribosomal protein L48
MSigDB	molecular signature database
Myo5a	myosin 5a
NA	noradrenaline
Negr1	neuronal growth regulator 1
Nell2	nel-like 2 homolog

NES	normalized enrichment score
Nr2f6	nuclear receptor subfamily 2, group F, member 6
Nr4a3	nuclear receptor subfamily 4, group A, member 3
NRG1	neuregulin 1
Ntrk2	neurotrophic tyrosine kinase, receptor type 2
Ntrk3	neurotrophic tyrosine kinase, receptor type 3
OD	optical density
P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6
Pcdh17	protocadherin 17
Pcdhac2	protocadherin alpha subfamily C, 2
PCR	polymerase chain reaction
PDE	phosphodiestherase
Pdpk1	3-phosphoinositide dependent protein kinase 1
Pex2	peroxisomal biogenesis factor 2
PFC	prefrontal cortex
Pink1	PTEN induced putative kinase 1
Pla2g2c	phospholipase A2, group 2C
Pou3f2	POU domain, class 3, transcription factor 2
Ppia	cyclophilin A
Ppp3r1	calcineurin B
Prdx1	peroxiredoxin 1
Psma6	proteasome (prosome, macropain) subunit, alpha type 6
Psme1	proteasome (prosome, macropain) 28 subunit, alpha
PUMA	propagating uncertainty in microarray experiments
Pyy	peptide yy
Rgs9	regulator of G-protein signaling 9
Rims1	RAB3 interacting molecule 1
Rora	RAR-related orphan receptor alpha
Rph3a	rabphilin 3A
Rpl14	ribosomal protein L14
Rpl32	ribosomal protein L32
Rpl37	ribosomal protein L37
Rpl8	ribosomal protein L8
Rps23	ribosomal protein S23
Rps27a	ribosomal protein S27a
Rps3a	ribosomal protein S3a
S27a	ribosomal protein S27a
SAL	saline
SEM	standard error of the mean
SERT	serotonin transporter
SHAM	sham-surgery
Sipa1l1	signal-induced proliferation-associated 1 like 1
Slc1a3	high-affinity glial glutamate transporter
Slc2a4	facilitaded glucose transporter, GLUT4

Slc38a5	solute carrier family 38, member 5
Slco1a5	solute carrier organic anion transporter family, member 1a5
Slick	sodium- and chloride-activated ATP-sensitive potassium channel
SNRI	selective serotonin and noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
Stat3	signal transducer and activator of transcription 3
Sv2b	synaptic vesicle glycoprotein 2b
Syn2	synapsin II
Synj2	synaptojanin 2
Syt8	synaptotagmin 8
Tap1	transporter 1, ATP-binding cassette, sub-family B
TCA	tricyclic antidepressant
TPH	tryptophan hydroxylase
Txn1	thioredoxin 1
Ucp3	uncoupling protein 3
Unc13b	unc-13 homolog B
Vamp1	synaptobrevin 1, vesicle associated membrane protein 1
Vdac1	voltage-dependent anion channel 1
VLX	venlafaxine
VMAT	vesicular monoamine transporter
Zfp180	zinc finger protein 180
Zfp36l2	zinc finger protein 36, C3H type-like 2
Zfp462	zinc finger protein 462
Znf313	zinc finger protein 313

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2. INTRODUCTION

2.1.MDMA

2.1.1. General aspects

Among the European population approximately 12.3 million users have already tried or will try the amphetamine derivate, ±3,4-methylenedioxy-methamphetamine (MDMA) in their lifetimes, mostly in the form of ecstasy tablets [1].

At the end of the last decade a transient drop of ecstasy use in the European population was observed as a result of the poor quality of the tablets, but recently usage patterns recovered, because of the substantially improved quality and the new administration forms, like the MDMA powder, which may be inhaled and the crystal form with definitely higher purity [1].

In Hungary the drop arrived earlier and usage remained continuously lower among teenagers [2]. An explanation may be that in 2006/2007 a book was published [3], aimed to teenagers, parents and teachers and promoted by the media about the symptoms of ecstasy abuse, the dangers of its use and the additional risks caused by contaminations and unknown active ingredients in the tablets.

In the US drug usage patterns mimic those in most European countries (except Hungary), where recently an increasing demand for the tablets is observable. A recent work of Maxwell analyzing drug use patterns among young individuals concluded that MDMA is currently returning to the market and again, ever more users are turning to the drug for its acute effects [4]. While MDMA is usually considered responsible only for a limited number of drug related deaths, new psychobiological and functional findings emphasize the long-term threats imposed by its use [3], a serious concern because of the recovering illegal markets in many countries.

2.1.2. Acute effects

The effects of ecstasy in rodents and humans are biphasic (for a review see [5]). In the first phase MDMA causes a release of serotonin (5-hydroxytryptamine, 5-HT), noradrenaline (NA) and dopamine by reversing transmembrane transporter functions [6-10]. In serotonergic terminals the serotonin transporter (SERT) is responsible for the

acute reuptake of 5-HT into the cytoplasm, which is normally released into the synaptic cleft as a result of neuronal activity [10]. From the cytoplasm 5-HT molecules will be transported by the vesicular monoamine transporter (VMAT) into the vesicles [10]. Both processes are reversed by MDMA, thus, the uptake of MDMA into the cells is paralleled by the acute release of 5-HT [9, 11-13]. Similar processes happen in dopaminergic and noradrenergic terminals [6, 8, 14]. The acute increase of the monoamine levels in the synaptic clefts is responsible for the initially observable so called "positive" effects.

Among these positive effects, euphoria is a consequence of the dopamine release in the reward circuitry [15], but it has to be noted that MDMA is able to further modulate its own effects within the mesolimbic dopaminergic system through different 5-HT receptors activated by the released 5-HT [16-18]. Additionally, differentially from other amphetamine derivates, MDMA is able to cause an entactogenic effect, a feeling of elevated sociability, which is a unique feature of this drug among similar compounds [19]. This elevated sociability is usually accompanied by elevated anxiety, decreased aggressiveness [16, 20-22] and increased locomotor activity [16, 23-25]. These acute effects, i.e. euphoria, elevated sociability and the tirelessness made MDMA a common party drug at rave and techno parties in the late twentieth century and are contributing to the current rise in its popularity [7].

At the same time, neurotoxicity may also occur even in this early phase. The autoregulation of the cerebral blood flow is normally responsible for the "use-dependent" supply of neurons with nutrients and oxygen. Administration of MDMA in rats results in the impairment of this mechanism and while blood flow within several brain regions is reduced, glucose demand is elevated [26]. These processes may result in an acute lack of nutrients and oxygen within neurons accompanied by a possible functional impairment. MDMA acutely also causes an elevation in body temperature [16, 25-29], which may further add to its neurotoxic properties [7]. The hot environment in "rave" and "techno" parties, the lack of proper hydration together with the elevated physical activity may further exacerbate the stress the neurons have to cope with in human users.

Besides the impact on the central nervous system, MDMA may cause adverse effects at the kidneys [30], in the cardiovascular system and at the heart [31, 32] and can

also induce hepatotoxicity [32]. The latter effects are usually responsible for the deaths involving MDMA [33], but acute serotonergic toxicity caused by the concomitant use of other 5-HT releasing agents may also play a decisive role [34, 35].

2.1.3. Long-term serotonergic toxicity

Beside of the acute neurotoxicity described above, in the long-run, both in experimental animals and in human users a decrease in serotonergic markers was reported, which was interpreted as a selective vulnerability of the 5-HT system for MDMA [36-38]. These serotonergic markers include SERT and tryptophan hydroxylase (TPH), the latter being the key enzyme in 5-HT synthesis. The decrease in TPH and SERT mRNAs in rats could be observed in the cortical regions both after 7 and 21 days following a single dose MDMA administration [37], paralleled by a decrease in paroxetine binding sites [22]. Anatomical innervation supports the involvement of the cortical regions in 5-HT impairments, since serotonergic projections originating from the dorsal raphe nucleus (DR) and to a lesser extent from the median raphe nuclei in the brainstem [39] innervate upper brain structures, e.g. the frontal cortical regions [40, 41].

The frontal lobe plays major roles in differential functions of the adult brain. It is involved in cognitive tasks, like risk evaluation [42], executive functions [42, 43], and memory [42-46] and impairments within these regions also strongly associate with neuropsychiatric diseases [42, 47]. As a probable consequence of the decreased serotonergic tone and nonspecific impairments, corresponding functional deficits were observed in humans and rodents following MDMA administration, e.g. sleep disturbances, increased anxiety and impulsivity levels, aggression, and foremost, impaired decision making, learning/memory deficits and depression [7, 37, 48-52].

Besides cognitive functions and the modulation of affective circuitries frontal cortex (FC) is also implicated in motor system functions. Accordingly, MDMA caused functional deficits in motor related tasks. In humans, elevated reaction time [53] and tremor during movements [54] were reported, while previous polydrug (incl. MDMA) users showed impairments in motor speed dexterity compared to controls, which could not be explained by the use of other substances [55]. On the other hand, no group differences were found in simple visual reaction time [56] or the finger tapping test [57] between MDMA users and controls. The parallel measurements of brain activation via functional magnetic resonance

imaging (fMRI) and performance in an event-related motor tapping test resulted in changes of brain activity in the basal ganglia-thalamocortical circuitry, however, no significant group differences in motor performance could be observed [58]. Furthermore, heavy users often complain about unconscious motor movements as a probable result of MDMA abuse (for a review see [51]). In rats, two weeks following a single dose MDMA administration elevated motor activity could be demonstrated [59]. In summary, all the latter changes suggest that MDMA may negatively influence motor functions.

Besides the serotonergic and accompanying functional impairments, neuroprotective mechanisms also may occur and recovery processes can begin. Heat-shock proteins (HSPs) are responsible for ameliorating the damage caused by cellular stress of different origin, including hyperthermia or ischemia [60]. Three days after a single dose MDMA administration elevated levels of HSP 27 have been demonstrated in the FC, while this elevation disappeared 7 days after treatment [36]. Another protein, brain-derived neurotrophic factor (BDNF), a member of neurotrophic factors, is implicated in dendritic arborization, synaptogenesis, etc. and may play a role in memory formation and other cognitive processes [61, 62]. Evaluating MDMA's effects on BDNF mRNA expression an elevation was reported in FC up to 7 days following administration [63]. These results suggest a partial reinstatement of the cortical networks.

2.1.4. The therapeutic potential of MDMA

While the above results support a long-lasting serotonergic deficit with possible accompanying consequences, functional impairments following MDMA use are usually moderate. This has resulted in previous and recent criticism of reports underlining neurochemical impairments, arguing that in human users, serious, long-lasting consequences are rare. In a paper published in 2004, Green also discussed possible implications in drug-assisted psychotherapy, especially following serious psychic trauma [64]. This form of application is based on MDMA's entactogenic effect, through which MDMA could raise the possibility of a more successful psychotherapy (despite or rather besides the fact that it may cause mood disorders on the long-run). The Multidisciplinary Association for Psychedelic Studies (www.maps.org) supports several ongoing studies investigating the role of MDMA in drug-assisted psychotherapy of war veterans and other patients suffering from post-traumatic stress disorder with the approval of the U.S. Food and Drug Administration. These studies are

the continuations of the report published in 2010 [65]. In the latter study Mithoefer et al. demonstrated an 83% clinical response to psychotherapy in chronic post-traumatic stress disorder patients receiving MDMA compared with 21% in the control subjects. While the latter results are impressive, MDMA-caused serotonergic deficits, though region- and time dependent, seem to be obvious and subsequent molecular events, which may further characterize its functional impairments remained so far poorly examined leaving room for progress (for a review in humans see [66] and in animals see [5] and [52]).

The studies examining molecular events except the decrease in the serotonergic markers (described earlier) are scarce. The few reports, which evaluated alterations in mRNA levels of genes after MDMA administration mostly measured those which were thought to be related to MDMA-caused molecular alterations [37, 50, 67-69]. While these studies are valuable tools, they are only limitedly able to reveal so far unknown pathways, which may be altered by the drug on the long-run. If we assume that even smaller changes in neurotransmitter levels can alter the level of downward signaling molecules, the molecular pattern arising on the ground of the 5-HT damage may manifest in complex and hardly predictable changes. Thus, genome-wide gene expression analysis could provide new aspects about the chronic use of MDMA, besides, through the recent advance of network scale analysis, binding the caused functional deficits to intracellular changes in expression levels.

2.2. Venlafaxine

2.2.1. General aspects

Major depressive disorder (MDD) is a psychiatric disease, characterized by diminished interest, anhedonia, depressed mood and negative thoughts, sleeping problems and tiredness [70]. In the 1960's, based on the observation of already effective antidepressants, Schildkraut proposed the so called "monoamine hypothesis" of mood disorders, namely, that affective disorders are inherently related with the imbalance of monoamines within the brain [71]. This concept formed the basis of MDD ever since and fostered the development of generations of antidepressants, the older tricyclic antidepressants (TCAs), acting at multiple targets, and the newer selective serotonin

reuptake inhibitors (SSRIs) and selective serotonin/noradrenaline reuptake inhibitors (SNRIs), among others.

These substances elevate the levels of monoamines acutely in the synapses by inhibiting their reuptake from the synaptic cleft. Because TCAs, with their dual 5-HT and NA reuptake inhibition, were more effective than SSRIs in the treatment of MDD [72, 73] and the combination of fluoxetine (FLX, an SSRI) and desipramine (basically a NA-reuptake inhibitor [NRI]) resulted in elevated antidepressant effects [74, 75], SNRIs were synthetized. These act both at serotonergic and noradrenergic neurotransmitter reuptake combining TCAs multiple mechanisms of action and greater efficacy, but limiting their side effects by acting exclusively at these targets. A prominent member of SNRIs is venlafaxine (VLX), which, indeed, seems to be superior to SSRIs in the treatment of MDD in terms of remission rates, earlier onset of action and economic costs [76, 77].

While all of these medications may be effective in the treatment of affective disorders, there is an ongoing debate about the exact role of monoamines in the disease. The main argument of the sceptics is that all of the medications elevate monoamine levels acutely, still, their therapeutic efficacy is only obvious after weeks, which led to the hypothesis that adaptive changes within the 5-HT system are responsible for their therapeutic efficacy [78].

Another argument of the critics is that the response rate to these medications is unpredictable and widely varies. Approximately 30-40% of patients do not respond to current pharmacotherapeutic efforts suggesting that sole monoaminergic manipulations are insufficient in the treatment of the disease.

Furthermore, monoaminergic theory could also not provide an answer why the combination of psychological and pharmacological therapies provides better results than any of these alone.

2.2.2. Synaptic theory of depression

The above mentioned discrepancies have led to other theories trying to unravel the underlying molecular changes behind MDD. The role of BDNF was raised and supported by some [79-81] and opposed by other authors [82-85] causing the scientific community to conclude that the effects of BDNF are probably region dependent having

beneficial effects especially in the hippocampus [86, 87]. Other proposed mechanisms involved the immunological and neuroendocrine systems [suggesting a malfunction in hypothalamus-pituitary-adrenal axis (HPA)] or the epigenetic modifications of the DNA, through which changes in the expression levels of several genes may occur (for a review see [88]).

Recent findings from antidepressant treatments, studies in MDD patients and animal experiments suggested that neuronal network plasticity may be substantially changed during MDD and also, nonetheless reversely, in subsequent therapeutic efforts (for a review see [89]). Studies have demonstrated morphological abnormalities, i.e., differences in gray matter volume, neuronal organization, electrophysiological activity and receptor pharmacology in the circuitry connecting the medial prefrontal cortex (PFC), amygdala and hippocampus of depressed patients (for reviews see [90-92]). All these findings suggested structurally abnormal networks in the brains of MDD patients. Consequently, the emerging synaptic theory (or network hypothesis) of depression also suggested that pharmacological treatment (primarily via manipulating monoaminergic neurotransmission) may result in the enhancement of synaptic plasticity and that under these circumstances positive environmental stimuli, like psychotherapy, could reinstate optimal network functions [89].

Thus, the theory was in-line with both clinical experiences in MDD patients and also explained so far unexplained spots in antidepressant treatments. At the same time, however, it also suggested other useful applications for antidepressants, e.g. in diseases, where a damage to neuronal networks is obvious.

2.2.3. The use of antidepressant medications in post stroke recovery

Some antidepressants were, indeed, recently successfully applied for post stroke motor recovery.

In a study, ten stroke patients received reboxetine, a NRI, in a double-blind placebo-controlled design. Reboxine induced improvements in motor functions, like tapping speed and grip strength, but left dexterity and thumb movements, evoked by transcranial magnetic stimulation, unchanged [93].

The beneficial effects of FLX were also demonstrated in human experiments. Dam et al. demonstrated improvements in walking and daily activities in FLX-treated

severely disabled stroke patients [94] and Pariente et al. also reported FLX-induced improvements in motor skills in patients with subcortical motor stroke [95]. Additionally, in the FLAME clinical trial, the efficacy of FLX was tested in 113 ischemic stroke patients with hemiplegia and hemiparesis (patients with previously diagnosed MDD were strictly excluded). FLX was administered 3 months long starting within 5-10 days following the onset of stroke and all patients had physiotherapy beside the pharmacological intervention. The motor improvement and the number of independent patients at day 90 were significantly higher in the FLX group when compared to the placebo treatment [96]. In addition, meta-analyses both from human and animal subjects further supported the efficacy of SSRIs after stroke [97, 98].

But not only SSRIs or NRIs may be beneficial in post-stroke motor recovery. In a randomized, double-blind, crossover study 7 days long VLX treatment improved finger-tapping rate in a motor task compared to the placebo group and a positive correlation was reported with the activation of motor and sensory cortices [99].

Besides improving motor functions FLX was also able to reduce the number of post stroke depression cases in the FLAME trial [96]. Similarly, VLX could induce a statistically significant reduction in the symptoms of post stroke depression patients [100].

All of the latter results suggest that chronic treatments with 5-HT and/or NA reuptake inhibitor antidepressants are able to beneficially influence post-stroke motor recovery besides reductions in post-stroke depressive symptomatology.

2.2.4. Unresolved questions about venlafaxine's effects

Studies investigating the molecular alterations underlying VLX's effects are limited in number. Gene expression studies in animals, which could reveal important alterations at the molecular level, were usually investigating VLX's mechanisms of action in one of the regions of the depressive circuitry (amygdala, hippocampus, PFC) [101-105]. However, these studies may provide only limited information, since it was demonstrated by human brain imaging and autopsy studies that several other brain areas including for example the FC, cingulate cortex, the striatal structures or thalamus may mediate the symptoms of depression [106].

Indeed, supporting the involvement of the FC region which participates in motor functions, a recent study found associations between depressed mood and altered locomotor patterns [107]. In addition, MDD also occurs in diseases affecting the FC, e.g. frontal lobe atrophy [108] or multiple sclerosis related depression [109] and FC is also involved in cognitive functions, which are impaired in MDD.

We have already discussed the connection between monoaminergic manipulations and motor recovery or the role of the serotonergic innervations of the FC in previous chapters (see 1.2.1). All these facts suggest that studies investigating antidepressant effects within this region may be of importance in understanding the consequences of the treatments.

Beside regional limitations, most of the studies investigating gene expression behind VLX's effects were addressing acute effects, which are plausibly less relevant in the drug's main therapeutic actions [76, 110].

Additionally, these studies were hypothesis-driven excluding the possibility of the discovery of new pathways and actions of the drug. A hypothesis-free approach would be especially important because following chronic treatment diverse and heterogeneous changes may occur, also suggested by the synaptic theory of depression.

2.3.MDMA and venlafaxine

2.3.1. Important interaction possibilities

As a releaser, MDMA, causes highly elevated levels of monoamines and through the vasoactive properties of the released substances induces disruption of local cerebral blood flow from glucose and oxygen demand and hyperthermia [16, 26, 36, 69]. Since it is transported into the neuron terminals it can also attenuate the function of mitochondria-attached monoamine oxidase B enzymes and via these mechanism elevates free radical production within the cells (for a review see [111]). The antidepressant VLX also releases NA and 5-HT, but in lesser extent, thus, it lacks the accompanying negative effects.

While there are undoubtedly similarities in the acute actions of the two drugs, they have markedly different effects on the long-term. While MDMA causes a selective 5-HT deficiency (as discussed in 1.1), TCAs, SSRIs and SNRIs all elevate

monoaminergic synaptic transmission, as a particular consequence of the desensitization of presynaptic autoreceptors, mainly 5-HT1A serotonergic- [112-114], but possibly also 5-HT1B serotonergic and α2 adrenergic receptors (for a review see [115]). Thus, MDMA and VLX act fundamentally different at the molecular level in the long-term (Fig 1).

The previously discussed alterations also suggest that MDMA and VLX may counteract each other functionally on the long-run, e.g. in motor- and cognitive functions. MDMA administration, as discussed in previous chapters, was usually related with negative effects on motor functions, like elevated reaction time [53], tremor or motor speed dexterity [55], while VLX treatment was associated with elevations in motor performance in healthy individuals [99].

Cognitive functions following MDMA use were also impaired, like decision making, learning and memory [7, 49, 52]. At the same time, pre-training administration of VLX showed heterogeneous effects in memory tasks, but post-training treatment improved performance in rats (for a review see [116]), while positive effects were also suggested on affective cognition (for a review see [117]).

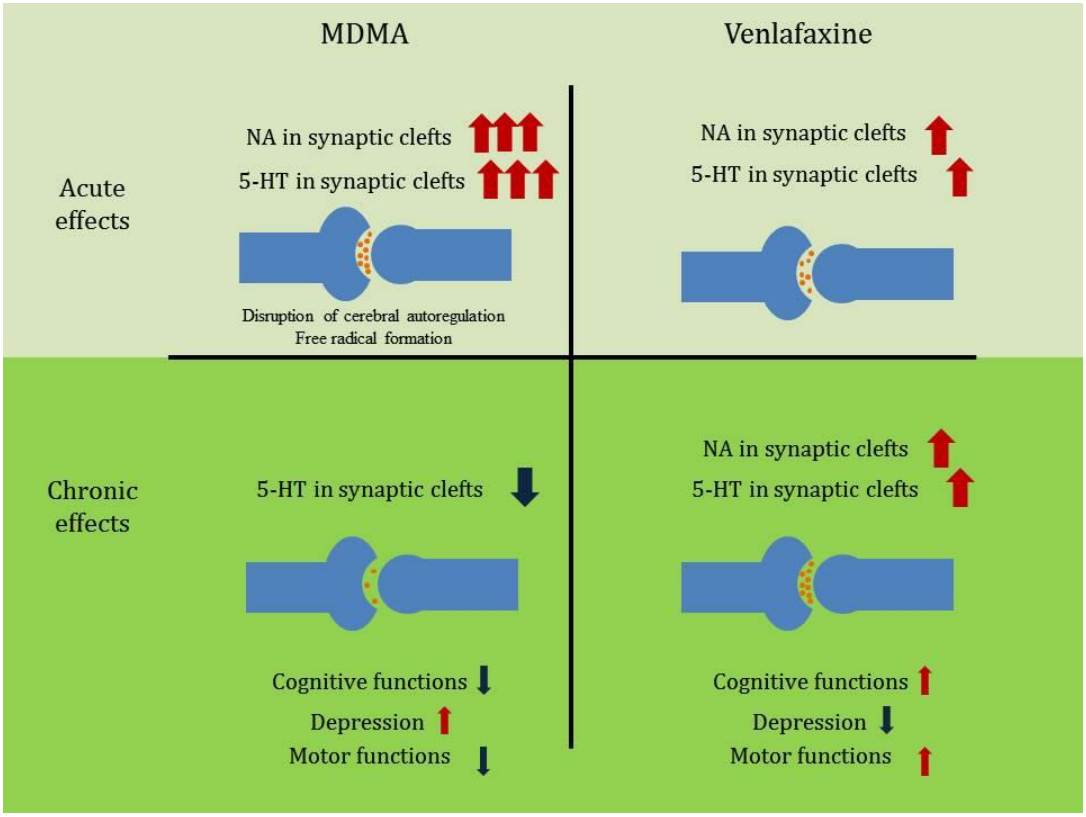

Figure 1 The acute and chronic consequences of MDMA and venlafaxine administration in the serotonin and noradrenaline content of synaptic clefts and the possible subsequent functional alterations.

Acutely, both 3,4-methylenedioximethamphetamine (MDMA) and venlafaxine (VLX) enhance serotonin (5-HT) and noradrenaline (NA) levels, though in a different extent, in the synaptic clefts of frontal cortical neurons. On the long-term, they have markedly different effects. Even a single-dose MDMA administration causes a selective serotonergic neurotoxicity and thus, decreases in the 5-HT concentrations, while chronic VLX treatment, through the desensitization of 5-HT1A autoreceptors elevates synaptic 5-HT levels in the synaptic clefts. At the bottom of the figure some of the possible functional consequences of the altered serotonergic tone were listed. For references and further details see main text.

In addition, MDD in previous MDMA users is more common than among the general population [118, 119] thereby exposing them to antidepressant treatments later in life.

While it has been demonstrated by numerous studies that pretreatment with antidepressants may reduce MDMA-induced effects, probably by interfering with SERT (in the case of SSRIs) and NA transporter (in the case SNRIs and NRIs) [120-126], antidepressant administrations following MDMA use were only scarcely investigated. Thompson et al. showed that 5-weeks long chronic FLX treatment was able to reverse MDMA-induced elevated anxiety and depression levels in the emergence test and in the forced swimming test (FST), respectively, but left reduced social interactions unchanged in rats [127]. At the same time, Durkin et al. showed reduced efficacy of chronic FLX treatment following previous MDMA administration [128]. Paroxetine binding sites were also altered in cortical regions as a response to a previous exposure of the drug [22]. Dark Agouti (DA) rats represent the human poor metabolizer phenotype. Since these animals have a less active variant of the CYP2D1 enzyme, corresponding to the human CYP2D6 responsible for MDMA metabolism, they are model animals for those human individuals, who are especially vulnerable to the neurotoxic effects of the drug [5, 28]. In DA rats, a challenge with FLX 6 months after an initial MDMA administration resulted in altered responses, namely, an elevated time of aggressive behavior, suggesting that SSRI antidepressant effects may be altered even after a single dose of MDMA and such a long time [129].

The above studies all impose the possibility that effects of antidepressants may be altered by previous MDMA use. The lack of further studies evaluating the interactions of previous MDMA and later antidepressant administration is surprising, since these aspects are not only important scientific questions, but may also influence therapeutic approaches in addicts. Antidepressants with a dual mechanism of action remained so far completely uninvestigated in similar experimental setups, despite the fact that they may be superior to SSRIs through their extended mechanism of action.

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Because of the complex nature of possible alterations, and the mass of involved pathways and functions suggest a need again for a hypothesis-free approach. Thus, microarrays, able to measure whole-genome gene expression levels and create hypotheses following data acquisition instead before it, may have advantages over classical hypothesis-driven experimental setups for such purposes [130].

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3. OBJECTIVES

1. Selective serotonergic toxicity and functional alterations on the long-run following a single-dose of MDMA were already described in the literature, but underlying chronic molecular changes remained so far uninvestigated in the FC region. In order to reveal which genes or pathways may be involved in the effects 21 days after MDMA administration, we performed a genome-wide microarray analysis in the FC of DA rats, the latter representing the vulnerable human individuals to the drug's effects.

2. The real therapeutic efficacy of VLX treatment is only obvious after weeks suggesting adaptive processes on the molecular and network levels of cortical neurons, which remained poorly understood after chronic use. To fill this gap, we have performed a genome-wide microarray-analysis after a chronic 3-weeks long VLX treatment regimen in the FC of the DA rat strain to characterize the transcriptional background behind the drug's therapeutic effects.

3. Following the analysis of MDMA's and VLX's effects individually (see 1. and 2.), we also investigated the genome-wide molecular changes after the combined treatment of a single-dose of MDMA and a subsequent 3-weeks-long VLX treatment in the FC of DA rats to reveal, whether a consecutive VLX treatment could compensate for the MDMA-induced changes, and to report, if and how a previous MDMA treatment may influence the effects of VLX on the molecular level.

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4. METHODS

4.1. Animals

The animal experiments and housing conditions were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC), and the National Institutes of Health Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985) as well as special national laws (the Hungarian Governmental Regulation on animal studies, 31 December 1998 Act). The National Scientific Ethical Committee on Animal Experimentation and the Food Chain Safety and Animal Health Directorate of the Central Agricultural Office, Hungary (permission number: 22.1/3152/001/2007) approved the experiments.

Altogether 42 male DA rats (Harlan, Olac Ltd, Shaw's Farm, Blackthorn, Bicester, Oxon, UK) were used, aged circa 8 weeks [126.71 ± 3.30 g (mean + SEM) at the beginning of the experiment]. The animals (four per cage) were kept in standard cages and under controlled environmental conditions (temperature 21 ± 1 °C, humidity: 40-50%, 12 hour light-dark cycle starting at 6:00 a.m.). Food and drinking water were available for them *ad libitum*.

4.2. Drug Administration and Experimental Design

The animals were randomly assigned to four groups according to the treatment regimens (SAL/SHAM, MDMA/SHAM, SAL/VLX, MDMA/VLX, see. Fig. 2). MDMA (Sanofi, Hungary, purity >99.5%), dissolved in 0.9% NaCl (SAL) at an equivalent dose of 15 mg/kg free base was administered intraperitoneally (i.p.) in a volume of 1 ml/kg to the MDMA treated animals. Control animals received SAL i.p. in equivalent volumes (1 ml/kg).

VLX (Egis Pharmaceuticals, Hungary) was dissolved similarly in 0.9% NaCl solution and Alzet 2001 osmotic minipumps (Durect Corp., CA, USA) were filled with the solution. In VLX treated groups these Alzet osmotic minipumps were inserted subcutaneously under the back skin of the animals half day after the initial injections to avoid acute serotonin-syndrome in previously MDMA treated rats. The pumps delivered 40 mg/kg VLX each day. All surgery was performed under halothane anesthesia, and all

efforts were made to minimize suffering of the animals. The control group underwent sham surgery/osmotic minipump insertion (containing saline) in a randomized manner (which will be abbreviated by SHAM to avoid confusion with the MDMA control group). The surgical procedures had to be repeated each week for 3 weeks, due to the limited volume of the osmotic pumps. After surgery, animals were always returned to their cages and were kept there until the next procedure.

During surgery two animals died and one has lost its pump, thus, altogether 39 animals went through the entirety of these procedures.

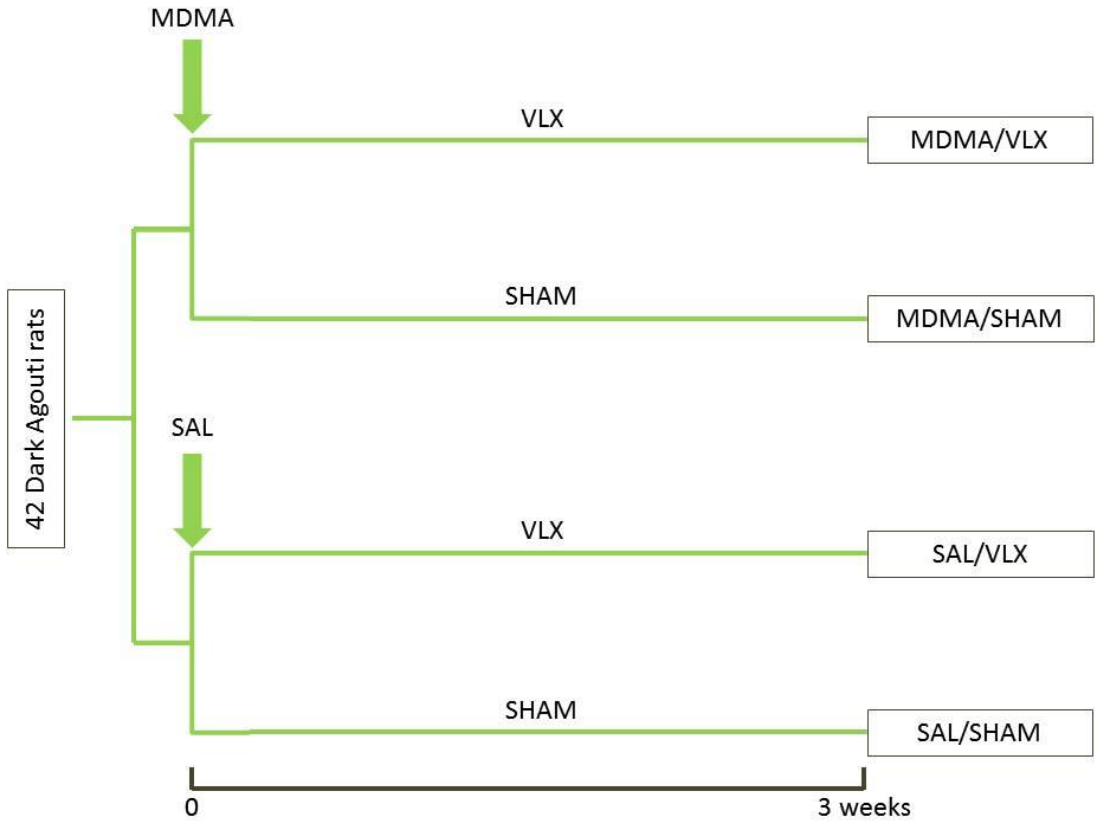

Figure 2 Experimental groups and setup.
Altogether 42 Dark Agouti (DA) rats were divided initially into two groups. One group of the animals received a single 3,4-methylenedioxy-methamphetamine (MDMA) injection (in a dose of 15 mg/kg), while the other saline. Thereafter animals were further divided into two groups, receiving either chronic venlafaxine treatment (VLX, 40 mg/kg/die via osmotic minipumps) or undergoing sham surgery/implantation of saline filled minipumps. Thus, at the end of the experiments altogether 4 groups: SAL/SHAM, MDMA/SHAM, SAL/VLX and MDMA/VLX were created based on the different combinations of treatments. SAL – saline, SHAM – sham operation/treatment with saline filled minipumps.

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4.3.RNA Extraction and Sample Preparation

The rats were sacrificed quickly by decapitation 3 weeks after the surgery. The brains were removed and 2 mm thick coronal sections were cut, and the FC regions were dissected according to Paxinos and Watson [131] (from bregma +1.7 to -0.3 mm) and stored at -80°C. TRIzol reagent (Ambion, TX, USA) in a volume of 1 ml was used for homogenization, according to the manufacturer's instructions. Then the homogenized samples were centrifuged at 12000 x g at 4°C for 10 minutes, supernatant transferred to Eppendorf tubes and incubated at room temperature for 5 minutes. Chloroform was added in a volume of 200 µl, the mixture was vortexed and incubated at room temperature for 2-3 minutes. After centrifugation at 12000 x g at 4°C for 15 minutes the upper aqueous phase was separated, mixed with 500 µl of isopropranol and incubated for another 10 minutes at room temperature. Following the centrifugation of the samples at 12000 x g at 4°C for 10 minutes the supernatant was removed and 1 ml 75% ethanol was added. The centrifugation was repeated at 7500 x g at 4°C for 5 minutes, the supernatant was removed, and again, 1 ml 75% ethanol was added. Following the third centrifugation step (at 7500 x g at 4°C for 5 minutes), ethanol was removed and RNA pellets dried. Pellets, dissolved in 20 µl diethylpyrocarbonate treated-dH2O, were stored at -80°C until further processing.

Thereafter 1-2 µl of the solution were used for optical density (OD, 260/230 and 260/280 ratios) measurements to assess the quality and quantity of the samples. The OD ratios were measured for all samples, and in addition, randomly repeated to determine reliability of the quality measurements. No differences were observed. Samples with lowest RNA concentrations were excluded from further analysis in a way that finally all groups consisted of 8 animals. Two randomly selected samples from the same treatment group were pooled resulting in 4 pooled samples per treatment group. Thus, altogether 16 pooled samples (4 per treatment group) were sent for microarray analysis with the Illumina (San Diego, CA, USA) RatRef-12 v1 beadarray expression chip to Service XS (Leiden, Netherlands). At Service XS a purification process and quality control measurements with Agilent Bioanalyzer and Nanodrop spectrophotometer were made and one pooled sample from the MDMA/VLX treated group had to be excluded due to degradation.

4.4.Data Analysis

The raw data returned from Service XS were processed with beadarray [132], preprocessCore [133] and puma [134] Bioconductor [135] packages for R statistical programming language [136] as described in [137]. Beadarray package was used to analyze bead level data and to give the flexibility to use other options than the standard Illumina methods provided by Illumina's BeadStudio software. The standard Illumina summarization method provides a point estimate for each expression level of each bead, whereas the uncertainty of such estimates remains excluded from further analysis. Since microarray experiments are associated with low precision probe-level measurements (called probe-level measurement error) relevant results may be biased by the resulting noise. To avoid such bias, we used the puma (Propagating Uncertainty in Microarray Analysis) package, which uses probe-level measurement error in subsequent analyses, however, has the prerequisite of special data formats.

According to our original aims, to reveal molecular alterations related to pharmacological manipulations we have chosen wider inclusion criteria and used "log = TRUE; n = 10" settings by creating the summary data for each bead via the createbeadsummaryData, allowing excluded outliers by standard Illumina method to be included in our downstream analyses, (similar settings were also used by others [137]). For normalization, the quantile normalization method was used via the preprocessCore package. The backgroundCorrect method in the puma package used was set to "minimum", meaning that any intensity equal or less than zero after background subtraction was set equal to half the minimum of the positive corrected intensities for that array. Additionally, pumaComb, pumaDE, and write.rslts functions with default settings were used to create summary tables and statistical values used in the subsequent analyses. These calculations resulted in the so called minimum probability of positive log-ratio (MinPplr), a Bayesian-based value for significance [138], besides also providing the fold change values between different comparisons. MinPplr has been shown earlier to be superior to classical methods, as a result of the inclusion of probe-level errors in microarray experiments [138]. To further address the problems of multiple testing, which is the result of the high number of statistical hypothesis tests

(more than 22000 in our experiment) performed [139], we used a stricter criteria and changes were considered statistically significant only when the MinPplr was below 0.001 in the MDMA and double treated group, and 0.005 in the VLX group. This is a usual reduction used in microarray experiments. While several methods exist to correct for multiple hypothesis testing, like the Bonferroni-method or the family-wise error rate, which may compensate for the false positive results, they may be very restrictive because of the assumption that every hypothesis test is independent from the others (for example the Bonferroni-correction divides the significance criterion [usually 0.05] by the number of tests performed [139]). Thus, we considered them inappropriate for our original aims, namely, the discovery of so far undiscovered pathways in the effects of the pharmacological agents. In case of the individual genes we haven't used correction for multiple testing, rather decreased the limit for significance to the mentioned levels.

In summary, we used 3 different methods in the analysis of individual genes to reduce the possible biases known to be attributed to microarray experiments. First, we used BeadArray chips, which were designed to reduce spatial artefacts on microarray plates because of the random localization of the beads on the plate. Second, we included probe-level errors in downstream analyses with the puma package to address the problem of single point estimations of expression values, thereby enhancing the accuracy of the results. This method also allowed us to calculate the MinPplr value, which is a better predictor of the probability of a different expression than conventional p-value. Third, we have reduced the significance criteria to lower levels to address the elevated possibility of false positive results resulting from multiple hypothesis testing.

Please note, that throughout the dissertation genes will not be presented, which were only unconfirmed *loci* at the time of the analysis. We assumed that such genes may unnecessarily add to the complexity of the presented data and raise concerns about the reliability of results, since these genes were only annotated based on sequence homology or similarity with known proteins without any further validation.

4.4.1. Pathway analysis of the MDMA treatment

Besides calculating individual significance levels for each gene, gene set enrichment analysis (GSEA) was performed using GSEA version 3.1 from the Broad Institute at MIT (http://www.broadinstitute.org/gsea) [140, 141]. GSEA is a widely used

method for the discovery of altered molecular pathways instead of individual genes. The basis of GSEA is the use of the so called gene sets, which contain genes grouped together by similarities between them, like common regulatory patterns, biological functions, etc. Based on these similarities multiple gene set databases exist, called molecular signature databases (MSigDBs). After the definition, which gene set database to use, in the next step, GSEA creates a ranked list (based on the respective t-values of the genes after performing hypothesis tests for every transcript between two treatment groups,) to order all the genes on the microarray platform and thereby evaluating their relative expression compared to other transcripts. Thereafter a cumulative value for a given gene set is created; this is the so called nominal enrichment score (NES), which is a summary of the relative expression values of the individual genes belonging to a given gene set [139, 140]. The NES can, thus, be used to assess the magnitude of the alterations of genes grouped by certain criteria.

In the current analysis, gene identifiers used both in the array dataset and in the gene sets, were gene symbols. The entries in the data set were collapsed to gene symbols with the median expression value used for the probe sets. Gene sets were restricted to contain between 15 and 500 genes, since smaller or larger sets may result in statistical artefacts. T-test was used for ranking genes and the permutation type was the gene set, because of the small sample size per treatment group. Number of permutations was set to 1000 with the seed of permutation: 149. Other basic and advanced fields were left as default.

Gene sets (GMT format) from the MSigDB for C5 category (gene ontology [GO] gene sets) were used and in addition, neuronal function related gene sets were selected from the GO homepage (www.geneontology.org) manually.

Normalized enrichment score (NES), nominal p-value and false discovery rate (FDR) were calculated for the gene sets. The latter represents a correction for multiple testing (discussed in 3.4) and basically gives an estimation about how much of the significant results may be false positive [139, 142]. A gene set with a nominal p-value <0.05 and FDR <0.25 was considered statistically significant.

Network visualization and analysis using enrichment results was done using Cytoscape 2.8.3. with its plugin “Enrichment Analyzer”. Following cut-off rates were

used: similarity coefficient cut-off 0.1, p-value cut-off 0.05 and FDR cut-off 0.25 [143, 144].

4.4.2. Pathway analysis of the VLX treatment

Following the evaluation of the effects of MDMA, in case of the VLX treated groups, besides of the above mentioned methodology, we also used textmining methods in NCBI's medical databases to create individual gene sets related to VLX's well-known positive effects in neuropathic pain and migraine [145, 146]. To estimate the relations between genes and the latter diseases the hits of Pubmed queries "<gene name> AND (pain OR neuropath* OR nocicept* OR migraine)" were counted. We wrote R scripts [136] with the genome wide annotation database for Rattus Norvegicus [147] and the GO annotation database [148] from Bioconductor [135] and reversely mapped the genes with hits into the GO hierarchy. In addition we also used the Pain Genes Database (http://www.jbldesign.com/jmogil/enter.html) to create two additional gene sets [149]. This database contains pain related results from knockout mice. We have created one gene set from the genes resulting in decreased nociception (PAIN_DB_DOWN) and one including those genes which knockout resulted in elevated nociception (PAIN_DB_UP) in mice. All the resulting gene sets (altogether 1781, among them 1454 from the MSigDB C5 and 327 individually selected ones) were included in the analysis widening the possibility of the discovery of new pathways.

4.4.3. Analysis of the combined treatment

Following the analyses of the individual treatments we addressed if and how MDMA and VLX may interact in the FC of DA rats. The GSEA results of the double treated animals with the above mentioned methodology resulted almost exclusively in seemingly additive effects. Therefore, we tried to identify those genes which could reflect interactions between the two treatments by ANOVA.

We have used the R package "lmdme: linear model decomposition for designed multivariate experiments" [150], which uses linear models to compare expression values between treatments and calculate interactions between them. The method was eligible for the use of ANOVA in unbalanced designs, the latter being a limitation of

several other methods [150]. The input table contained the normalized results of the experiment obtained following the preliminary filtering of the raw data according to the description in previous chapters. We used a significance criterion 0.001 to obtain statistically relevant results to reveal which genes (unconfirmed *loci* were excluded) may reflect interactions in the double treated group. The figure representing the result was created with the R package ggplot2 [151].

4.5.PCR Validation

Altogether 19 RNA (Table 1) products were validated on a Fluidigm GEx real-time polymerase chain reaction (PCR) 96x96 array (San Francisco, CA, USA).

Table 1 Validated RNA products from the polymerase chain reaction experiment as presented on the GEx array.
The table shows the validated genes for the polymerase chain reaction experiment on the plate. The mRNAs validated were selected based on the results of the microarray experiment of the single-dose 3,4-methylenedioxy-methamphetamine treatment and on the preliminary results for the venlafaxine treatment. Cyclophilin A (PPIA) was selected as internal control. Camk2b - calcium/calmodulin-dependent protein kinase II beta; Camk2g - calcium/calmodulin-dependent protein kinase II gamma; Camk2n2 - calcium/calmodulin-dependent protein kinase II inhibitor 2; Cnr1 - cannabinoid receptor type 1; Dao1 - D-amino acid oxidase; Gria3 - glutamate receptor, ionotropic, AMPA3; Grin2b - glutamate receptor, ionotropic NMDA2B; Hsd11b - hydroxysteroid 11-beta dehydrogenase 2; Igf2 - insulin like growth factor 2; NAC – non assay control, Nr2f6 - nuclear receptor subfamily 2, group F, member 6; NRG1 - neuregulin 1; P2ry6 - pyrimidinergic receptor P2Y6, G-protein coupled, Pla2g2c - phospholipase A2, group 2C; Sipa1l1 - signal-induced proliferation-associated 1 like 1; Slc38a5 - solute carrier family 38, member 5; Slco1a5 - solute carrier organic anion transporter family, member 1a5; Syt8 - synaptotagmin 8; Tap1 - transporter 1, ATP-binding cassette, sub-family B.

	1	2	3	4
A	NAC	NAC	NAC	NAC
B	CNR1	IGF2	PPIA	TAP1
C	SLCO1A5	P2RY6	NRG1	CAMK2N2
D	GRIA3	CAMK2G	CAMK2B	SIPA1L1
E	NAC	NAC	NAC	NAC
F	GRIN2B	DAO1	HSD11B1	NR2F6
G	SLC38A5	NAC	NAC	NAC
H	SYT8	PLA2G2C	NAC	NAC

Table 2 Fold change values for selected genes presented in the thesis on the beadarray and PCR platforms on a logarithmic scale.
The table shows base 2 logarithmic values of fold changes when compared to the SAL/SHAM group for validated and discussed genes on the two different platforms, the Illumina RatRef-12 v1 beadarray expression chip and the Fluidigm GEx real-time polymerase chain reaction (PCR) 96x96 array, respectively. Camk2b - calcium/calmodulin-dependent protein kinase II beta; Camk2g - calcium/calmodulin-dependent protein kinase II gamma; Gria3 - glutamate receptor, ionotropic, AMPA3; Grin2b - glutamate receptor, ionotropic NMDA2B; MDMA - 3,4-

methylenedioximethamphetamine, single-dose, 15 mg/kg i.p.; PCR – polymerase chain reaction; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Genes	log2 fold change on microarray (vs. SAL/SHAM)			log2 fold change in PCR (vs. SAL/SHAM)		
	MDMA	*VLX*	*MDMA/VLX*	*MDMA*	*VLX*	*MDMA/VLX*
Camk2g	0.941	1.047	1.173	0.705	0.689	1.164
Grin2b	0.446	0.579	0.471	0.28	0.287	0.42
Camk2b	0.935	1.139	1.091	0.704	0.289	0.392
Gria3	0.492	0.856	1.001	0.327	0.51	0.941

Genes were selected based on analyses of the MDMA and preliminary results of the VLX treatments. The Taqman Gene Expression assays for the appropriate RNAs were obtained from Applied Biosystems (Carlsbad, CA, USA) and directly sent to Service XS since the validation experiment was also performed by them. Each sample was used *in duplo* (200 ng and 500 ng) following quality control measurements (samples with already degraded or insufficient amount of RNA were excluded). Upon receiving the raw results individually written R scripts built around the cor.test function with default settings were used for the comparison of the fold change values between the microarray and PCR data.

The Pearson correlation coefficients were 0.384 (0.491) and 0.389 (0.465) in the MDMA-, 0.438 (0.552) and 0.421 (0.572) in the VLX-, 0.421 (0.589) and 0.441 (0.602) in the MDMA/VLX for the 200 ng and 500 ng samples, respectively (the Spearman rank correlation coefficients are given in parentheses). In all cases p-values for Pearson's correlation coefficients were below 0.05 (and 0.005 for Spearman rank correlation). For genes discussed and explicitly presented in the thesis the fold change values on a logarithmic scale measured by the two different methods are given in Table 2.

4.6.Availability of supporting data

As a usual requirement of microarray experiments [152], data supporting the results have been deposited in NCBI's Gene Expression Omnibus (GEO) under the GEO Series accession number GSE47541 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47541).

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5. RESULTS

5.1.MDMA

5.1.1. Differentially Expressed Genes after MDMA treatment

Three weeks following an MDMA administration 155 genes were differentially expressed, among them 66 were up- and 89 downregulated, respectively. After the correction for unconfirmed *loci* altogether 87 genes remained, 51 down- and 36 upregulated, respectively (MinPplr < 0.001). Significantly upregulated genes included some related to calcium signaling pathways (Camk2g and Camk1g) and an NMDA-type glutamate receptor, NMDA2B (Grin2b). At the same time, genes related to response to neuronal stress, like the alpha subunit of the heat-shock protein 1 (Hspca) and the heat-shock factor 2 (Hsf2) were downregulated, in addition to the high-affinity glial glutamate transporter (Slc1a3).

5.1.2. Gene Set Enrichment Analysis following the MDMA treatment

According to the GSEA analysis 55 gene sets were enriched after the single-dose MDMA treatment, (including literature-based and Msig DB C5 gene sets). The upregulated gene sets were the “response to hyperoxia”, “positive regulation of synapse assembly”, “regulation of synaptic plasticity”, “growth factor activity” and “dendrite development” gene sets. Thus, dendrite and synapse development and growth factor activity were among the upregulated processes 3 weeks after MDMA administration [153].

At the same time, gene sets related to protein synthesis and localization (e.g. “translation”, “macromolecule biosynthetic process” or “protein localization”), transmembrane transport (e.g. “organic acid transport”, “carboxylic acid transport”, “amino acid transport”) nucleocytoplasmic transport (e.g. “nucleocytoplasmic transport”, “pattern specification process”, “protein import into nucleus”) were downregulated, along with those involved in chromatin maintenance and oxidoreductase activity (see Table 3 for changes with the highest and lowest NESs). To give a biological perspective to these alterations, the gene sets were grouped by biological and neuronal aspects (Table 4). Thus, it became clearly visible that the most prominent alterations were related to protein synthesis and protein localization (with 21 significantly dysregulated GO sets) and 10 gene sets were involved in transport processes [153].

(Please note, individually selected gene sets used in all analyses can be downloaded from the Supplementary table S2 from [153] or at http://www.ncbi.nlm.nih.gov/ pmc/articles/PMC3902429/)

Table 3 The significantly enriched top gene sets in the frontal cortex of Dark Agouti rats 3 weeks after a single dose of MDMA earlier.
The table shows gene sets altered by a single dose of 3,4-methylenedioxi-methamphetamine (MDMA, 15 mg/kg) 3-weeks earlier. In the table Gene Ontology (GO) identifiers, their names, the nominal enrichment scores (NES), nominal p-values (nomP) and the false discovery rates (FDR) are presented. The NES gives an overall estimation about the magnitude of the dysregulation for given set. Adapted from [153].

Significantly enriched gene sets in the frontal cortex after single-dose MDMA administration

GO ID	GO Cathegory	NES	nomP	FDR
GO:0015849	ORGANIC ACID TRANSPORT	-1.750	0	0.175
GO:0046942	CARBOXYLIC ACID TRANSPORT	-1.737	0	0.122
GO:0006412	TRANSLATION	-1.715	0	0.102
GO:0009059	MACROMOLECULE BIOSYNTHETIC PROCESS	-1.702	0	0.081
GO:0007389	PATTERN SPECIFICATION PROCESS	-1.650	0	0.147
GO:0003723	RNA BINDING	-1.640	0	0.137
GO:0016836	HYDRO LYASE ACTIVITY	-1.637	0	0.122
GO:0006886	INTRACELLULAR PROTEIN TRANSPORT	-1.632	0	0.118
GO:0006325	ESTABLISHMENT AND OR MAINTENANCE OF CHROMATIN ARCHITECTURE	-1.624	0	0.116
GO:0016358	DENDRITE DEVELOPMENT	1.383	0.049	0.142
GO:0008083	GROWTH FACTOR ACTIVITY	1.387	0	0.165
GO:0048167	REGULATION OF SYNAPTIC PLASTICITY	1.480	0.025	0.088
GO:0051965	POSITIVE REGULATION OF SYNAPSE ASSEMBLY	1.521	0	0.073
GO:0055093	RESPONSE TO HYPEROXIA	1.547	0.034	0.097

Table 4 Biologically relevant processes according to the gene set enrichment analysis and individual considerations in MDMA treated animals.
Following gene set enrichment analysis (GSEA) gene sets were ordered into relevant biological processes to provide a more comprehensive overview about the effects of a single dose of 3,4 methylenedioxy-methamphetamine (MDMA) 3-weeks earlier, when compared to the control group. In the table biological processes, the number of related gene sets and the directions of alterations are presented (red arrow – upregulations, blue arrow – downregulations) Adapted from [153].

Biologically relevant processes with the number of enriched gene sets in the frontal cortical region

Related biological process	Number of enriched gene sets related to the term	Direction
Protein synthesis and localization	21	↓
Transmembrane transport	10	↓
Nucleocytoplasmic transport	7	↓
Cell growth	6	↑ ↓
Others	4	↑ ↓
Chromatine maintenance	3	↓
Dendrite and synapse development	3	↑
Oxidoreductase acitivity	2	↓

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5.2. Venlafaxine

5.2.1. Differentially expressed genes following chronic venlafaxine treatment

Comparison of the individual transcripts resulted in 381 differentially expressed genes, and 222 remained following the correction for unconfirmed *loci* in the VLX treated group compared to the SHAM controls (MinPplr < 0.005). Among them 23 were already experimentally linked to depression or its therapy. From these, Ace, Cox17, Faah, Gfap, Pyy, Vdac1 were downregulated, while Ascl1, Bcl2, Camk2b, Camk2g, Cd47, Gad2, Gnaq, Gria3, Grin2b, Hcn1, Negr1, Ntrk2, Ntrk3, Ppp3r1, Sv2b, Syn2, Synj2, Vamp1 were upregulated [154].

In addition, 23 genes could have been identified with a possible role in MDD based on the pathomechanism or current antidepressant therapies (candidate genes). The Cntn2, Dpp4 were downregulated while Cdh22, Clstn2, Enpp1, Epha5a, Gas2, Glp1r2, Grin2a, Kif1b, Kif2b, Kif5a, Lphn1, Mmp9, Myo5a, Pdpk1, Pex2, Prdx1, Rims1, Rph3a, Slc2a4, Ucp3, Unc13b [154]. See Table 5 for genes with the highest and lowest fold change values within significantly altered genes. Some prominent genes altered by other antidepressant treatments, like galanin and its receptors, or Galp remained unaltered in the FC of DA rats [155].

Table 5 The top 10 down- and upregulated genes in the frontal cortex of venlafaxine treated animals when compared to the appropriate control. The table shows the top 10 down- (in blue) and upregulated genes (in red) in the chronically venlafaxine treated group (VLX, 40 mg/kg/die via osmotic minipumps) following comparison with the appropriate controls according to their fold change. The table shows the official gene symbols, descriptions, the fold change and minimum probability of positive log ratio (MinPplr), the latter being considered as a measure of significance.

Symbol	Definition	FoldChange	MinPplr
Ap2b1	Rattus norvegicus adaptor-related protein complex 2, beta 1 subunit (Ap2b1), mRNA.	-0.76499185	0.004129173
Ppp1r9b	Rattus norvegicus protein phosphatase 1, regulatory subunit 9B (Ppp1r9b), mRNA.	-0.516356474	0.0000131
Xpmc2h	PREDICTED: Rattus norvegicus XPMC2 prevents mitotic catastrophe 2 homolog (Xenopus laevis), mRNA.	-0.504683165	0.000000612
Rpl37	Rattus norvegicus ribosomal protein L37 (Rpl37), mRNA.	-0.479527801	0.0000602
RGD620382	Rattus norvegicus Nucleoside 2-deoxyribosyltransferase domain containing protein RGD620382, mRNA.	-0.463271622	0.000225963
Mrpl44	PREDICTED: Rattus norvegicus mitochondrial ribosomal protein L44 (Mrpl44), mRNA.	-0.456330866	0.000404078
Pdia3	Rattus norvegicus protein disulfide isomerase associated 3 (Pdia3), mRNA.	-0.455381421	0.000114862
Ict1	PREDICTED: Rattus norvegicus immature colon carcinoma transcript 1 (Ict1), mRNA.	-0.439411351	0.001488574
Faah	Rattus norvegicus fatty acid amide hydrolase (Faah), mRNA.	-0.423527445	0.000783626
Rpl31	Rattus norvegicus ribosomal protein L31 (Rpl31), mRNA.	-0.420025147	0.00000451
Wdfy1	Rattus norvegicus WD repeat and FYVE domain containing 1 (Wdfy1), mRNA.	1.033940597	0.000303253
Mgll	Rattus norvegicus monoglyceride lipase (Mgll), mRNA.	1.051836447	0.000243907
Negr1	Rattus norvegicus neuronal growth regulator 1 (Negr1), mRNA.	1.0643769	0.0000493
Camk2b	Rattus norvegicus calcium/calmodulin-dependent protein kinase II beta subunit (Camk2b), mRNA.	1.07303988	0.000145499
Kcnd2	Rattus norvegicus potassium voltage gated channel, Shal-related family, member 2 (Kcnd2), mRNA.	1.115278532	0.0000809
Rora	PREDICTED: Rattus norvegicus RAR-related orphan receptor alpha (Rora), mRNA.	1.142647751	0.000000151
Myo5a	Rattus norvegicus myosin Va (Myo5a), mRNA.	1.16232232	0.00007
Nfix	PREDICTED: Rattus norvegicus nuclear factor I/X (Nfix), mRNA.	1.186860596	0.000271313
Sv2b	Rattus norvegicus synaptic vesicle glycoprotein 2b (Sv2b), mRNA.	1.23938002	0.000377632
Cdh22	Rattus norvegicus cadherin 22 (Cdh22), mRNA.	1.367400821	0.000327713

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5.2.2. Network analysis following chronic venlafaxine treatment

Besides the analysis of individual genes, again, GSEA was performed for the identification of important pathways [154]. As a result, 525 gene sets were found to be significantly enriched. To be able to interpret this enormous amount, the interactome was clustered and in the following subsections these subnetworks will be presented (see Fig 3).

Neurotransmitter release and uptake. The network contained 20 upregulated gene sets. Functions of the sets are related to neurotransmitter transport and secretion, synaptic endo- or exocytosis and the regulation of these processes. The top 3 gene sets with highest NES values were: “regulation of exocytosis” (NES=1.96), “exocytosis” (NES=1.85), “synaptic vesicle” (NES=1.77).

Neuronal function. The cluster contained 76 different gene sets representing various neuron parts, processes and functions, e.g. synaptic plasticity, synaptosome, neuron migration, neuronal death, terminal button, glutamate signaling, GABA-signaling, Ca-signaling, memory, learning and cognition. The top 3 gene sets with highest NES values were: “terminal button” (NES=2.36), “pallium development” (NES=2.16) and “regulation of long term neuronal synaptic plasticity” (NES=2.15). In addition, two downregulated gene sets were also significant: “response to iron ion” (NES=-1.72) and “negative regulation of neuronal projection development” (NES=-1.45). The second one basically means a downregulation of a negatively regulating gene set, thus in fact, stimulating neuronal projection growth.

Insulin signaling. The interactome was composed of 5 upregulated gene sets and four were directly linked with insulin. These were “insulin receptor binding” (NES=1.55), “phosphoprotein phosphatase activity” (NES=1.28), “protein dephosphorylation” (NES=1.26), “regulation of glycogen biosynthetic process” (NES=1.89).

Mitochondrial antioxidant activity. The cluster contained 9 downregulated gene sets related to superoxide metabolism. From these the top 3 sets were: “superoxide metabolic process” (NES=-1.82), “response to oxygen radical” (NES=-1.78) and “mitochondrial inner membrane” (NES=-1.77). See Fig. 4 for the results of the clustering.

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Other pathways. We have also observed changes in other pathways, like the upregulations of “Wnt receptor signaling” (NES=1.35), “peptide hormone secretion” (NES=1.91) or “cyclic nucleotide phosphodiesterase activity” (NES=1.71).

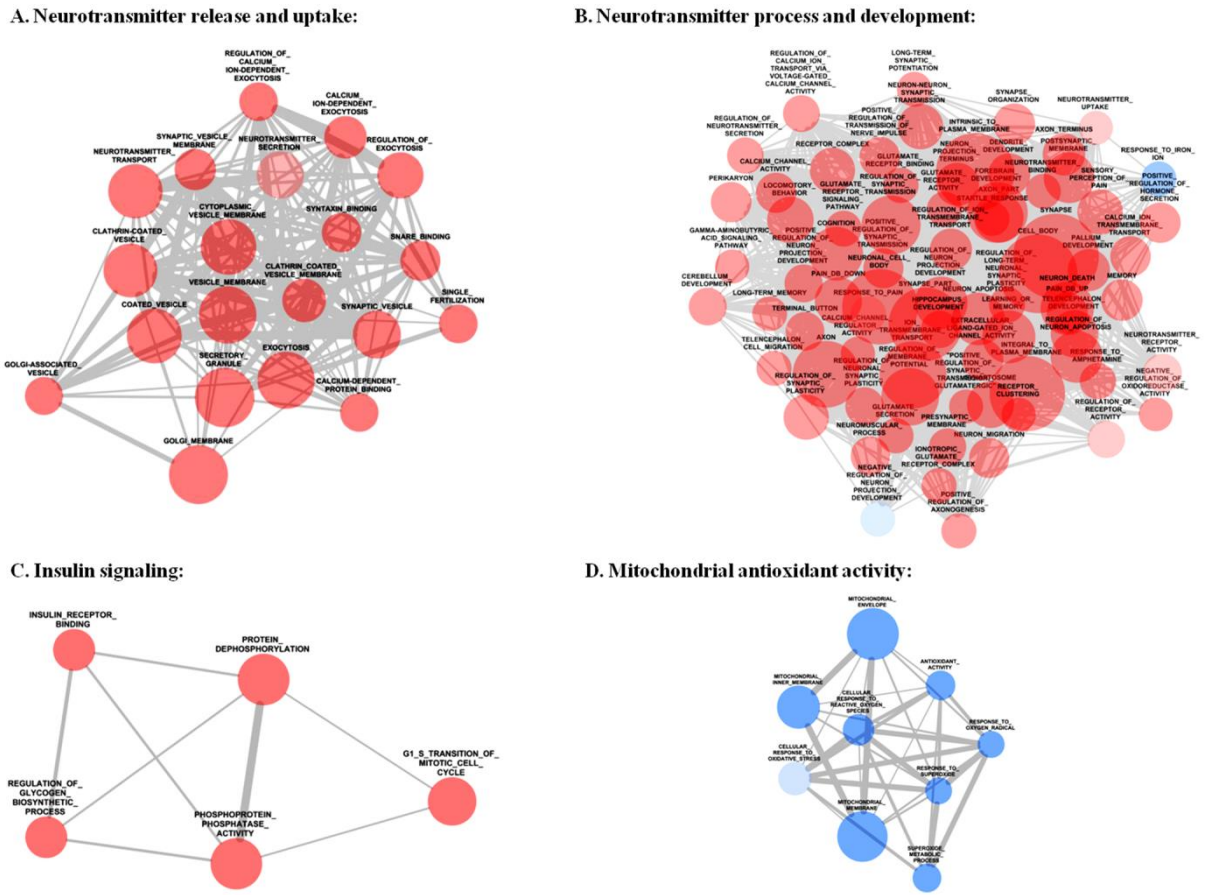

Figure 3 Network analyses of significantly enriched gene sets after three-week-long venlafaxine (40 mg/kg/die) administration in rats when compared to the appropriate control.

Gene Ontology terms (GO) are represented as nodes (red nodes – upregulated sets, blue nodes – downregulated sets). The size of the nodes show the relative size (the number of genes) within a gene set in the cluster, while thickness of the grey edges the number of common genes between the sets. Dysregulations were related to neurotransmitter release and uptake (A), neuronal processes and development (B), insulin signaling (C) and mitochondrial antioxidant activity (D). Some other processes are not represented on the figure. See text for further details. Adapted from [154].

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5.3. The double treatment

5.3.1. Results of the MDMA/VLX vs. MDMA/SHAM comparison

In the double treated group, when compared to the animals which received only a single dose of MDMA, altogether 27 genes were significantly changed (MinPplr < 0.001). Among these only 21 remained following correction for unconfirmed *loci*, 8 downregulated, while 13 upregulated, respectively (See Table 6 for genes with the highest and lowest fold change values). Downregulated genes included: Eif4ebp1, Erp29, while among upregulated genes were Pink1, Il1rapl1, Pou3f2, Slick and Col4a3bp.

Table 6 The altered genes in the frontal cortex of Dark Agouti rats following a combined treatment with a single dose of MDMA and a subsequent 3-weeks long VLX administration and compared to the MDMA treated animals.

Genes were ordered by their fold change values and those with the highest differences from their control counterparts are presented in the table. At the end of the table fold change and as a measure of significance, the minimum probability of positive log ratio (MinPplr) were also given besides the official symbols and descriptions of the genes (blue – downregulated genes, red – upregulated genes). MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Symbol	Definition	FoldChange	MinPplr
Eif4ebp1	Rattus norvegicus eukaryotic translation initiation factor 4E binding protein 1 (Eif4ebp1), mRNA.	-0.648196067	0.000276176
Erp29	Rattus norvegicus endoplasmic retuclum protein 29 (Erp29), mRNA.	-0.54535893	1.98E-05
Ankrd9	PREDICTED: Rattus norvegicus ankyrin repeat domain 9 (Ankrd9), mRNA.	-0.495828864	0.000323866
Banf1	Rattus norvegicus barrier to autointegration factor 1 (Banf1), mRNA.	-0.47524388	0.000763813
Dmp1	Rattus norvegicus dentin matrix protein 1 (Dmp1), mRNA.	-0.427912114	0.000336834
G10	Rattus norvegicus maternal G10 transcript (G10), mRNA.	-0.40642888	0.000137087
Abcc12	Rattus norvegicus ATP-binding cassette, sub-family C (CFTR/MRP), member 12 (Abcc12), mRNA.	-0.386340065	0.000476125
RGD1305622	PREDICTED: Rattus norvegicus hypothetical LOC287173 (RGD1305622), mRNA.	-0.261644942	0.000882308
Mdh1	Rattus norvegicus malate dehydrogenase 1, NAD (soluble) (Mdh1), mRNA.	0.3250082	0.000787173
Pink1	PREDICTED: Rattus norvegicus PTEN induced putative kinase 1 (Pink1), mRNA.	0.33272576	0.000139443
Olr1248	Rattus norvegicus olfactory receptor 1248 (Olr1248), mRNA.	0.353995499	0.000646683
Pum1	PREDICTED: Rattus norvegicus pumilio 1 (Drosophila) (Pum1), mRNA.	0.357560137	3.67E-05
Tbx15	PREDICTED: Rattus norvegicus T-box 15 (Tbx15), mRNA.	0.41250369	0.000622008
Il1rapl1	PREDICTED: Rattus norvegicus interleukin 1 receptor accessory protein-like 1 (Il1rapl1), mRNA.	0.424356406	0.00023561
Pou3f2	PREDICTED: Rattus norvegicus POU domain, class 3, transcription factor 2 (Pou3f2), mRNA.	0.436395703	0.000987391
Slick	Rattus norvegicus sodium- and chloride-activated ATP-sensitive potassium channel (Slick), mRNA.	0.490739038	0.000822134
Col4a3bp	PREDICTED: Rattus norvegicus procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein (Col4a3bp), mRNA.	0.506727796	0.000154309
Eltd1	Rattus norvegicus EGF, latrophilin and seven transmembrane domain containing 1 (Eltd1), mRNA.	0.532711396	0.000147244

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GSEA revealed altogether 47 enriched gene sets in the double treated group when compared to the MDMA treated animals. The upregulated gene sets were (in descending order according to their respective NESs) “dendritic shaft”, “golgi membrane”, “coagulation”, “presynaptic membrane”, “synaptic vesicle membrane”, “positive regulation of tyrosine phosphorylation of stat3 protein”, “cytoskeleton dependent intracellular transport”, “wound healing” and “tyrosine phosphorylation of stat3 protein”.

Besides the 9 upregulated gene clusters, 38 sets were downregulated following comparison of the double treated group to the MDMA-treated group. Spectral clustering of these gene sets resulted in 3 major and some minor networks. The first network was related to *mitochondrial functions* and contained 1 upregulated and 11 downregulated gene sets, among them “hydrogen ion transmembrane transporter activity” (NES=-2.03), “proton transport” (NES=-1.93) and “mitochondrial inner membrane” (NES=-1.87), while the only upregulated gene set was “cytoskeleton dependent intracellular transport” (NES=1.78).

The second major interactome was related to *responses to oxidative stress* with 1 up- and 11 downregulated gene sets, among them “thyroid hormone metabolic process” (NES=-1.87), “cellular response to oxidative stress” (NES=-1.81), “glutathione metabolic process” (NES=-1.81) had the lowest NESs, while the only upregulated gene set within the cluster was “golgi membrane” (NES=1.99).

The third cluster was related to *ribosomal functions* and all of the 7 dysregulated gene sets were downregulated. “Structural constituent of ribosome” (NES=-2.89), “cytosolic small ribosomal subunit” (NES=-2.67) and “cytosolic large ribosomal subunit” (NES=-2.66) showed the lowest NESs among them.

From the smaller clusters one with 5 downregulated gene sets contained primarily gene sets implicated in *biosynthetic processes*, among them with the lowest NESs were: “RNA binding” (NES=-2.19), “ribosome biogenesis” (NES=-1.96) and “hormone activity” (NES=-1.93).

Also a small cluster was formed by the “dendritic shaft” (NES=2.16), “presynaptic membrane” (NES=1.80) and “synaptic vesicle membrane” (NES=1.79) upregulated gene sets.

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A small, homogenous cluster related to a specific pathway of cytokine signaling was formed from two upregulated gene sets: “positive regulation of tyrosine phosphorylation of stat3 protein” (NES=1.79) and “tyrosine phosphorylation of stat3 protein” (NES=1.75).

Fig. 4 summarizes the GSEA results after clustering.

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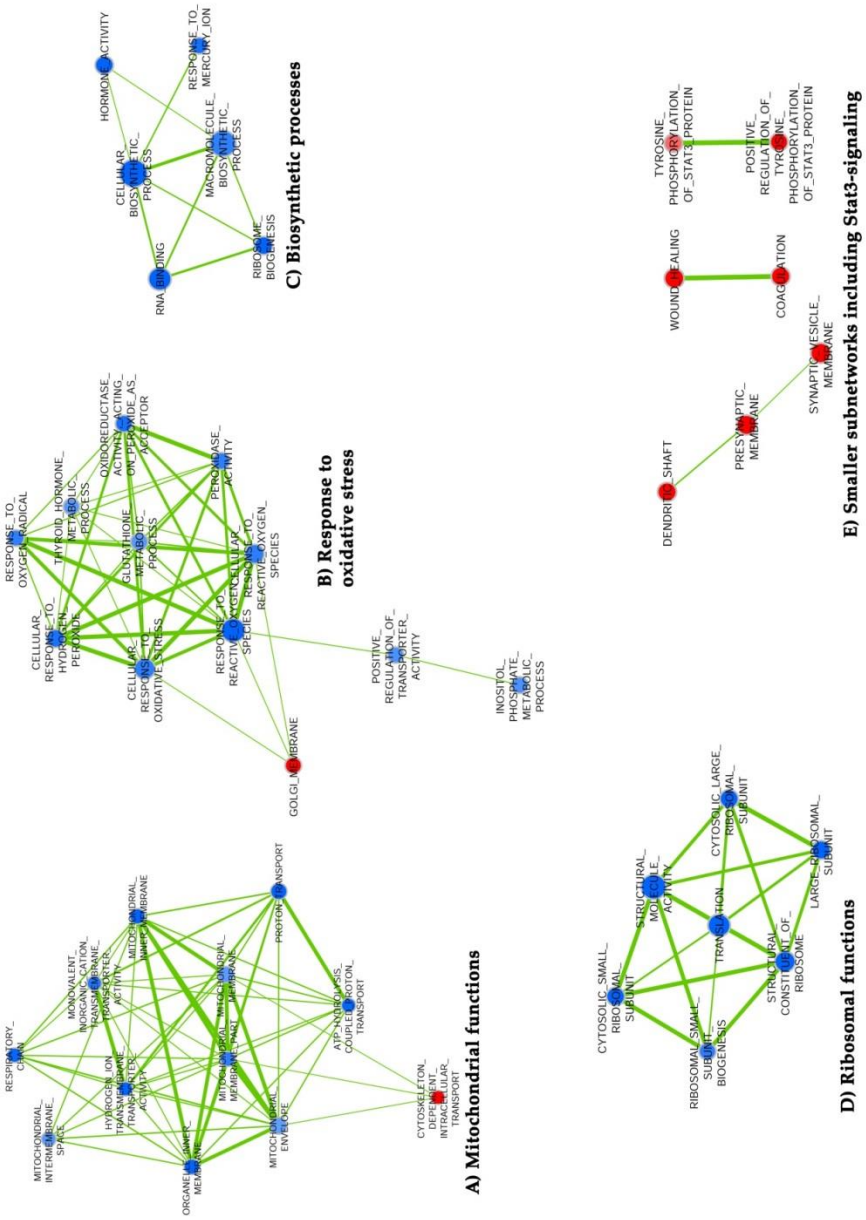

Figure 4 Results of the GSEA after clustering in the frontal cortex of the double treated (single-dose MDMA treatment and subsequent 3-weeks long VLX treatment) Dark Agouti rats when compared to MDMA treated animals.
The subnetworks represent clusters of the gene set enrichment analysis (GSEA) obtained by spectral clustering. The double treatment downregulated gene sets related to mitochondrial functions (A), responses to oxidative stress (B), biosynthetic processes (C) and ribosomal functions (D), while some smaller sets including phosphorylation of Stat3 signaling were upregulated (E). Nodes are gene sets and the thickness of the edges varies with the number of common genes between the sets. For a detailed description see text (red nodes – upregulated gene sets, blue nodes – downregulated gene sets). MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

5.3.2. Results of the MDMA/VLX vs SAL/VLX comparison

When comparing the MDMA/VLX group to those animals, which only received VLX-treatment 31 genes showed altered patterns. Following correction for unconfirmed *loci* only 20 remained and among these 7 were down- and 13 upregulated, respectively (MinPplr < 0.001). The downregulated genes included a few ribosomal proteins (*Rps27a*, *L32*) and thioredoxin 1 (*Txn1*). Genes involved in transcription regulation, like zinc-finger protein (*Znf313*) and the Msx-interacting zinc-finger (*Miz1*), in G-protein signaling, like the regulator of G-protein signaling 9 (Rgs9), and in other processes, like alkaline phosphatase (Alpl) and carbonic anhydrase 2 (Ca2) were upregulated, among others. (Table 7 demonstrates genes with highest and lowest fold change values.)

Table 7 The most altered genes in the frontal cortex of Dark Agouti rats following a combined treatment with a single dose of MDMA and a subsequent 3-weeks long VLX administration and compared to the VLX treated animals.

Genes were ordered by their fold change values and those with the highest differences from their control counterparts are presented in the table. At the end of the table exact values of fold change and, as a measure of significance, the minimum probability of positive log ratio (MinPplr) for the genes, marked by official symbols and description, are also given (blue – downregulated genes, red – upregulated genes). MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Symbol	Definition	FoldChange	MinPplr
Txn1	Rattus norvegicus thioredoxin 1 (Txn1), mRNA.	-0.694702686	0.000140015
Rps27a	Rattus norvegicus ribosomal protein S27a (Rps27a), mRNA.	-0.54282847	0.000103675
Chchd1	PREDICTED: Rattus norvegicus coiled-coil-helix-coiled-coil-helix domain containing 1 (Chchd1), mRNA.	-0.5035197	0.000534769
Srp14	PREDICTED: Rattus norvegicus signal recognition particle 14 (Srp14), mRNA.	-0.48192115	0.000371098
Rpl32	Rattus norvegicus ribosomal protein L32 (Rpl32), mRNA.	-0.47759893	0.000130415
Sec11l3	Rattus norvegicus Sec11-like 3 (S. cerevisiae) (Sec11l3), mRNA.	-0.34156798	0.000482195
Gp1bb	Rattus norvegicus peanut (Drosophila)-like 1 (Gp1bb), mRNA.	-0.27949355	0.000339514
Gm1012	PREDICTED: Rattus norvegicus gene model 1012, (NCBI) (Gm1012), mRNA.	0.270123023	0.000499969
Znf313	PREDICTED: Rattus norvegicus zinc finger protein 313 (Znf313), mRNA.	0.286993948	0.000470716
Olr816	Rattus norvegicus olfactory receptor 816 (Olr816), mRNA.	0.316458759	0.000491524
Usp13	PREDICTED: Rattus norvegicus ubiquitin specific protease 13 (isopeptidase T-3) (Usp13), mRNA.	0.333699851	0.0000216
Olr1248	Rattus norvegicus olfactory receptor 1248 (Olr1248), mRNA.	0.352398154	0.000773262
Alpl	Rattus norvegicus alkaline phosphatase, tissue-nonspecific (Alpl), mRNA.	0.36531257	0.000120945
Miz1	Rattus norvegicus Msx-interacting-zinc finger (Miz1), mRNA.	0.36573897	0.00030819
Pum1	PREDICTED: Rattus norvegicus pumilio 1 (Drosophila) (Pum1), mRNA.	0.388218713	0.00000779
Ca2	Rattus norvegicus carbonic anhydrase 2 (Ca2), mRNA.	0.41194117	0.000856971
Rgs9	Rattus norvegicus regulator of G-protein signaling 9 (Rgs9), mRNA.	0.560238557	0.0000224

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The GSEA revealed 14 downregulated gene sets in the MDMA/VLX treated group, when compared to the SAL/VLX treated animals. No upregulated gene sets could have been found. The downregulated gene sets were (in descending order according to their respective NESs): “structural constituent of ribosome”, “cytosolic large ribosomal subunit”, “cytosolic small ribosomal subunit”, “large ribosomal subunit”, “RNA binding”, “structural molecule activity”, “translation”, “ribosome biogenesis”, “proteasome core complex”, “macromolecule biosynthetic process”, “transferase activity transferring one carbon groups”, “threonine-type endopeptidase activity”, “ribosomal small subunit biogenesis” and “ribonucleoprotein complex”. Table 8 depicts the results of the GSEA.

Table 8 Dysregulated gene sets in the frontal cortex of Dark Agouti rats following a combined treatment with a single dose of MDMA and a subsequent 3-weeks long VLX administration when compared to the VLX treated animals.
The table represents the results of the gene set enrichment analysis (GSEA). Only downregulated gene ontology terms were found in this comparison. In the table besides the nominal enrichment score (NES), which represents the magnitude and direction of changes, nominal p-values (nomP) and false discovery rates (FDR) are also presented. MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Significantly enriched gene sets in the frontal cortex following double treatment when compared to VLX-treated animals

GO ID	GO Cathegory	NES	nomP	FDR
GO:0003735	STRUCTURAL CONSTITUENT OF RIBOSOME	-2.892	0	0.000
GO:0022625	CYTOSOLIC LARGE RIBOSOMAL SUBUNIT	-2.632	0	0.000
GO:0022627	CYTOSOLIC SMALL RIBOSOMAL SUBUNIT	-2.559	0	0.000
GO:0015934	LARGE RIBOSOMAL SUBUNIT	-2.545	0	0.000
GO:0003723	RNA BINDING	-2.346	0	0.000
GO:0005198	STRUCTURAL MOLECULE ACTIVITY	-2.255	0	0.001
GO:0006412	TRANSLATION	-1.988	0	0.040
GO:0042254	RIBOSOME BIOGENESIS	-1.951	0	0.059
GO:0005839	PROTEASOME CORE COMPLEX	-1.933	0.002252	0.065
GO:0009059	MACROMOLECULE BIOSYNTHETIC PROCESS	-1.909	0	0.074
GO:0016741	TRANSFERASE ACTIVITY TRANSFERRING ONE CARBON GROUPS	-1.903	0.002169	0.070
GO:0004298	THREONINE-TYPE ENDOPEPTIDASE ACTIVITY	-1.902	0.004175	0.065
GO:0042274	RIBOSOMAL SMALL SUBUNIT BIOGENESIS	-1.843	0.004149	0.110
GO:0030529	RIBONUCLEOPROTEIN COMPLEX	-1.827	0	0.120

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5.3.3. The results of the MDMA/VLX vs. SAL/SHAM comparison

From the 258 dysregulated genes 131 remained after the correction for unconfirmed *loci*. From these 131 significantly altered genes 40 were downregulated and 91 were upregulated, respectively (MinPplr < 0.001).

The downregulated genes included several ribosome constituents (*Rps23*, *Rpl37*, *Rps3a*, *Rps27a*, *Rpl14*, *Rpl8*, *Rpl32*) and a cytochrome c oxidase subunit (*Cox4i1*), mitochondrial ribosome constituents (*Mrpl48*, *Mrpl42*) and genes involved in proteasome functions (*Psme1*, *Psma6*), in addition to glutathione peroxidase 1 (*Gpx1*) and thioredoxin 1 (*Txn1*).

Collagen-related genes (*Col5a1*, *Col4a2*, *Col27a1*, *Col4a3bp*), cadherins and protocadherins (*Cdh7*, *Pcdhac2*, *Pcdh17*), transcriptional regulators (Zinc-finger proteins: *Zfp36l2*, *Zfp180*, *Zfp462*, but also others: *Nr4a3*, *Pou3f2*, *Rora* and *Sipa1l1*), genes related to GABAergic- (*Nell2*, *Gabrb2*, *Gabrb3*), glutamatergic- (*Gria3*) or G-protein related intracellular mechanisms and signaling (*Gnao*, *Gnaq*) were upregulated, besides potassium channels (*Kcnd2*, *Kcnc2*) and calcium/calmodulin dependent kinases (*Camk2b*, *Camk2g*). The dysregulated genes with highest or lowest fold change values can be found in Table 9.

Besides changes in individual genes, the combined treatment of MDMA and VLX also caused marked upregulations on the level of gene sets. From 258 dysregulated gene sets 220 were upregulated and 38 downregulated in the MDMA/VLX treated group compared to the control animals. Like in the case of the other complex comparisons, we separated these results by spectral analysis to provide a more sophisticated overview and present these clusters in the next section.

Table 9 The most altered genes in the frontal cortex of Dark Agouti rats following a combined treatment with a single dose of MDMA and a subsequent 3-weeks long VLX administration and compared to the control animals.

Genes were ordered by their fold change values and those with the highest fold change differences compared to the control animals are presented in the table. Official gene symbols, the descriptions and fold change and as a measurement of significance, the minimum probability of positive log ratio (MinPplr) values were given. (Blue – downregulated genes, red – upregulated genes), MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Symbol	Definition	MinPplr	FoldChange
Rps23	Rattus norvegicus ribosomal protein S23 (Rps23), mRNA.	0.0001249	-0.856034974
Stmn3	Rattus norvegicus stathmin-like 3 (Stmn3), mRNA.	5.23E-06	-0.837873349
Xpmc2h	PREDICTED: Rattus norvegicus XPMC2 prevents mitotic catastrophe 2 homolog (Xenopus laevis) (Xpmc2h), mRNA.	8.04E-11	-0.831580305
Gpx1	Rattus norvegicus glutathione peroxidase 1 (Gpx1), mRNA.	0.0002528	-0.76760237
Ndufs5b	PREDICTED: Rattus norvegicus NADH dehydrogenase (ubiquinone) Fe-S protein 5b, 15kDa (NADH-coenzyme Q reductase) (Ndufs5b), mRNA.	0.000055	-0.72778929
Rpl37	Rattus norvegicus ribosomal protein L37 (Rpl37), mRNA.	0.0008172	-0.72065918
Txn1	Rattus norvegicus thioredoxin 1 (Txn1), mRNA.	0.000061	-0.715637811
Ranbp1	PREDICTED: Rattus norvegicus RAN binding protein 1 (Ranbp1), mRNA.	0.0000121	-0.705967796
Rps3a	Rattus norvegicus ribosomal protein S3a (Rps3a), mRNA.	8.31E-06	-0.65360496
Ppp1r14a	Rattus norvegicus protein phosphatase 1, regulatory (inhibitor) subunit 14A (Ppp1r14a), mRNA.	0.0000596	-0.639672973
Usp45	PREDICTED: Rattus norvegicus ubiquitin specific protease 45 (Usp45), mRNA.	2.01E-06	1.038499588
Cpd	Rattus norvegicus carboxypeptidase D (Cpd), mRNA.	6.44E-06	1.044686078
Gria3	Rattus norvegicus glutamate receptor, ionotropic, AMPA3 (alpha 3) (Gria3), mRNA.	0.0000237	1.067333548
Camk2g	Rattus norvegicus calcium/calmodulin-dependent protein kinase II gamma (Camk2g), mRNA.	0.0000133	1.11797036
Rora	PREDICTED: Rattus norvegicus RAR-related orphan receptor alpha (Rora), mRNA.	3.92E-06	1.118281641
Gabrb2	Rattus norvegicus gamma-aminobutyric acid receptor, subunit beta 2 (Gabrb2), mRNA.	4.06E-06	1.128507597
Bmpr2	PREDICTED: Rattus norvegicus bone morphogenic protein receptor, type II (serine/threonine kinase) (Bmpr2), mRNA.	0.0001985	1.199518775
Negr1	Rattus norvegicus neuronal growth regulator 1 (Negr1), mRNA.	0.0000245	1.21098146
Kcnd2	Rattus norvegicus potassium voltage gated channel, Shal-related family, member 2 (Kcnd2), mRNA.	0.0000325	1.264834572
Myo5a	Rattus norvegicus myosin Va (Myo5a), mRNA.	0.0000355	1.3352515

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Development of neuronal connectivity

Within those clusters which could be related to the development of connections between neurons the first cluster was related to *axono- and neurogenesis and dendrite development*. The cluster contains 9 significantly upregulated gene sets, among them "regulation of dendrite development" (NES=2.15), "dendrite development" (NES=2.01) and "dendrite morphogenesis" (NES=1.97) gene sets in addition to several other gene sets delineating similar processes.

An additional cluster containing 4 upregulated and a downregulated gene set could be bound to *dendrite projection development*. The "postsynaptic density" (NES=2.17), "dendritic spine" (NES=2.05) and "neuron spine" (NES=2.04) gene sets were among the most upregulated gene sets, while "β-amyloid binding" was downregulated (NES=-1.73).

A smaller cluster representing *cytoskeleton organization and related processes* contains 3 upregulated gene sets, "cytoskeleton dependent intracellular transport" (NES=1.59), "microtubule based process" (NES=1.56) and "cytoskeleton organization and biogenesis" (NES=1.45).

A cluster containing 4 similar processes focusing on *actin cytoskeleton* was also upregulated, including the "actomyosin" (NES=1.73), "stress fiber" (NES=1.63) and "actin cytoskeleton" (NES=1.50) gene sets.

Gene sets related to *synapse formation* and organization were also upregulated and clustered by spectral clustering into one group: "synapse organization" (NES=1.67), "regulation of synapse organization" (NES=1.65) and "positive regulation of synapse assembly" (NES=1.58).

A more heterogeneous group of 5 upregulated gene sets was formed and partially related to *neuron shape and projection development* contained "response to amphetamine" (NES=1.75) "neuronal cell body" (NES=1.67), and "basal plasma membrane" (NES=1.63) gene sets, among others.

Finally, a cluster tightly related to the *development of neuronal connectivity* was upregulated. The 3 gene sets with the highest NESs within the cluster were "cell morphogenesis involved in differentiation" (NES=1.85), "neuron projection

morphogenesis" (NES=1.84) and "cell morphogenesis involved in neuron differentiation" (NES=1.84).

Summary of the clusters related to the development of neuronal connectivity can be seen on Fig. 5.

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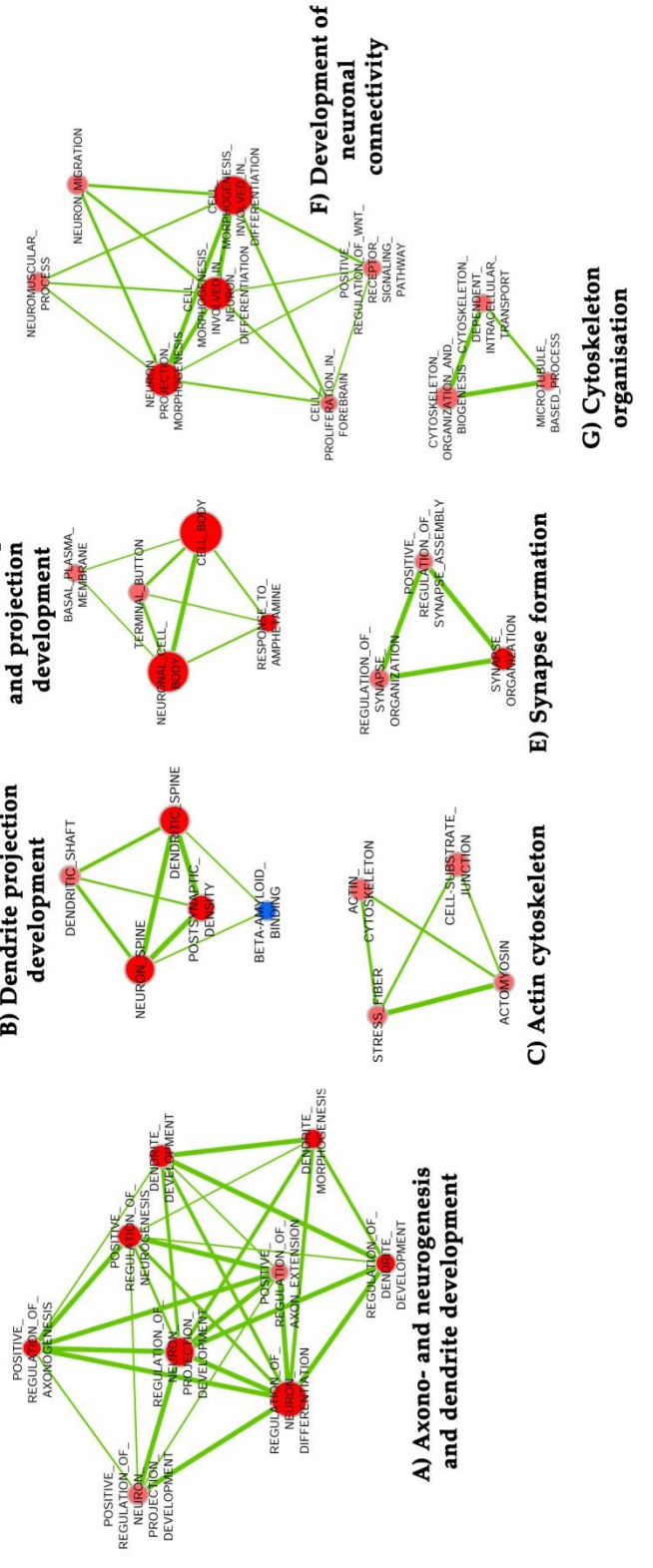

Figure 5 The results of the GSEA analysis on different processes related to neuronal connection development following the combined treatment with a single dose of MDMA and a 3-weeks long VLX treatment and compared to the control group.
Altogether 32 genes sets related to neuronal connection development were upregulated following a combined treatment with a single dose of 3,4-methylenedioximethamphetamine (MDMA, 15 mg/kg, i.p.) and a subsequent 3 weeks-long venlafaxine (VLX) treatment (40 mg/kg via osmotic minipumps) in the frontal cortex of Dark Agouti (DA) rats. These gene sets could be clustered to smaller subnetworks related to axono- and neurogenesis and an overall development of neuronal projections and connectivity (A, B, D, E, F), and as possible underlying processes, to actin cytoskeleton (C) and cytoskeleton organization (G). Nodes and edges visualize gene sets and common genes between the different gene sets, respectively. (red nodes – upregulated gene sets, blue nodes – downregulated gene sets). GSEA – gene set enrichment analysis.

Growth factors, transcription factors and development of brain structures

Altogether 5 clusters formed by exclusively upregulated gene sets seemed to be connected with different developmental processes, transcription factors and growth factors.

A cluster representing 5 upregulated gene sets were related to *transcription factors* including the following 3 gene sets with the highest NES values: “RNA polymerase II. transcription factor binding” (NES=1.75), “RNA polymerase II. transcription cofactor activity” (NES=1.70) and “protein binding transcription factor activity” (NES=1.63).

A heterogeneous cluster from 5 up- and 1 downregulated gene sets could be associated with *growth factor stimulus*. The highest NES was attributed to the gene set: “maternal process involved in female pregnancy” (NES=1.66), which seems to be irrelevant and was probably a result of genes overlapping between the sets (since the base for clustering was the similarity between them). Thus, we provide here the following 3 upregulated gene sets with the highest NESs: “vascular endothelial growth factor receptor signaling pathway” (NES=1.63), “response to growth factor stimulus” (NES=1.53) and “cellular response to growth factor stimulus” (NES=1.51).

Additional clusters were related to differential developmental processes, best described by the following names: inner ear development, development of differential brain structures and embryonic development. While relevance could be attributed to these processes, they probably (similarly to the “maternal process involved in female pregnancy”) only represent that some factors stimulate growth both in the adult brain and during embryonic development. Except these additional clusters see Figure 6 for details.

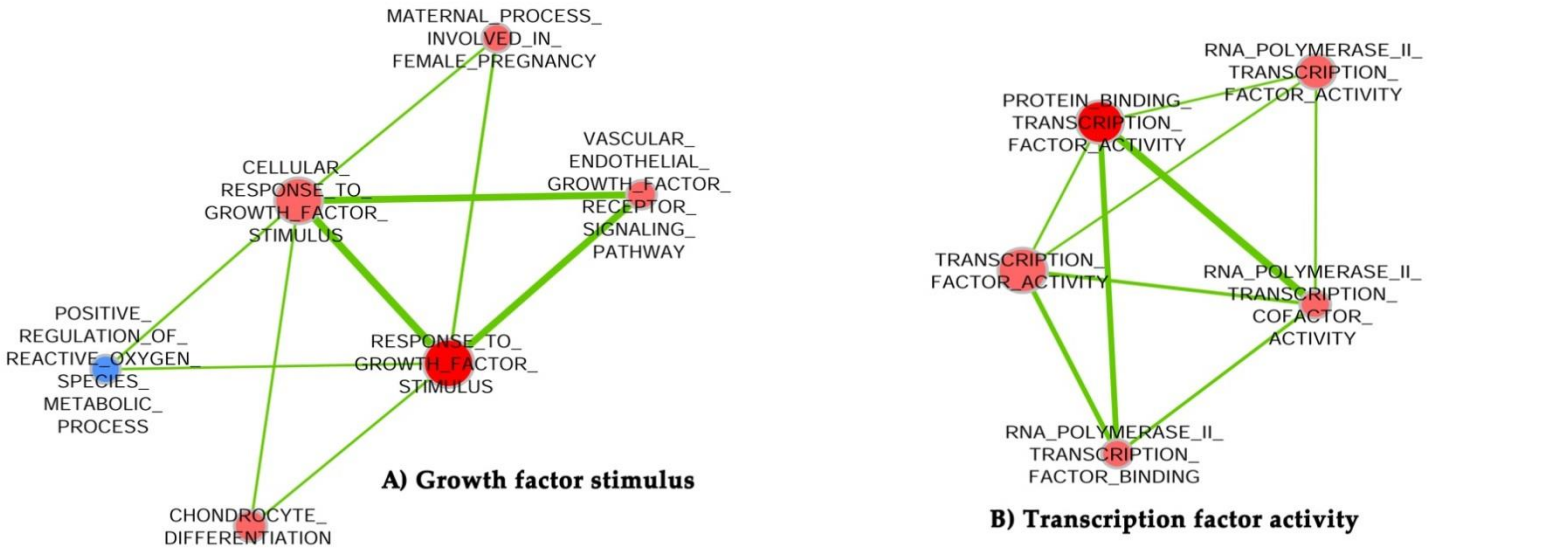

Figure 6 Clusters of growth factor stimulus and transcription factor activity related gene sets in Dark Agouti rats after a combined treatment with a single dose of MDMA and a 3-weeks long VLX treatment and compared to the control group.

Gene sets related to growth factor stimulus (A) and transcription factor activity (B) were almost exclusively upregulated after the combination treatment with 3,4-methylenedioxy-methamphetamine (MDMA, 15 mg/kg, i.p.) and a subsequent 3 weeks-long venlafaxine (VLX) treatment (40 mg/kg via osmotic minipumps) in the frontal cortex of Dark Agouti (DA) rats, as measured by gene set enrichment analysis. The only gene set downregulated "positive regulation of reactive oxygen species metabolic process" is rather related to oxidative processes than to growth factor stimuli and is probably a result of overlapping genes between sets.

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Neurotransmitter release and function

Besides connectivity of neurons, processes of different aspects of synaptic signaling were also upregulated.

A cluster of 6 gene sets related primarily to n*eurotransmitter transport* contained the following 3 gene set with the highest NES: “regulation of exocytosis” (NES=1.77), “syntaxin binding” (1.73) and “snare binding” (NES=1.69).

The synaptic vesicle related 4 upregulated gene sets formed a smaller cluster, the following sets showing the highest NESs: “synaptic vesicle membrane” (NES=1.98), “clathrin coated vesicle membrane” (NES=1.90) and “synaptosome” (NES=1.80).

Accordingly, another cluster related to *cell-cell signaling and synaptic transmission* contained 6 upregulated gene set and among them the highest NESs were attributed to “presynaptic membrane” (NES=1.91), “synaptic transmission” (NES=1.68) and “transmission of nerve impulse” (NES=1.60).

A cluster of 8 gene sets contained only upregulated gene sets and could be related to the *positive regulation of synaptic transmission and synaptic plasticity*. Among the 8 gene sets, the most enriched were the “regulation of long-term neuronal synaptic plasticity” (NES=2.06), “regulation of neuronal synaptic plasticity” (NES=2.05) and “regulation of synaptic transmission” (NES=1.97) sets.

At the same time gene sets related to the *negative regulation of synaptic transmission* formed a smaller cluster, in which all 4 gene sets were upregulated, inclusive “negative regulation of synaptic transmission” (NES=1.79), “negative regulation of neurological system process” (NES=1.79) and “negative regulation of transmission of nerve impulse” (NES=1.73).

Clusters related to neurotransmitter release and functions are presented on Fig. 7.

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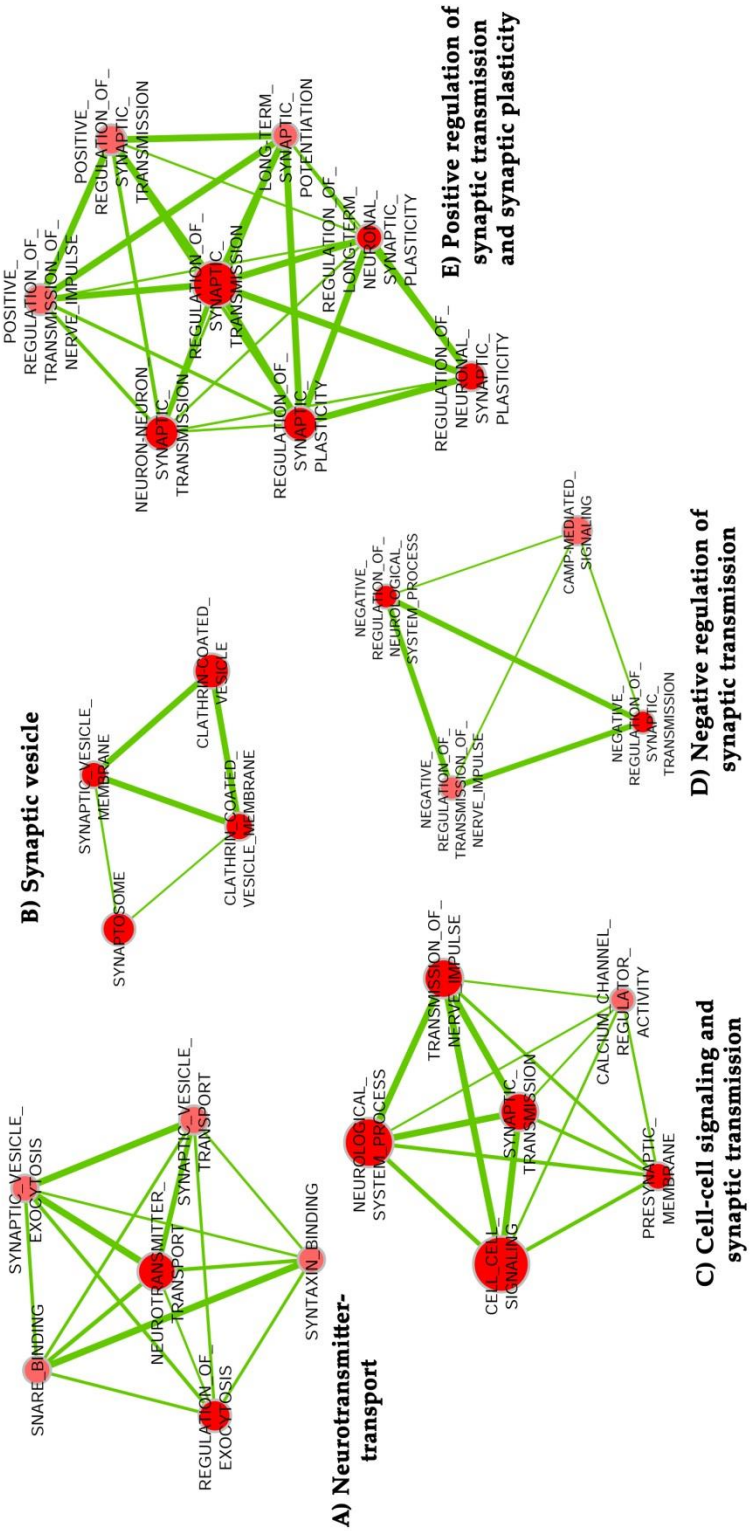

Figure 7 Clusters of the GSEA results related to neurotransmitter release and functions in the frontal cortex of Dark Agouti rats following a combined treatment with a single-dose MDMA (15 mg/kg) and a subsequent 3-weeks long VLX administration (40 mg/kg) when compared to the control (SAL/SHAM) group.

Altogether 28 different gene ontology sets linked to neurotransmitter release and function were obtained by gene set enrichment analysis (GSEA) and were further analyzed by spectral clustering. Results show 5 different clusters related to neurotransmitter transport (A), synaptic vesicles (B), cell-cell signaling and synaptic transmission (C), negative- (D) and positive regulation of synaptic transmission (E). In the resulting networks nodes and edges are gene sets and common genes between the different gene sets, respectively. (red nodes – upregulated gene sets, blue nodes – downregulated gene sets). MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Protein kinases

Altogether 9 different clusters (all of them basically upregulated) were inherently related to kinases.

The *Wnt signaling* cluster contained 4 upregulated gene sets including "negative regulation of protein binding" (NES=1.86), "regulation of protein binding" (NES=1.59) and "Wnt receptor signaling pathway" (NES=1.53) with the highest NES values.

Another specific cluster of 4 upregulated gene sets was related to *phosphorylation of Stat3*, which contained "positive regulation of protein phosphorylation" (NES=1.77), "positive regulation of tyrosine phosphorylation of Stat3 protein" (NES=1.59) and "tyrosine phosphorylation of Stat3 protein" (NES=1.58).

A smaller cluster of 3 exclusively upregulated gene sets represented *MAP-kinase cascades*: "regulation of MAP kinase activity" (NES=1.54), "MAPKKK cascade" (NES=1.50) and "protein kinase cascade" (NES=1.47)

A cluster of 3 upregulated gene sets was also formed, which included the "*calmodulin-dependent protein kinase activity"* (NES=1.67), the "protein serine/threonine kinase activity" (NES=1.85) and the "protein amino acid phosphorylation" (NES=1.79) gene sets.

Other bigger, but less specific clusters included those related to *serine/threonine kinase activity* [one with 5 upregulated gene sets: "protein kinase activity" (NES=1.87), "protein autophosphorylation" (NES=1.83), "kinase activity" (NES=1.77) and another with 5 upregulated gene sets: "protein serine/threonine kinase activity" (NES=1.82), "phosphorylation" (NES=1.67), "receptor signaling protein serine/threonine kinase activity" (NES=1.55)] and *thyrosine kinase activity* [with 3 upregulated gene sets: "transmembrane receptor protein tyrosine kinase signaling pathway" (NES=1.83), "enzyme linked receptor protein signaling pathway" (NES=1.70), "transmembrane receptor protein tyrosine kinase activity" (NES=1.46)]. In addition, another cluster related to *transmembrane receptor protein kinases* contained 4 upregulated gene sets [the 3 with the highest NES values: "phosphotranferase activity alcohol group as acceptor" (NES=1.86), "growth factor binding" (NES=1.77), "transmembrane receptor protein kinase activity" (NES=1.71)]. A heterogeneous cluster containing 3 gene sets was also formed by the analysis: "regulation of lipid kinase activity" (NES=1.64), "cell

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surface receptor linked signal transduction" (NES=1.46) and "positive regulation of oxidoreductase activity" (NES=-1.76).

Some clusters related to kinases are presented on Fig. 8.

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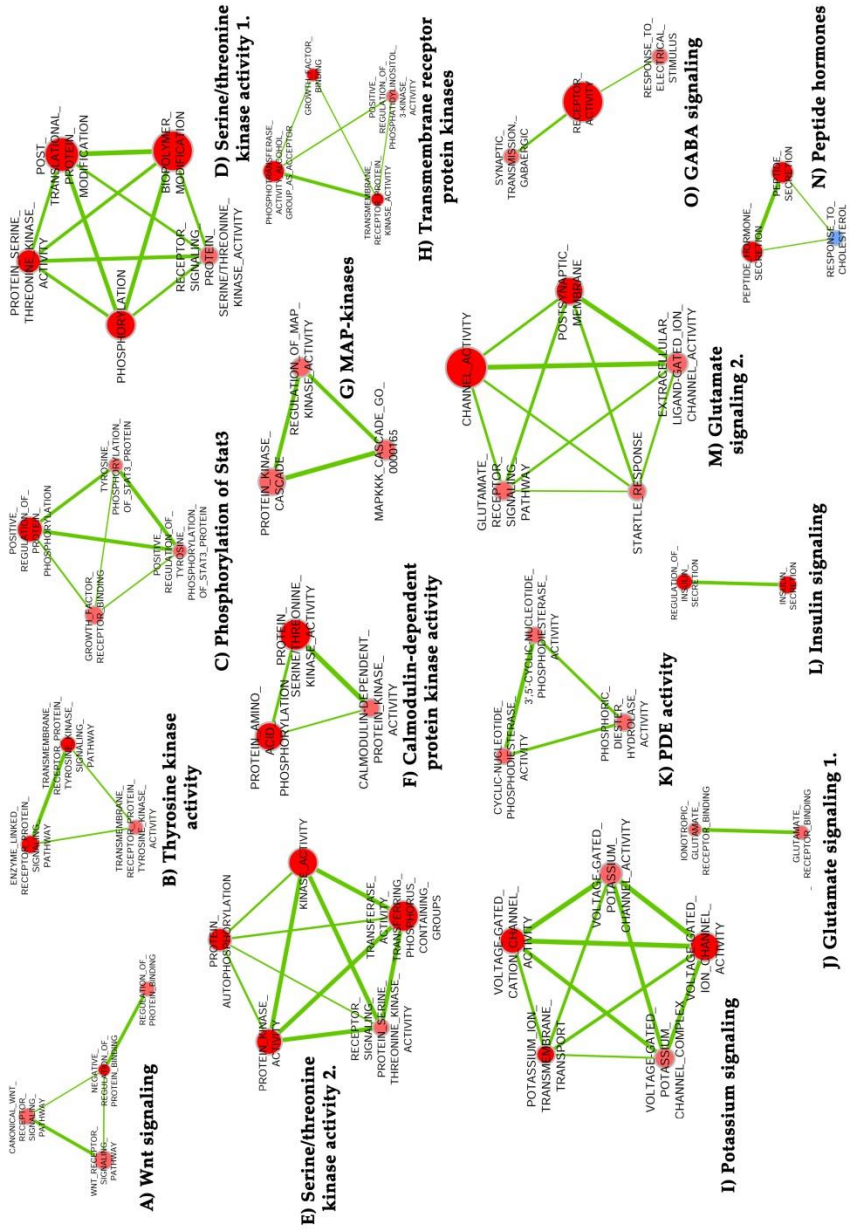

Figure 8 Upregulated clusters of the GSEA results related to various processes in the frontal cortex of Dark Agouti rats following a combined treatment with a single-dose MDMA and a subsequent 3-weeks long VLX administration and compared to the control group.
Smaller clusters, represented here, were composed of significantly enriched gene sets, which could have been linked to some specific pathways, like kinase activity of different types (Wnt signaling (A), thyrosine kinase activity (B), phosphorylation of Stat3 (C), serine/threonine kinase activity (D,E), calmodulin-dependent protein kinase activity (F), MAP-kinase (G), transmembrane recptor protein kinases and growth factor binding (H)) or potassium signaling (I), phosphodiesterase (PDE) activity (K), insulin signaling (L), glutamate signaling (J, M), peptide hormones (N) and GABA signaling (O). Nodes are gene sets and edges symbolize common genes between the different gene sets. (red nodes – upregulated gene sets, blue nodes – downregulated gene sets). GSEA – gene set enrichment analysis; MAP – mitogen activated protein; MDMA – 3,4-methylenedioxy-methamphetamine; single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

Specific signaling pathways

Several clusters could be connected with specific signaling pathways.

One cluster formed from 5 upregulated gene sets was related to *potassium signaling*: "potassium ion transmembrane transport" (NES=1.89), "voltage-gated potassium channel complex" (NES=1.71), "voltage-gated cation channel activity" (NES=1.69) were the 3 gene sets with the highest NESs.

Another smaller cluster composed of 2 up- and 1 downregulated gene set could be connected to *peptide hormones*: "peptide hormone secretion" (NES=1.76), "peptide secretion" (NES=1.70), and "response to cholesterol" (NES=-1.60).

A cluster containing 3 upregulated gene sets was related to *phosphodiesterase (PDE) activity*: "cyclic-nucleotide phosphodiesterase activity" (NES=1.61), "3',5'-cyclic-nucleotide phosphodiesterase activity" (NES=1.60) and "phosphoric diester hydrolase activity" (NES=1.51).

Insulin signaling was represented by a cluster of 2 upregulated gene sets ["insulin secretion" (NES=1.78) and "regulation of insulin secretion" (NES=1.59) and is mentioned here for an adequate comparison with the effects of the VLX-treatment.

Glutamate signaling was represented by multiple clusters [one with 2 upregulated gene sets: "regulation of receptor activity" (NES=1.51) and "ionotropic glutamate receptor complex" (NES=1.51); and another with 2 upregulated gene sets: "ionotropic glutamate receptor binding" (NES=1.54) and "glutamate receptor binding" (NES=1.57); while a bigger cluster with 5 upregulated gene sets was also found: "postsynaptic membrane" (NES=1.75), "startle response" (NES=1.65), "extracellular ligand-gated ion channel activity" (NES=1.57), but also including "glutamate receptor signaling pathway" (NES=1.54)].

GABA signaling was also upregulated, however, was clustered along with 2 less specific gene sets: "synaptic transmission, GABAergic" (NES=1.61), "response to electrical stimulus" (NES=1.55) and "receptor activity" (NES=1.53).

Some clusters related to specific pathways are presented on Fig. 8.

There were additional clusters which will not be described here, because 1) they could not be related to any neuronal processes or 2) were too heterogeneous to provide clear directions or 3) contained less than 3 gene sets besides one of the above conditions. (We

also have to note that the classification of these gene sets and clusters are entirely subjective and several other possibilities also exist. The current classification, however, may help to present the main alterations following the combined treatment.)

Downregulated clusters of gene sets

Among the downregulated gene sets 3 clusters were formed.

Gene sets related to *translation* were clustered into a group which consisted of 7 downregulated and 1 upregulated gene sets. The 3 most downregulated gene sets according to their NESs: “cytosolic large ribosomal subunit” (NES=-2.83), “large ribosomal subunit” (NES=-2.81) and “translation” (NES=-2.48).

In addition, another cluster could also be connected to the latter, contained exclusively downregulated gene sets related to *ribosomal functions and subunits*. Gene sets with the lowest NES values were: “structural constituent of ribosome” (NES=-3.15), “cytosolic small ribosomal subunit” (NES=-2.83) and “RNA binding” (NES=-2.74).

Another cluster formed by 4 downregulated gene sets was related to the *regulation of the response against oxidative stress*: “cellular response to oxidative stress” (NES=-1.94), “oxidoreductase activity acting on peroxide as acceptor” (NES=-1.86) and “peroxidase activity” (NES=-1.85).

Downregulated clusters are represented on Fig. 9.

Other clusters were not presented here based on grounds discussed earlier.

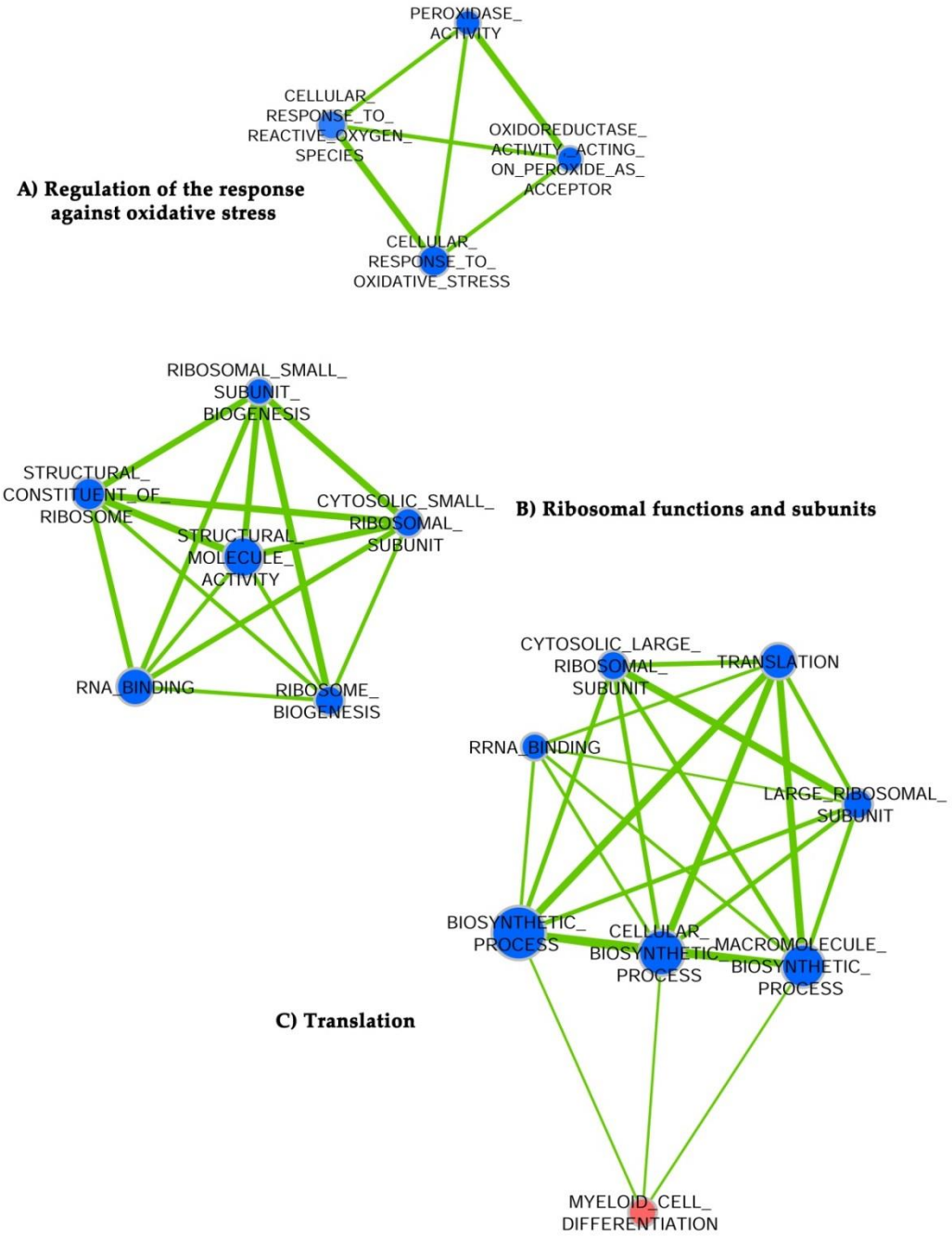

Figure 9 Clusters of mainly downregulated gene sets provided by the GSEA in the frontal cortex of Dark Agouti rats following a combined treatment with a single-dose MDMA and a subsequent 3-weeks long VLX administration and compared to the control group.

Downregulated clusters were composed of significantly enriched gene sets and centered on three basic biological pathways: regulation of the response against oxidative stress, ribosomal functions and subunits and translation. Nodes and edges are gene sets and common genes between the different gene sets, respectively (red nodes – upregulated gene sets, blue nodes – downregulated gene sets). GSEA – gene set enrichment analysis; MDMA – 3,4-methylenedioxy-methamphetamine, single-dose, 15 mg/kg i.p.; VLX – venlafaxine, 3 weeks long, 40 mg/kg via osmotic minimpumps.

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5.3.4. The results of linear models

To assess which genes may mirror interactions between the pretreatment with MDMA and treatment with VLX, we have fitted linear models. Surprisingly, only one gene Tbp, a TATA-box binding transcription factor reflected such interactions with a p-value of 0.0006 and showed the lowest expression in the double treated group. All the other genes showed simple additive effects at the used significance criterion. The mean intensities of Tbp signal after normalization can be seen on Fig. 10.

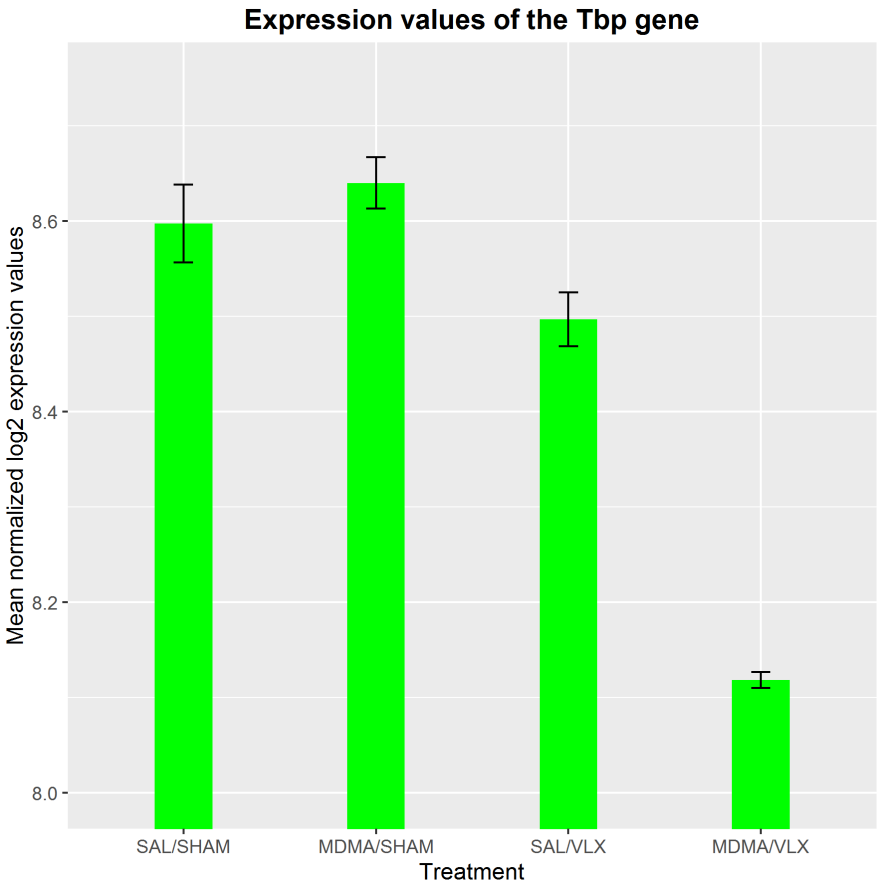

Figure 10 The normalized mean expression values for the Tbp gene in the different treatment groups.
The figure shows the normalized expression values (and SEM) in the control group (SAL/SHAM), the 3,4 methylenedioxy-methamphetamine treated group (MDMA/SHAM, 15 mg/kg MDMA i.p. 3 weeks earlier), the venlafaxine treated group (SAL/VLX, 40 mg via osmotic minipumps for 3 weeks) and in the group with both MDMA injection and subsequent VLX administration as measured with whole-genome microarrays. In the latter group an interaction effect was found with ANOVA and a downregulation is evident. Tbp – TATA binding box protein

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6. DISCUSSION

6.1. The MDMA/SAL vs. SAL/SHAM comparison

Following 3 weeks after a single neurotoxic dose of MDMA we report downregulations of gene sets involved in chromatin organization, nucleocytoplasmic transport, ribosome-related functions, protein synthesis/folding and transmembrane transport processes in the FC region of DA rats (Fig. 11) [153]. These alterations may reflect long-term consequences of the acute neurotoxic effects of the drug, like the toxic metabolite formation, the disturbed autoregulation of the cerebral blood flow or the hyperthermic effect and free radical production (the latter directly supported by the upregulation of the response to hyperoxia gene set). However, besides these negative effects, upregulation of new neurite and synapse formation was also observed.

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Figure 11 Schematic representation of MDMA's actions following a single-dose 3 weeks earlier in the frontal cortex of Dark Agouti rats.
This figure summarizes the effects of a single-dose (15 mg/kg, i.p.) 3,4-methylenedioxy-methamphetamine (MDMA) administration 3 weeks earlier in the frontal cortex of Dark Agouti rats. Significantly downregulated gene sets were ordered according to their main sites of action into the text boxes and red crosses mark the basic sites of alterations suggested by our results. All the changes point to a wide-scale impairment of the cellular machinery. TRP – transport. See text for further details. Adapted from [153].

Thiriet et al. have examined the levels of 1176 mRNAs in the FC of Sprague-Dawley rats following a single-dose MDMA administration at various time points until 7 days [156]. Several alterations could have been observed, though they were usually restricted to close time points after MDMA injections. Plausibly due to differences in the strain, time-scale and dosage regimen between the two studies no common changes can be reported. Martinez-Turillas et al. could observe elevations in BDNF levels within the FC of Wistar rats up to 7 days following MDMA administration, but these changes also diminished by 7 days [63] and again, we were unable to demonstrate similarities in our experimental setup.

We report wide-scale downregulations of biosynthetic processes in the FC of DA rats, following a single neurotoxic dose of MDMA. Besides the requirement for proteins in every intracellular process, translation and its regulation are important contributors to synaptic plasticity and network functions [157, 158]. For the early phase in the potentiation of the connections between neurons the posttranslational modifications of already synthetized proteins are enough, but for long-lasting interactions between cells changes in the levels of macromolecules are a prerequisite. It has been shown that dendrites are main sites for protein synthesis in neurons and these processes are substantially modulated by incoming signals concentrating at the dendritic synapses [159]. Thus, the downregulation in the synthesis of these macromolecules points towards the possibility of an impaired network functioning as a result of neurotoxic effects in the FC of DA rats following an MDMA injection 3 weeks earlier.

One might argue that no specific alteration or pathway could have been identified in the current experiment with a genome-wide approach. Indeed, serotonergic pathways remained unaltered which contradicts the results of previous experiments with the same rat strain and dosing regimen [36, 37]. However, as noted previously, 5-HT damage in the FC may be mild, which was also supported by the fact, that in earlier experiments the decrease was only significant by finely measuring grain densities, but not by autoradiography signal following in-situ hybridization [37]. This suggests that MDMA does not exert its effect at specific targets in the FC at the examined time supporting the possible contribution of the acute, non-specific mechanisms, like free

radical production and disruption of local cerebral blood flow, in its long-term consequences.

The FC region dissected in the present experiment contained parts from primary and secondary motor cortices and some parts of the PFC and is involved in motor functions, cognitive processes and depression pathophysiology [45, 108, 160]. Therefore, the observed alterations may provide the ground of functional consequences of MDMA on the long term. Indeed, studies in the same rat strain reported chronic alteration in motor functions following single-dose MDMA administration [59, 161, 162]. In an fMRI study changes in the right supplementary motor area were also present in human MDMA users accompanied by elevated tremor and increased reaction times [58]. Cognitive decline is also a common result of heavy MDMA use in human addicts and is supported by animal experiments [49, 51, 52, 163]. These functional deficits are in line with our current results, based on the important contribution of frontal lobe functions in cognitive tasks [42, 160]. Third, MDMA users have a greater risk for depression during their lifetime (though causality between the two remains uncertain) [118, 119]. All the above processes require network functionality instead of individual neurons and support rather altered interaction possibilities of neurons than impairments of individual cells.

On the other hand, the latter consequences (MDD, cognitive decline, motor disturbances) are usually only obvious in heavy users suggesting that normally the human brain may, at least partially, compensate for these effects. The upregulation of neurite and synapse formation related gene sets and growth factor activity gene set indicate a partial recovery of FC networks, however, limitedly 3 weeks after a single dose of the drug. Similar processes were supported earlier by others [164] and these changes may be involved in the lack of 5-HT related alterations in the current experiment. The upregulation of some calcium/calmodulin dependent kinases (Camk2g, Camk2b) points toward similar conclusions. Since these proteins have well-established roles in synaptic plasticity, long-term potentiation and cognitive functions (for a review see [165]), they may propose a mechanism, by which a reinstatement of network functions after MDMA-caused damage may occur.

The DA rats used in the current study represent the human poor metabolizer phenotype [5, 28] and the dose used in the experiments corresponds to heavy use in

humans. Our results suggest that MDMA causes neurotoxic effects in such users via the downregulation of gene sets related to biosynthetic processes. These alterations may, through decreased network functionality, lead to the commonly observed functional consequences, while previously reported 5-HT impairments remained insignificant in the current setup. At the same time, upregulation of the gene sets related to synapse/dendrite formation indicates new synapse formation and reorganization in the FC of DA rats, ongoing processes possibly trying to compensate for the neurotoxic effects of the drug 3 weeks after its administration.

6.2. The SAL/VLX vs. SAL/SHAM comparison

To evaluate VLX's effects at a therapeutically relevant time point we analyzed transcriptomic changes in the FC of DA rats after 3 weeks-long VLX treatment (40 mg/kg/die via osmotic minipumps). Chronic VLX administration had positive effects on neurotransmitter release and upregulated neuroplasticity, axonogenesis and cognitive function related gene sets, besides significantly elevated expression of individual genes related to these processes. Interestingly, VLX was also able to alter the expression of genes involved in mitochondrial antioxidant activity and the insulin signaling pathways, suggesting so far unidentified mechanisms of action.

6.2.1. Neurotransmitter release

Gene sets related to neurotransmitter release and reuptake, receptor exo- and endocytosis were upregulated, meaning that most of the genes in the particular gene set were also upregulated. These results suggest that synaptic neurotransmission, thus, concentrations and balance of neurotransmitters in the synapses of the FC were substantially altered. Besides the gene sets, individual genes also supported these results, with marked upregulations in synaptic vesicle-related genes. Synj2, has been shown to be involved in membrane trafficking and its expression was decreased in the temporal cortex of patients with MDD, while in our study the antidepressant VLX upregulated its transcription [166]. Vamp1, a synaptic vesicle docking and/or fusion protein's expression has been increased in rat FC after chronic imipramine or sertraline

treatment [167, 168]. Syn2, a neuronal phosphoprotein involved in the coating of synaptic vesicles and regulating neurotransmitter release was upregulated following lithium treatment [169]. Sv2b was upregulated after imipramine treatment in the FC regions of rats [168]. All of the latter three showed upregulations in our study. In addition, the expression of Ppp3r1, a calcineurin regulatory subunit was also increased. Calcineurin is known to interact with the SERT modulating its plasma membrane expression and influencing 5-HT uptake [170], while the inhibition of calcineurin in the PFC has been shown to induce depressive-like behavior [171]. Other vesicle-membrane related upregulated genes included kinesin-family member proteins (Kif1b, Kif2b and Kif5a) responsible for the transport of organelles, synaptic vesicle precursors, receptors, cell signaling molecules, cell adhesion molecules [172-174], but expression of Myo5a, a myosin V heavy-chain gene was also elevated by chronic VLX, the latter being responsible for vesicle exocytosis and transport [175, 176]. Furthermore, VLX also upregulated Rims1, Rph3a and Lphn1, which regulate synaptic vesicle exocytosis and neurotransmitter release [177-179].

These results suggest that synaptic neurotransmission is significantly elevated following long-term antidepressant use and are in line with the long-proposed theory of adaptive processes in neurotransmitter levels of cortical networks in the effects of antidepressants [78]. While direct serotonergic alterations were not confirmed, our results approved a specific pathway long suggested to be involved in MDD. Faah, fatty-acid amid hydrolase is the well-known enzyme responsible for the degradation of e.g. anadamide, the endogenous cannabinoid. The inhibition of this enzyme was already associated with the suppression of stress and anxiety [180], while its mRNA level was decreased by the chronic VLX treatment applied in our study. Additionally, as another pathway, potassium channels were also upregulated in the present paradigm, namely Kcnk1, Kcnc2 and Kcnd2, the latter identified as a candidate gene in a study of bipolar disorder [181].

6.2.2. Synaptogenesis, neuron migration

VLX upregulated several synaptogenesis and neuronal migration related gene sets, like “neuron migration” (NES=1.72) or “regulation of neuron projection development” (NES=1.44) gene set.

On the gene level Negr1 has already been studied in cerebrospinal fluid of MDD patients and showed upregulations [182]. VLX elevated the mRNA level of this gene in the FC. While this may be a discrepancy between our study and that of Maccarrone et al. there is growing literature about the selective expression of genes in different brain regions, even for individual neurons within distinct areas [183].

Cdh22, a cadherin involved in the axon guidance in spatio-temporal cortices of mice [184]; Eph5a, implicated in the synapse formation and long-term potentiation through the modulation of glutamate signaling [185]; Gas2, found in the subventricular stem cell niche and involved in the apoptosis of neurons [186]; Pex2, a gene involved in abrupt neuronal migration in Zellweger syndrome, when mutated [187] were all upregulated. Chronic VLX treatment downregulated TAG-1 (Cntn2), which serves as migration target for GABAergic interneurons [188]. By blocking it, VLX may act as an inhibitor of neuronal migration for these neurons.

6.2.3. Synaptic plasticity

In the FC of DA rats following chronic VLX treatment in a therapeutic dose “regulation of synaptic plasticity (NES=1.79)”, “synapse organization (NES=1.59)”, “neuron-neuron synaptic transmission (NES=1.71)” or “neuron projection terminus (NES=1.67)” were upregulated.

We found increases in the expression of Trk genes (Ntrk2, Ntrk3). These tyrosine kinases are transmembrane receptors stimulated by neurotrophins (e.g. BDNF, NT-3 or NT-4). They promote neuron survival, while polymorphisms and decreased expression of these genes showed associations with mood disorders [189, 190]. Abnormalities in glutamatergic neurotransmission paralleled MDD [191] and chronic treatment with antidepressants have been shown to influence the glutamatergic system through the AMPA3 receptor in the hippocampus [192], which was also upregulated by our paradigm. Additional genes coding NMDA-receptors were also upregulated, namely

Grin2a and Grin2b, while a polymorphism in the latter was associated with MDD [193]. Besides such direct relations, glutamatergic signaling via these receptors is involved in synaptic plasticity and long-term potentiation [194, 195]. Glutamatergic cation channels, like NMDA-channels activate second messenger systems, related primarily to Ca-signaling. Our results show that Camk2b and Camk2g, calcium/calmodulin dependent kinases were upregulated following chronic VLX treatment. Since these transcripts showed elevated expression in MDMA treated animals, these alterations are probably unrelated to the changes in glutamatergic genes. Whatever the underlying cause, the fact that Camk2 activation accompanies antidepressant-like effects further support the importance of the elevated mRNA levels of these genes in the current experiment [196]. In addition to signaling via ion channels, Gnaq and Gnao showed elevated expression in the FC following chronic VLX treatment. Gnao represents the $G\alpha_0$ unit of G-protein coupled receptors and its activation causes a decrease in intracellular cAMP levels; Gnaq codes the $G\alpha_q$ subunit coupling to 7-transmembrane receptors and is involved in second-messenger systems related to intracellular signaling via phospholipase Cβ [197]. Consequently, G-protein coupled receptors are probably involved in the wide scale changes following chronic VLX treatment. The Cd47 protein participates in the regulation of neuronal networks and Cd47-deficient mice showed prolonged immobility (depression like behavior) in the FST [198]. Mmp9, another gene also induced by VLX treatment, is involved in synaptic plasticity and cognitive processes. Mice over-expressing Mmp9 showed enhanced performance in the novel object recognition and the Morris water-maze tasks and these effects were paralleled by increased dendritic spine density in the hippocampus and the cortex [199]. Astroglial cells may also play a role in the effects of VLX. Gfap, the glial fibrillary acidic protein is involved in the regulation of the shape and function of astroglia [36]. Reductions of Gfap in astrocytes seemed to be involved in MDD [200]. In our study Gfap was downregulated underlining the need for further experiments delineating the exact role of Gfap and astroglia in the pathophysiology of MDD.

6.2.4. Behavior, learning and memory

Many memory associated, significantly altered sets, such as „long term synaptic potentiation" (NES=1.400), „long term memory" (NES=1.65) or "glutamate signaling pathway" (NES=1.699) were upregulated in the FC following chronic VLX administration to DA rats.

On the gene level in addition to the glutamatergic changes discussed in the previous chapter, Gad2, the rate limiting enzyme for the conversion of glutamate to gamma-aminobutyric acid (GABA) was elevated. GABA depletion via reductions in Gad2 levels was suggested to be in connection with MDD in the cingulate cortices of human subjects [201]. Furthermore, GABA and glutamate balances in different brain regions may be substantial in maintaining cognitive functions [202]. Grin2b polymorphisms (a gene already discussed in the previous chapter) were associated with MDD and, thus, suggest a mechanism through which patients may experience cognitive deficits [193]. The upregulation of this gene may be involved to counteract such deficits and improve memory functions, an effect of VLX already proven on the functional level [203]. The inhibition of the brain renin-angiotensin system may have antidepressant effects. On the other hand, Ace, the angiotensin converting enzyme, also plays a role in the degradation of substance P and the elevated levels of the latter were pro-depressive [204]. Thus, downregulation of Ace in our study is a contradictory finding and has to be unraveled in the future. Downregulation of Clstn2, calsyntenin 2, can cause episodic memory deficits in humans [205]. Hence, the upregulation observed in our experiment may contribute to the pro-cognitive effects of VLX. Hcn1 encodes a protein, which controls the way how neurons respond to synaptic input, and is also a "pacemaker protein" because of its oscillatory activity [206]. Additionally, this gene may be important for memory functions, which assumption was supported by the fact, that deletion of this gene caused impaired motor learning and memory deficits in mice [207]. Hcn1 was upregulated in our study. However, the downregulation of this gene in the hippocampus was associated with antidepressant effects [208], emphasizing the importance of region specific gene expression studies.

Genes, which expression levels are altered following learning are numerous. Ascl1 (Achaete scute complex-like 1), was augmented following a Morris water maze paradigm in animals [209] and also showed elevated mRNA levels in our study.

Glp1r2-, Glucagone like peptide 1 receptor deficient mice lacking Glp1r2 in their hippocampus show learning deficits, while mice overexpressing it in the same region show enhanced learning and memory capabilities [210]. Glp1r2 gene was also upregulated in our study.

The sum of alterations related to synaptogenesis, synaptic plasticity, learning and memory is compelling and point to an involvement of these processes in VLX's positive effects within the FC of DA rats. These results strongly support the synaptic theory of depression by suggesting the maintenance and formation of cortical networks following antidepressant use. Furthermore, besides antidepressant properties, these changes may also inherently be involved in the reinstatement of network functionality of motor functions during stroke recovery.

6.2.5. Mitochondrial antioxidant activity

Mitochondrial function was attributed an important role in MDD. Oxidative damage was increased in postmortem brains of human MDD subjects, while the activity of mitochondrial complex I was decreased [211]. Therefore, it is surprising that several gene sets related to mitochondrial functions and reactions to oxidative radicals were downregulated. In addition to the network level, VLX unexpectedly downregulated a member of the terminal mitochondrial respiratory chain complex IV, the copper chaperone (Cox17) and Vdac1, the voltage-dependent anion channel, a mitochondrial outer membrane protein. All these results suggest negative effects of the chronic VLX treatment on these processes (in line with the GSEA results). However, VLX upregulated Bcl-2, an antiapoptotic factor, and Prdx1, peroxiredoxin 1, an antioxidant, suggesting a partially positive effect on mitochondrial functions. Accordingly, in a previous study Bcl-2 was downregulated in the FC of bipolar patients [212], while in mononuclear cells of lithium responder MDD patients lithium could increase the expression of Bcl2 [190].

Thus, on a gene level, VLX seems to stimulate genes, which may have a positive influence, while on the network level (i.e. gene sets) its effects are negative on mitochondrial functions.

6.2.6. Insulin signaling

Patients suffering from diabetes have a higher risk for developing depression and cognitive deficits [213]. There is also a well-known correlation between elevated blood glucose and peripheral neuronal damage [214]. In our experiments, VLX upregulated gene sets related to insulin signaling, e.g. “insulin receptor binding” (NES=1.55) or “G1 S transition mitotic cell cycle” (NES=1.49). On the level of individual genes, the mRNA level of serine exopeptidase, dipeptidyl-peptidase 4 (Dpp4), was downregulated, while its reduced levels fostered neuronal insulin receptor functions and cognitive processes in rats with insulin resistance [215]. Insulin also induced the synthesis of Pdpk1, which was an inducer of PSD-95, the latter being an adapter molecule for ion channel and neurotransmitter receptor clusters and causes enhancement of synaptic transmission in the hippocampus [216]. An elevation of Pdpk1 mRNA levels could be observed in our experimental paradigm. Several other genes, related to insulin functions or signaling were also upregulated. Among them were, Enpp1, which modulates insulin sensitivity [217], Slc2a4, the type 4 glucose transporter [218], Ucp3, the uncoupling protein 3, which prevents glucose-induced transient membrane hyperpolarization in mitochondria, the formation of reactive oxygen radicals and apoptosis [219] or Glp1r2, already discussed previously.

Insulin, via these molecules and also others, is implicated in the normal functions of the cortical neurons and cognitive processes, thus, this pathway may provide a new target in the investigations of current antidepressants and future therapies.

Molecular changes and their nexus in the FC after 3-weeks long chronic VLX treatment are presented on Fig. 12.

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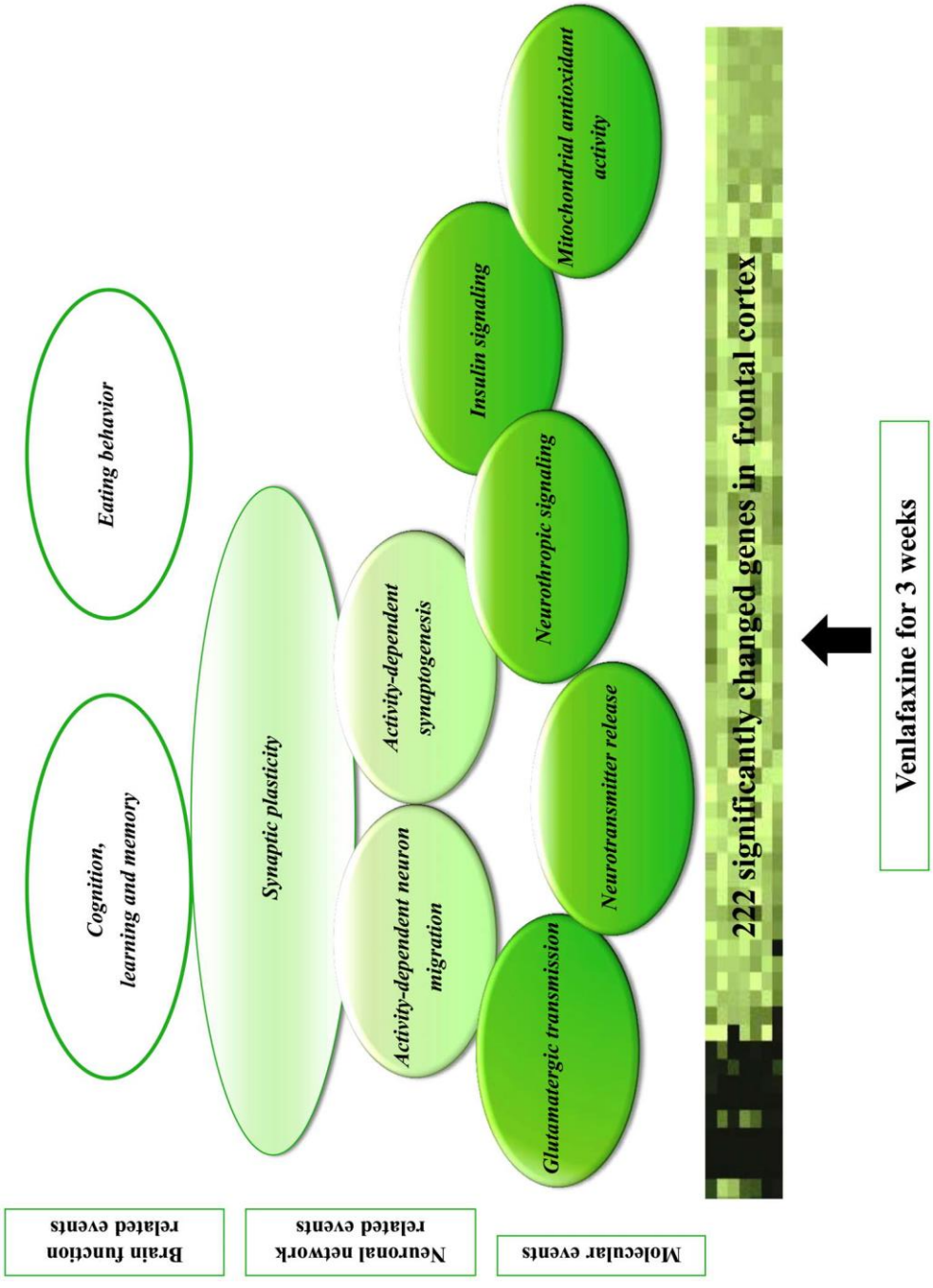

Figure 12 Tiered representation of the effects of a 3-weeks long VLX treatment in the frontal cortex of Dark Agouti rats.
The figure shows how molecular events, e.g. changes in glutamate-, insulin- and neurotrophic signaling may lead to elevated neuron migration and synaptogenesis. As a result, complex functions of the frontal cortex, like cognition will be improved. Adapted from [154].

6.2.7. Other pathways

The elevations in the expression of gene sets related to peptide hormones were implicated in the effects of VLX. In a small human MDD sample mirtazapine altered hormone (among them peptide hormone) levels (including leptin, ghrelin, and cortisol) following 4-weeks long treatment [220]. Nocturnal leptin levels were elevated in patients, suggesting important roles for this peptide in MDD [221], while ghrelin was involved in memory retention providing a potential link for cognitive decline observed in MDD and SSRI treatment [222]. However, these peptides remain largely unevaluated following antidepressant use and mirtazapine has different mechanisms of action than VLX [223]. Here, we demonstrated that (peptide) hormone secretion may be an important contributor to the effects of VLX and that further studies centered on VLX should address it.

Both serotonergic and noradrenergic receptors are G-protein coupled receptors with the exception of 5-HT3. Thus, the upregulation of PDEs which are responsible for the degradation of intracellular second-messengers of G-protein signaling and NO-signaling, like cAMP and cGMP, may reflect the chronic alterations within these pathways. Genes encoding the Gq- and Go-subunit were upregulated after VLX treatment, as already discussed, supporting G-protein signaling involvement in the effects of VLX. However, PDE inhibitors were shown to have pro-cognitive effects and through such consequences may find application in MDD [224]. The upregulation of these gene sets in our experiments may reflect pro-depressive changes. Further studies are definitely required to address the functional implications of these changes and their relations to the NO-pathway and G-protein signaling.

Attenuated Wnt signaling was observed in neuropsychiatric disorders [225] and stimulation of canonical and non-canonical Wnt pathways were also demonstrated to be part of antidepressant effects in the hippocampus [225]. Our results demonstrate that VLX shares this mechanism of action in the FC of rats.

Galanin and its three receptors, GalR1-3 [226-228] and alarin, the product of the Galp gene [229], are all proposed to be involved in antidepressant effects [228, 230, 231]. The SSRIs FLX and sertraline were able to induce changes in galanin or GalR1-3 levels in different brain regions [232-235], thus, the lack of similar effects of VLX in the current experiment (and in other brain regions) [155] may propose a distinction of

VLX's mechanisms of action from those of the SSRIs. Alarin, a product of alternative splicing of the Galp gene, also showed antidepressant properties [230, 231] and remained similarly unaltered in our experiments showing that VLX leaves the galanin system genes unchanged at a therapeutically relevant time point [155].

In summary, besides elevated expression of neurotransmission- and neurotransmitter-related genes and pathways, 3 weeks long VLX treatment stimulated the expression of genes and gene sets of synaptogenesis, synaptic plasticity and cognitive processes. These results suggest that a therapeutically more efficient antidepressant than SSRIs may be able to enhance network functionality and cognitive processes in FC regions of rats. Furthermore, we also identified insulinergic pathways in the FC, which may be a novel mechanism employed by VLX in the latter effects, and excluded the galanin system as a possible explanation for elevated efficacy of VLX compared to SSRIs. In contrast to these positive effects, mitochondrial functions-related genes and sets were downregulated after 3 weeks long treatment, which may underline the need of further experiments focusing on the consequences of these possibly negative alterations.

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6.3. The double treatment

6.3.1. The MDMA/VLX vs. MDMA/SHAM comparison

To evaluate whether the treatment with VLX may compensate for the damage caused by single dose MDMA on the molecular level, we investigated, what changes occur in double treated (MDMA + VLX) rats compared to the MDMA group. The comparison between these animals resulted in the downregulation of mitochondrial functions, biosynthetic processes and translation, besides the upregulation of synaptic vesicle composition and stat3 phosphorylation.

VLX used in the current therapeutic dose is known to cause free radical production in-utero in rats, and as a result, substantial reductions in neocortical thickness and induction of apoptosis [236]. Elevations of Bax, a pro-apoptotic protein was also observed in the same experiment [236]. At the same time, 7 days-long 10 mg/kg VLX treatment was shown to reduce free radical production and restored glutathione levels and catalase activity in whole brain samples of mice, alterations related to the nitric oxide system [237]. In case of the single treatment with VLX in our experiment, Bcl-2, an antiapoptotic factor and prdx1, an antioxidant were also upregulated [154], suggesting a heterogeneous effect on mitochondrial functions and free radical elimination. We have found no compensatory factors (like Bcl-2) among individual genes, which would modulate free radical production and mitochondrial functions when comparing the double treatment to the MDMA/SHAM group. The attenuated responses to free radical burden in the FC may be a consequence of the effects of MDMA 3 weeks earlier, since it is well established that MDMA may cause such effects, even worsened by the acute hyperthermia in human users [5, 7, 238]. In the current setup MDMA alone could only limitedly influence these mechanisms (oxidoreductase activity and electron transport were downregulated in the MDMA treated animals), however, might have prepared the ground for VLX to exert a stronger impact. This means that VLX when administered after a prior MDMA administration, may downregulate mitochondrial functions and the elimination of free radicals further than MDMA alone.

The downregulation of biosynthetic processes has been observed by the sole treatment with MDMA and was identified as primary consequence of MDMA toxicity earlier. Our results demonstrate that an additional VLX treatment may even worsen these effects with 12 additional downregulated gene sets when compared to MDMA alone. One of the key mechanisms for neuronal survival, formation of new synapses and maintaining plasticity in neurons, is translation and protein synthesis [157, 158]. The regulation of translational processes and protein synthesis involve translation factors and their regulatory proteins, e.g. eukaryotic translation initiation factor 4E-binding protein (Eif4ebp1) [158, 239]. Eif4ebp is involved in synaptic plasticity and long-term memory related translation initiation [240]. This gene was significantly downregulated after the combined MDMA/VLX treatment compared to MDMA/SHAM treated animals, supporting our previously discussed findings on the gene set level and emphasizing VLX's contribution to such effects.

A cluster of 3 gene sets implicated in the membrane composition was upregulated. We have already proven such effects following 3 weeks long VLX treatment. These results point out that VLX may enhance synaptic signaling even after previous MDMA administration and treatment with the latter 3 weeks earlier is unable to induce similar effects.

Gene sets implicated in the regulation of Stat3 thyrosine phosphorylation were upregulated in the current comparison without alterations after the individual treatments. Janus kinases and signal transducer and activator of transcription (Stat) cascades are important second messenger systems for the receptors of inflammatory cytokines [241]. Following activation, receptor-associated Janus kinases phosphorylate Stats on a tyrosine residue. Tyrosine phosphorylation causes Stats to arrive in the nucleus, elevate their affinity to the DNA and start transcriptional processes [242, 243]. Stat3 has been implicated in MDD via mediating the effects of the pro-depressive interleukin 6 and thereby influencing SERT levels, while Stat3 inhibition induced antidepressant effects in mice [244]. Thus, the current upregulation of genes involved in tyrosine phosphorylation of Stat3 points toward a possible pro-depressive effect. On the other hand, not always are Stats involved in pathological states. In rats, axotomy in the regenerating facial and hypoglossal neurons, resulted in the upregulation of the Stat3-mRNA 3 hours later, measured by in-situ hybridization and PCR, and the tyrosine

phosphorylation of Stat3 remained evident even after 3 months [245]. Therefore, further studies are required to determine the exact role of the elevation of these gene sets in the effects of VLX following a pretreatment with MDMA.

In previous treatments, results on the gene set level were supported by individual genes, however, such support could only limitedly be found in the case of the comparison between MDMA/VLX and MDMA/SHAM groups. Beside the downregulation of Eif4ebp1, another gene, endoplasmic reticulum protein 29, Erp29, was also downregulated. Erp29 has been found to be involved in the protection of cortical neurons from apoptosis and in the induction of regeneration in the corticospinal tract following spinal cord transection in rats [246].

The PTEN induced putative kinase 1, PINK, was upregulated. Mutations within this gene were associated with psychiatric symptoms (inclusive MDD) in Parkinson patients [247]. Mutations in the interleukin 1 receptor accessory protein-like 1, Il1rapl1, were involved in mental retardation, while the protein is highly abundant in memory related areas within the brain [248]. Its upregulation in the current experiment may point to a possible reinstatement of memory functions by VLX following MDMA injection. Furthermore, Il1rapl1 is a part of the intracellular tail of interleukin 1 receptors and polymorphisms in the interleukin 1 gene were shown to modulate depressive phenotypes and anxiety [249]. The Slick is a Na^+-activated K^+-channel involved in the adaptation of neurons to prolonged stimuli and is usually widely expressed in the cortical layers of rats [250]. Since VLX causes prolonged elevated neurotransmission, Slick may be a part of the adaptive processes following VLX treatment in MDMA pretreated rats. Another mRNA of Col4a3bp, the procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein has also been upregulated. This gene was involved in ceramide regulation and brain development, besides roles in neurodegenerative disorders [251].

The VLX treatment failed to exert positive effects on neuronal function and synapse formation. The lack of such effects may reflect MDMA's similar, though possibly weaker effects (namely that MDMA was also able to induce new neurite and dendrite formation in the cortical networks). These slight alterations may have been enough to curtail VLX's wide-scale effects leaving only the negative consequences of the treatment significant.

As a summary, VLX's effects following a single-dose MDMA injection 3 weeks earlier included the downregulations of mitochondrial antioxidant activity and a more distinct impairment in biosynthetic processes when compared to the single-dose MDMA treatment. Therefore, we may conclude that VLX cannot compensate MDMA-induced similar impairments on a transcriptional level in the FC of DA rats, rather worsen them. Furthermore, probably due to the ongoing regeneration following an MDMA injection 3 weeks earlier, positive effects remained insignificant.

6.3.2. The MDMA/VLX vs SAL/VLX comparison

To further investigate, whether a previous treatment with MDMA is associated with substantial consequences on VLX's effects, the double treated animals were also compared to the VLX treated ones. In the comparison between MDMA/VLX vs SAL/VLX the 11 downregulated gene sets were all related to translation and ribosomal functions, while on the gene level we have found upregulations within antidepressant genes, like carbonic anhydrase 2 and Rgs9.

The downregulation of the gene sets related to translation is in line with our previous results, namely that MDMA's primary effects on the long-run were related to the downregulation of biosynthetic processes [153]. Apparently, MDMA-induced changes are independent of the subsequent VLX administration. Thus, it seems possible that the changes observed in the double treated group reflect those caused by MDMA 3 weeks earlier. However, in the comparison of the MDMA/VLX group with the MDMA/SHAM treated one reflected that VLX may cause similar downregulations within these important cellular functions. Consequently, MDMA-caused damage cannot be reversed, rather worsened by a subsequent VLX treatment.

The positive effects of MDMA on the gene set level, namely, the upregulation of growth factor stimulus and synapse and dendrite development could not be demonstrated in the current comparison. The lack of the significance of these results is a probable consequence of VLX's similar effect, since VLX treatment was used as control in the current comparison.

On the gene level a preceding MDMA administration downregulated the thioredoxin 1 (Txn1) gene. Txn1 was associated with neuronal survival during hypoxic conditions in the developing rat brain in a region dependent manner [252]. The present

downregulations may reflect negative changes associated with the earlier MDMA administration in the double treated animals. Beside Txn1 mRNA, some ribosomal proteins (Rps27a, L32*)* were also downregulated in line with the attenuated biosynthetic and translational processes.

The upregulated genes included zinc-finger proteins (Znf313 and Miz1), which are involved in transcription regulation. The overexpression of Alpl, the tissue-nonspecific alkaline phosphatase (upregulated in the present comparison), induced the expression of neurogenic differentiation markers and microtubule associated transcripts in a neuroblastoma cell line [253]. The upregulation of Alpl in the current setup suggests a positive effect of MDMA on neuron projection development at the gene level. Studies have also proposed roles for the carbonic anhydrase 2 activation in antidepressant mechanisms along synaptic plasticity and positive cognitive effects [254, 255]. A recent systematic review by Chow et al. concluded that carbonic anhydrases play a role in the positive cognitive consequences of SSRIs in Alzheimer patients [256]. The upregulation of a carbonic anhydrase is especially surprising, since no other comparison revealed an alteration of this gene. The Rgs9-2, a human variant of the Rgs9, was upregulated in the current comparison and was shown to modulate sensory and affective symptoms of neuropathic pain including depression [257]. Antidepressants of the SNRI class, like VLX, are known to attenuate neuropathic pain symptoms besides exerting antidepressant effects, binding the overexpression of Rgs9 with therapeutic effects [146].

The above results are somewhat contradictory. On the gene set level, MDMA seems to exert its negative effects on biosynthetic processes. This unfavorable influence is probably even worsened following VLX treatment, a consequence of the net reaction of both MDMA and VLX. On a gene level, however, multiple genes favor the notion that (if we assume that VLX is rather responsible for therapeutically positive effects than MDMA) VLX induces more positive alterations, at least in some gene-level aspects, following a previous MDMA injection. There are three possible explanations.

On one hand, a recent study of Willard et al. has shown that sertraline, an SSRI, prompted different changes in the hippocampus of depressed and non-depressed primates [258]. The authors concluded that antidepressants (or at least sertraline) may exert partially different changes in previously altered networks of depressed subjects

than in networks of healthy animals. While it is unlikely that the entirety of the molecular events behind VLX's effects is different in healthy and non-healthy animals, the beneficial genes which were upregulated may support these assumptions. In the cortical networks of MDMA-treated animals, VLX, at least on the gene level, seemed to show some additional beneficial alterations. Based on the above reasons VLX's main effects may be positively influenced by previously impaired networks.

Second, it seems also possible, that the compensatory changes, started after the initial MDMA injection, may manifest in the upregulation of these transcripts. If so, MDMA and VLX are together responsible for the observed alterations.

Third, gene sets showed no alterations and could not further support this conclusion, thus, the limited number of significantly altered genes suggests a mild and maybe an insignificant influence. Transcriptional microarray methodology is not primarily designed for the exact measurement of absolute expression levels (as already mentioned) and we have not validated the individual genes discussed in the current chapter with another method. However, we have validated our experiment comparing the microarray and PCR data, obtaining significant p-values in all correlations. Therefore, with the notion that further experiments are definitely required to validate the exact role of these genes in the effects of VLX and/or MDMA, they are discussed here, since the main purpose of the study was the exploratory analysis of the presented treatments.

In summary, on a gene set level, previous MDMA-treatment was clearly associated with negative consequences on translational and biosynthetic processes. Changes in the expression of individual genes may represent molecular evidence that previous impairment of cortical networks may stimulate some aspects of VLX-induced beneficial molecular events.

6.3.3. The MDMA/VLX vs SAL/SHAM comparison

Comparison between individual treatments and the double treated groups may reveal consequences of mutual interaction possibilities, but they are not informative about the net effects of the double treatment. To reveal how the combination of MDMA and VLX may influence the transcriptional activity of FC regions in rats, double treated animals were compared to the control group. The primary changes in the current

comparison overwhelmingly mimicked those observed following chronic VLX treatment, like the ones related to neuronal connectivity, neurotransmitter release, brain development, different phosphorylation pathways (e.g. calcium/calmodulin dependent kinases, serine/threonine kinases) or the ones related to specific neurotransmission pathways including glutamatergic, GABAergic neurotransmission, Ca-, potassium- and insulin-signaling. Downregulation in the gene sets related to translation, ribosomal functions or subunits and regulation of the response against oxidative stress could also be observed, as probable results of both MDMA pretreatment and VLX treatment. Differences from the effects of both individual treatments included the Stat3 signaling gene set and the Nr4a3 gene, among others. Interaction was only demonstrated at Tbp, TATA binding box protein, leaving all the other effects of the two treatments additive.

The upregulation of the gene sets also implicated in the effects of VLX show that MDMA is unable to arrest VLX's molecular actions in the FC. While altogether less gene sets were upregulated than in the case of the individual VLX treatment, the composition of these gene sets suggests similar consequences and even similar involved pathways. Glutamatergic, GABA-ergic, insulinergic and Ca-signaling were all upregulated. On network levels it is well-known that glutamate and GABA balance is an important contributor to cognitive skills and, as discussed earlier, imbalances in the glutamate/GABA ratio were associated with stressful reactions in the PFC of rats [202]. The upregulation of both signaling pathways may reflect a maintained balance, besides the activation of these pathways similar to the VLX treatment. While glutamate signaling may be accompanied with elevated intracellular calcium levels and a subsequent activation of calcium/calmodulin dependent kinases, this would require the activation of NMDA channels. However, in the current comparison only the AMPA3 receptors were upregulated, therefore, we assume that the elevated calcium signaling has different origin, like in the case of MDMA. Whatever the underlying cause, as already discussed, calcium/calmodulin dependent kinases are contributors to the maintenance of synaptic plasticity primarily via long-term potentiation. Thus, their upregulations after the combined treatment both on the gene level (Camk2b, Camk2g) and on the gene set level (calmodulin-dependent protein kinase activity) suggest that similarly to the VLX treatment, these kinases play a major part in VLX's effects even after a preceding MDMA administration. The same potassium channels were

upregulated like in the case of chronic VLX treatment, again proposing similar effects. Insulin signaling was also activated, though on a smaller scale. We have already discussed the possible involvement of this pathway in the beneficial therapeutic effects of VLX, however, the limited activation suggests that previous damage to the FC networks imposed by MDMA may limit this mechanism of action. Cadherins and protocadherins are known to influence neuronal connections [259], thus, fit well in the consequences of VLX on network functions.

At the same time, the negative effects of both the individual treatments were also observable. Chronic VLX treatment failed to compensate the downregulation of biosynthetic processes, rather worsened them, observed both on the gene (downregulation of Rps23, Rpl37, Rps3a, Rps27a, Rpl14, Rpl8, Rpl32 ribosomal proteins) and pathways levels (downregulation of translation and ribosomal constituents). Pretreatment with MDMA probably also added (as also discussed earlier) to the negative effects of VLX on mitochondrial processes. All these negative changes suggest additive effects between an acute dose of MDMA and a chronic treatment with VLX in the downregulated molecular pathways.

The overexpression of growth factor stimulus and transmembrane receptor protein kinases may be involved in the wide-scale upregulations observed following combined VLX treatment and MDMA pretreatment, similarly to the sole VLX treatment. It was proven earlier that BDNF levels are elevated following the same administration protocol with MDMA in the DA rat strain, like in the current study [69]. In the recent years it became widely accepted that downregulated trophic- and growth factors may be important participants in depressive symptomatology [260]. Hence, in the case of VLX, the complex network of different growth factors, trophic factors and their receptors, possessing kinase activity, may provide a basis for the restorative functions of VLX in cortical areas. The upregulation in the current comparison may reflect a common direction of changes of MDMA-induced recovery at 3 weeks and chronic VLX-induced therapeutic effects.

The elevated expression in Stat3 was already discussed earlier suggesting restorative functions or immunological activation in this region as a result of the combined treatment.

Some observed transcriptional effects remained unaltered in any other comparisons, but were found to be dysregulated by the combined treatment compared to the control animals. The overexpression of the transcription factor, Nr4a3, was only significant in the current comparison. Nr4a3 has been shown to associate with the attenuation of stroke symptoms and was proposed as a neuroprotective factor [261]. The accompanied upregulation of transcription related gene sets on the pathway level may be a possible consequence of the wide-scale changes in neurotransmission. Transcription is a requirement for the differences observed in the intracellular levels of proteins, which may accompany all the above discussed alterations. The observed downregulation of the translational machinery may even further stimulate transcription, given the fact that they are regulated simultaneously to determine final protein levels and thus, change in Nr4a3 may mirror a counteracting mechanism to the downregulations of translational processes [262].

As a summary, the changes are almost exclusively additive effects of the two individual treatments. MDMA acts after administration through the noradrenergic and serotonergic system, while VLX acutely influences the same monoamines to a lesser extent. At later time points MDMA is known to cause serotonergic toxicity and, at the same time, VLX was proposed to initiate elevated 5-HT and NA levels. Thus, the lack of interactions at 3 weeks is surprising. Therefore, to unequivocally exclude such effects between the two treatments, we have analyzed our data using ANOVA designed directly for microarray experiments. In line with the above results, with a significance criterion of 0.001 only one gene, Tbp showed an interaction effect and was downregulated in the double treated group. According to STRING, a protein interaction database [263], Tbp couples with several different transcription factors, e.g. those involved in rRNA transcription [264], which suggest that Tbp may be involved in the observed alterations of translational processes. In addition, mutations in Tbp gene have already been associated with the onset of schizophrenia and the measurable hypoactivation of PFC in a task examining the executive functions in such patients [265]. Furthermore, Tbp mutations were observed in spinocerebellar ataxia 17, in which psychotic symptoms also commonly occur [265, 266]. Therefore, an interaction culminating in Tbp and resulting in the downregulation of the transcription factor may mirror some negative interactive effects between the two substances.

The lack of other interactions, on the other hand, raises the possibility that the observed alterations are primarily the consequences of VLX's extended mechanism of action, namely the simultaneous influence on noradrenergic neurotransmission besides the 5-HT system on the long-run. Second, it is also possible that MDMA does not unleash long-lasting and serious serotonergic deficit in the FC or recovery processes may compensate for them, a hypothesis partially demonstrated by previous experiments in our laboratory [36, 69] and supported by the current one. Third, it is possible that VLX may also operate with lowered 5-HT levels and not necessarily require a completely intact serotonergic system or cortical networks. In fact, this is the case of MDD patients, in whom serotonergic neurotransmission is already impaired and network functions may be altered. Additionally, since adaptive mechanisms are long-proposed in the actions of different antidepressants, the initial actions at 5-HT and NA transmission may be exacerbated even by other additional pathways and transmitters, like sigma-1 receptors or the NOS-system, both demonstrated to be involved in VLX mechanisms of action [267, 268].

At the same time, it has been proposed that reductions in immobility and elevations in swimming time of Sprague-Dawley rats induced by chronic FLX administration in the forced swimming test were attenuated by pretreatment with MDMA [128]. Similar results were obtained after methylenedioxyamphetamine (MDA) administration and subsequent acute FLX treatment, however, elevated doses of FLX could reverse some effects [269]. Both of the latter studies suggest that prior serotonergic toxicity may undermine the effectiveness of SSRIs. We could not confirm counteracting effects of the two substances on the molecular level in the FC of DA rats. These results suggest that VLX may act differentially than the SSRIs, like in the case of the galanin system [155]. From a therapeutic perspective, through these alterations, the superiority of the SNRI VLX over SSRIs in certain pathological states, centered on the FC, is also raised.

However, besides the positive aspects, the SNRI VLX was clearly unable to counteract downregulations induced by MDMA, rather added its own negative effects on mitochondrial functions or even worsened them via Tbp. These alterations suggest that the MDMA-induced impairments may be lasting and combined serotonergic/noradrenergic manipulations are unable to reverse them. Of course, as net

effect a mixture of positive and negative functional consequences may occur; also suggested by Thompson et al. after FLX treatment in MDMA pretreated rats [127]. It seems possible that VLX may overcome these negative consequences of MDMA, since our results clearly demonstrate that VLX can exert its beneficial effects despite impairments caused by MDMA. Because VLX is a potent antidepressant able to induce positive molecular changes on network levels, the net effect of the combined treatment may be overwhelmingly positive, a promising conclusion for the use of VLX in MDMA users.

In summary, we failed to observe interactions between the two substances, MDMA and VLX, in the FC of DA rats except the transcription factor, Tbp. The main effects of MDMA and VLX are, thus, additive in the observed processes, with primarily positive effects of VLX in terms of synaptic plasticity, neurotransmitter release and cognitive processes and a possible combination of MDMA and VLX in elevated synapse formation. At the same time, neurotoxic consequences of MDMA and superposed negative effects of the antidepressant were also observable. Beside the lack of interactions, the two substances could not counteract each other via their additive effects. All these alterations emphasize the possibility that VLX may be used with therapeutic benefit in cases of cortical diseases in previous addicts, since the drug's main effects seem to remain mostly unaffected by previous MDMA administration.

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7. CONCLUSIONS

1. We could identify impaired protein synthesis and localization and reduced cellular transport processes as possible underlying molecular mechanisms of unselective toxicity triggered by a single dose of MDMA 3 weeks earlier in the FC of DA rats, while 5-HT markers remained unaltered. We could also demonstrate ongoing recovery processes marked by the upregulation of synapse development and growth factor activity.
2. At a therapeutically relevant time point VLX was shown to positively influence neurotransmitter release and synaptic vesicles related genes, supporting the long-term adaptive changes involved in antidepressant actions. It also induced synapse formation related gene sets and genes, supporting the synaptic theory of depression and the use of antidepressants following cortical damage. Insulin-signaling, as a novel pathway involved in the positive effects of VLX in the FC of DA rats was also demonstrated, besides the downregulated mitochondrial functions following 3 weeks-long chronic VLX treatment. Our results have identified the possible underlying mechanisms, by which VLX may exert its positive effects on the gene-, network- and functional levels in the cortex.
3. At a molecular level we have demonstrated through the comparison of MDMA/VLX vs. MDMA/SHAM animals that chronic VLX is unable to reverse MDMA induced neurotoxic effects. Rather a further impairment in mitochondrial functions and translational processes were observable suggesting that VLX can add to some of the negative consequences of MDMA in the FC of DA rats.
4. As demonstrated by the double treatment vs. the VLX treated animals, a previous MDMA treatment negatively regulated biosynthetic processes related gene sets, the main mechanism which may be responsible for the negative effects of the drug. Our results also proposed that VLX could not counteract these alterations on a gene set level. Surprisingly, some individual genes suggested that MDMA-induced toxicity may stimulate elevated expression pattern of antidepressant genes implicating that VLX may act differentially in healthy and impaired brain circuitries.
5. The double treatment compared to the control animals revealed almost exclusively additive changes, proposing that VLX effects on synaptic plasticity, neuronal

connectivity, neurotransmitter release, glutamatergic, GABAergic, calcium-, potassium and insulin signaling will be added to the effects of a pretreatment with MDMA in the FC of DA rats and that the basically positive effects of VLX will be maintained even after previous impairments. The dysregulated gene sets and genes in the double treated group, which remained unaltered by individual treatments, may provide the points where VLX may induce significant effects in the altered cortical networks, e.g. Stat3 signaling or Nr4a3.

6. At the same time, negative effects were also additive, leaving mitochondrial, biosynthetic- and translational processes downregulated. Moreover, they could be even worsened by the combined treatments of MDMA and subsequent VLX treatment in the FC of rats.
7. The only interaction observed was in the transcription factor Tbp. Given the protein's influence on other transcription factors involved in rRNA synthesis, the downregulation in the levels of Tbp may reflect an interaction possibility between the two substances.
8. The observed results suggest that VLX cannot reverse changes caused by a previous MDMA treatment and neither can be VLX counteracted substantially by MDMA on the molecular level in the FC of DA rats. These results point out that the two substances may act independently from each other, while the maintained positive transcriptional effects suggest that VLX may be effective in stroke or MDD patients who previously used MDMA.

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8. SUMMARY

The recreational drug, MDMA, causes non-selective and selective serotonergic toxicity, which influences cortical regions both on neuronal- and network levels. The antidepressant VLX, an inhibitor of 5-HT and NA reuptake, elevates monoamine levels, was shown to activate motor cortices and restored functional deficits mediated via the FC, e.g. in MDD. A previous generation of antidepressants, the SSRIs, were partially beneficial in the reinstatement of MDMA-induced impairments. However, on the molecular level at relevant time points, effects of MDMA, VLX or the combined treatment remained uninvestigated. To reveal interactions and alterations in molecular pathways 3-weeks following a single-dose MDMA administration, after a 3-weeks long chronic VLX treatment or the combination of both, we have performed whole-genome transcriptional microarray analysis in the FC of DA rats. Our results have shown that 1) MDMA caused downregulations in translational and biosynthetic processes and reinstatement of cortical networks; 2) chronic VLX treatment induced wide-scale upregulations in neurotransmission, synaptic plasticity, insulin signaling related gene sets and downregulated mitochondrial antioxidant activity; 3) the combined treatment downregulated biosynthetic processes and mitochondrial antioxidant activity related gene sets, but caused marked upregulations in neurotransmitter release and synaptic plasticity gene sets besides activating Stat3 pathway. Our experiment identified the downregulation of biosynthetic processes as a possible consequence of MDMA caused toxicity and an underlying cause for network dysfunctions. Both adaptive processes and synaptic theory could have been confirmed behind the therapeutic actions of VLX. The combined treatment showed almost exclusively additive effects meaning that VLX and MDMA act independently from each other except in the case of Tbp and downregulated gene sets after the combined treatment may point to impairments probably masked by the positive effects of VLX. We believe that these results revealed important aspects about the mechanisms of action of serotonergic/noradrenergic drugs and may provide the basis for the therapeutic application of SNRIs in stroke or depression even in previous MDMA addicts.

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9. ÖSSZEFOGLALÁS

Az MDMA olyan nem-szelektív és szelektív szerotonerg károsodásokat okoz, amelyek mind neuron-, mind hálózati szinten befolyásolják a kortikális területeket. A 5-HT és NA visszavétel gátló antidepresszáns VLX növeli a monoaminok szintjét, aktiválja a motoros kérget és képes helyreállítani a FC által mediált funkcionális károsodásokat pl. depresszióban. Az antidepresszánsok egy korábbi generációja, az SSRI-k részben hatékonynak mutatkoztak az MDMA-indukálta károsodások helyreállításában. Molekuláris szinten, a fenti hatásokra nézve releváns időpontban, az MDMA, a VLX és a kettő kombinációjának hatásai ismeretlenek. A két anyag közti interakciók és a molekuláris útvonalak változásának felderítésére 3 héttel korábbi MDMA adagolás, 3 hetes krónikus VLX kezelés, vagy a kettő kombinációja után, teljes genom transzkripciós microarray vizsgálatokat végeztünk DA patkányok FC régiójában. Eredményeink alapján 1) az MDMA csökkenti a transzlációs és bioszintetikus útvonalak expresszióját és helyreállító folyamatokat indukál, 2) a krónikus VLX kezelés széles-körű upregulációt okoz a neurotranszmisszió, a szinaptius plaszticitás és az inzulin jelátvitelhez köthető génszettekben és csökkenti a mitokondriális antioxidáns aktivitást, 3) a kombinációs kezelés downregulálta a bioszintetikus folyamatokhoz és mitokondriális antioxidáns aktivitáshoz köthető génszetteket, upregulációt okozott a neurotranszmitter felszabaduláshoz és szinaptikus plaszticitáshoz köthető génszettekben és fokozta a Stat3 jelátvitelt. Kísérletünk a bioszintetikus folyamatok csökkenését azonosította az MDMA-indukálta toxicitás lehetséges következményeként és a hálózati funkciók károsodása mögött meghúzódó okként. Mind adaptív változásokat, mind a szinaptikus teóriát sikerült igazolnunk a VLX terápiás hatásainak hátterében. A kombinált kezelés szinte kizárólag additív hatásokat mutatott, jelezve, hogy az MDMA és a VLX egymástól függetlenül hatnak kivéve a Tbp gén esetén, míg a downregulálódott génszettek olyan károsodásokra utalnak, melyeket a VLX pozitív hatásai elfedhetnek. Véleményünk szerint eredményeink fontosak lehetnek a szerotonerg/noradrenerg vegyületek hatásmechanizmusának megértésében és megalapozhatják az SNRI antidepresszánsok terápiás alkalmazását stroke-ban és depresszióban korábbi MDMA függőkben is.

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11. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

11.1. Journal articles related to the thesis

1. Petschner P, Vas S, Adori C, Ando DR, Balogh B, Gyongyosi N, Kirilly E, Kátai Z, Kovács G, Bagdy G (2010) **Az ecstasy által kiváltott neuronkárosodás és regeneráció funkcionális következményei**. *Addiktológia: Addictologia Hungarica*, **9**(2):103-124.
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11.2. Articles unrelated to the thesis

1. Horváth B, Vas S, Kátai Z, Kostyalik D, Molnár E, Petschner P, Gyertyán I, Bagdy G (2011) **Effect of acute escitalopram treatment on the quantitative EEG of rat in active wake and REM sleep**. *Neuropsychopharmacol Hung*, **13**(Suppl. 2):26-27.
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