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MAARJA KRASS

L-Arginine pathways and
antidepressant action

Department of Physiology, University of Tartu, Tartu, Estonia

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Supervisor: Vallo Volke, MD, PhD, Senior Research Fellow, Department of Physiology, University of Tartu

Reviewers: Enn Seppet, MD, PhD, Professor, Department of General and Molecular Pathology, University of Tartu

Anti Kalda, MD, PhD, Docent, Department of Pharmacology, University of Tartu

Opponent: Andrew Harkin, PhD, Senior Lecturer in Pharmacology & Director of Postgraduate Teaching and Learning, School of Pharmacy and Pharmaceutical Sciences, Trinity College, Dublin, Ireland

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LIST OF ORIGINAL PUBLICATIONS

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I.   **Krass M**, Wegener G, Vasar E, Volke V. Antidepressant-like effect of agmatine is not mediated by serotonin. Behavioural Brain Research 2008; 188: 324–328

II.   **Krass M**, Wegener G, Vasar E, Volke V. The antidepressant action of imipramine and venlafaxine involves suppression of nitric oxide synthesis. Behavioural Brain Research 2011; 218: 57–63

III. **Krass M**, Rünkorg K, Wegener G, Volke V. Nitric oxide is involved in the regulation of marble-burying behaviour. Neuroscience Letters 2010; 480: 55–58

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I.   The author conducted all the behavioural experiments, carried out the statistical analysis and participated in the manuscript development.

II.   The author participated in designing the study, performed the behavioural experiments and neurochemical studies, carried out the statistical analysis and was responsibe for writing the manuscript.

III. The author participated in designing the study, performed the behavioural experiment and neurochemical studies, performed the statistical analysis and was responsibe for writing the manuscript.

ABBREVIATIONS

ADP	adenosine diphosphate
AGAT	glycine amidinotransferase
ANOVA	analysis of variance
ATP	adenosine triphosphate
GAMT	guanidinoacetate N-methyltransferase
cGMP	$3'$–$5'$-cyclic monophosphate
CNS	central nervous system
DA	dopamine
FST	forced swim test
GAA	guanidinoacetate
GABA	γ-amino butyric acid
HPLC	high pressure liquid chromatography
5-HIIA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
L-Arg	L-arginine
MAOI	monoamine oxidase inhibitor
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	nonadrenergic, noncholinergic
NE	norepinephrine
NDRI	norepinephrine and dopamine reuptake inhibitor
7-NI	7-nitroindazole
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOx	nitrite plus nitrate
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
NOS	nitric oxide synthase
NRI	selective norepinephrine reuptake inhibitor
PCPA	*para*-Chlorophenylalanine methyl ester
SARI	serotonin antagonist/reuptake inhibitor
S.E.M	standard error of means
sGC	soluble guanylate cyclase
SNDI	serotonin and norepinephrine disinhibitor
SNRI	serotonin norepinephrine reuptake inhibitor
SSRI	selective serotonin uptake inhibitor
TCA	tricyclic antidepressant
TRIM	1-(2-trifluoromethylphenyl)imidazole
TST	tail suspension test

INTRODUCTION

Depressive disorders represent a major public health problem due to their high prevalence and psychosocial impact, and significant societal costs (Kessler *et al.*, 2003;Olesen *et al.*, 2008;Andlin-Sobocki *et al.*, 2005). There is evidence that only one third of patients respond favourably to the antidepressant drugs. One third does not respond at all, and in clinical trials, at least one third respond to the placebo (Silva, 2005). The percentage of patients exhibiting response and remission to either serotonin norepinephrine reuptake inhibitors (SNRI) or selective serotonin uptake inhibitors (SSRI) in an acute, randomized, double-blind clinical trial is approximately 60% and 35–40%, respectively (Lieberman *et al.*, 2005;Nemeroff, 2006). This observation has led to the almost universal view that although monoamine systems are integral to the mechanism of action of antidepressants, they are not the final common pathway of action. Identifying such pathways represents one future direction in the pharmacotherapy of mood disorders. Likewise, several new hypotheses of depression including impaired brain plasticity (D'Sa and Duman, 2002;Santarelli *et al.*, 2003) and role of neurotrophic factors (Shirayama *et al.*, 2002;Duman, 2004;Tanis *et al.*, 2007) have been proposed, but have so far not resulted in new clinically useful drugs (Pittenger and Duman, 2008). The limitations of current antidepressant drugs have warranted on-going research to identify pharmacological agents and strategies offering a greater therapeutic efficacy. NMDA/L-arginine/nitric oxide (NO) pathway has been implicated in the regulation of anxiety and depression by numerous preclinical studies (Wiley *et al.*, 1995;Volke *et al.*, 2003;Harkin *et al.*, 1999). It has previously demonstrated the possible indirect effect of some clinically used antidepressants in relevant clinical concentrations on nitric oxide synthase (NOS) activity in the hippocampus by means of *in vivo* microdialysis (Wegener *et al.*, 2003). NOS inhibitors have been shown to possess anxiolytic-like (Volke *et al.*, 1995), antidepressant like (Harkin *et al.*, 1999) and anti-psychotic-like properties in animal models. Interestingly, the other metabolite of arginine − agmatine (1-amino-4-guanidinobutane), has also been suggested to serve as a putative neurotransmitter in the brain (Reis and Regunathan, 2000). Administration of agmatine has effects on pain treshold, memory functions and possesses antidepressant- and anxiolytic-like effects in animals (Reis and Regunathan, 2000). The aim of the current study was to futher evaluate the role of L-arginine pathways in the mechanism of depression.

REVIEW OF LITERATURE
1. Major depressive disorder

1.1. Characteristics and prevalence of depression

Depression is a frequently seen psychiatric illness resulting in loss of psychosocial ability. It is a serious public health problem with high morbidity and mortality and it also increases the risk of co-morbidity. The prevalence of depression during life is 17–19% and the risk of committing suicide during depression is estimated to be 15% (Kessler *et al.*, 2003). The multiple symptoms of depression include anhedonia, depressed mood, inappropriate guilt, apathy, fatigue etc. Depressive disorders represent a major public health problem due to their high prevalence and psychosocial impact, and significant societal costs (Kessler *et al.*, 2003;Olesen *et al.*, 2008;Andlin-Sobocki *et al.*, 2005). Although the monoamine hypothesis has been the mainstay of depression research for many years (Schildkraut, 1965;Heninger *et al.*, 1996), the underlying pathophysiological mechanisms of depression remain obscure. The strength of monoamine hypothesis is that virtually all available antidepressant treatments, pharmacological as well as non-pharmacological (e.g., Electroconvulsive Therapy), affect the monoaminergic neurotransmission in the brain. More recently, it has been postulated that monoamines are merely modulators mediating the antidepressant effect of currently used drugs, and that downstream molecular events are more important in the pathophysiology of depression (Pittenger and Duman, 2008).

1.2. Antidepressants

Depression is a complex, heterogeneous disorder, and the mechanisms underlying its pathogenesis are not that clear and are subject of intensive investigation using pharmacological and genetic tools and animal models (Mcewen *et al.*, 2010).

The "monoamine hypothesis" of depression, which involves imbalances in serotonergic, noradrenergic and possibly dopaminergic functions, has dominated notions and explanations of the pathophysiology of depression since the empirical discovery of the antidepressant properties of monoamine oxidase inhibitors (MAOIs) and tricyclics about fifty years ago. Although the monoaminergic neurotransmitters serotonin (5-HT), noradrenaline (NA) and dopamine (DA) are undoubtedly involved, it is now recognized that monoamine deficits are only part of the story and may not be sufficient on their own to explain the mechanism of action of antidepressants.

Although a range of antidepressant medications are available, a substantial number of patients either do not respond adequately to these or are unable to tolerate their adverse effects. As a basic understanding of depression the monoamine hypothesis was formulated in the mid 1960s based on the anti-depressant efficacy of the monoamine reuptake inhibitors, the monoamine oxidase inhibitors and the depressogenic effects of reserpine as a monoamine depleter (Belmaker, 2007). This hypothesis suggests a deficiency or imbalances in the monoamine neurotransmitters, such as serotonin, dopamine and nore-pinephrine, as the cause of depression. Among therapeutic agents, many anti-depressants including tricyclics, monoamine oxidase inhibitors and selective serotonin reuptake inhibitors exert their therapeutic action through their ability to increase the synaptic content of monoamine neurotransmitters (Morilak and Frazer, 2004). However, antidepressants exert their therapeutic action only after chronic treatment, indicating that enhanced 5-HT or NE neurotransmission *per se* is not responsible for the clinical actions of these drugs. Second, anti-depressants are effective in less than 50% of patients (Nestler *et al.*, 2002; Berman *et al.*, 2000), which suggests that additional biological substrates could provide potential therapeutic targets. Novel molecules include other possible mechanisms like noradrenalin and selective serotonin antagonists or melatonin receptor agonist and 5HT2C receptor antagonist agomelatine (San and Arranz, 2008;Dubovsky and Warren, 2009). Many new targets are also suggested, e.g., small studies have suggested that NMDA receptor antagonist ketamine is able to improve the symptoms of depression within hours (Berman *et al.*, 2000). Due to multiple mechanisms of actions, the classification of antidepressants is complicated. One possible mechanism based classification of antidepressants has been given in Table 1.

Table 1. Classification of antidepressants (Stahl, 2008)

Mechanism	Abbrevations	Example
Serotonin selective reuptake inhibitors	SSRIs	Fluoxetine, Sertraline, Paroxetine, Fluvoxamine, Citalopram, Escitalopram
Serotonin norepinephrine reuptake inhibitors	SNRIs	Venlafaxine, Desvenlafaxine, Duloxetine, Milnacipran
Norepinephrine and dopamine reuptake inhibitors	NDRIs	Bupropion
Selective norepinephrine reuptake inhibitors	NRIs	Reboxetine, Atomoxetine
Alpha 2 antagonists as serotonin and norepinephrine disinhibitors	SNDIs	Mirtazapine
Serotonin antagonist/reuptake inhibitors	SARIs	Trazodone, Nefazodone
Classic antidepressants: monoamine oxidase inhibitors	MAOIs	Phenelzine, Tranylcypromine, Isocarboxazid, Amphetamines, Isocarboxazid, Amphatamines, Moclobemide
Classic antidepressants: tricyclic antidepressants	TCAs	Clomipramide, Imipramine, Amitriptyline, Nortriptyline, Protriptyline, Maprotiline, Amoxapine, Doxepine, Desipramine, Trimipramine, Dothiepin, Lofrepramine, Tianeptine

2. L-Arginine pathways

L-arginine has been classified as a 'semi-essential' or 'conditionally essential' amino acid (Wiesinger, 2001). This characterization alludes to the fact that arginine can be synthezised in a sufficient quantity in the healthy adult. However, it has to be extracted from the diet as a supplement to the endogenous synthesis in growing mammals, during a disease or trauma (Wiesinger, 2001). *De novo* synthesis of arginine depends on the presence of ornithine carbamoyltransferase which, together with carbamoylphosphate synthetase I is located in the mitochondrial matrix. It is one of the more metabolically versatile amino acids, giving rise to nitric oxide, urea, ornithine, citrulline, creatine, agmatine, glutamate, proline, and polyamines (Wu and Morris, 1998). It is therefore no surprise that its metabolism is complex and highly regulated.

De novo synthesis of arginine from citrulline occurs primarily in the proximal tubules of the kidney (Wu and Morris, 1998).

L-arginine can be catabolized by 4 sets of enzymes in mammalian cells: nitric oxide synthases, arginases, arginine:glycine amidinotransferase, and arginine decarboxylase. These L-arginine pathways have been outlined in Figure 1 and 2.
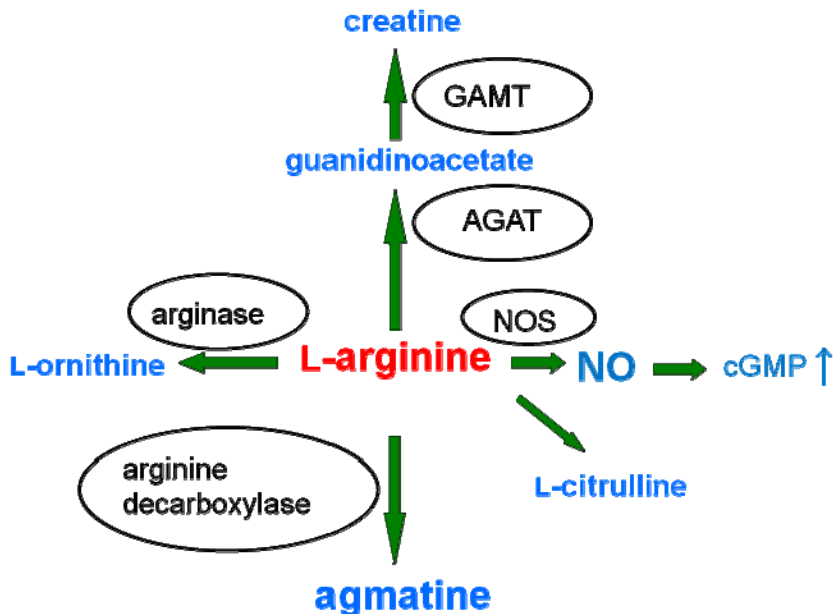
Figure 1. Pathways metabolizing L-arginine

Nitric oxide synthases:

$$\text{arginine} + O_2 \longrightarrow \text{citrulline} + NO$$

Arginases:

$$\text{arginine} \longrightarrow \text{ornithine} + \text{urea}$$

Arginine:glycine amidinotransferase:

$$\text{arginine} + \text{glycine} \longrightarrow \text{ornithine} + \text{guanidinoacetate}$$
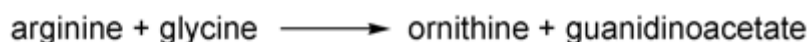
Arginine decarboxylase:

$$\text{arginine} \longrightarrow \text{agmatine} + CO_2$$

Arginine deiminase:

$$\text{arginine} \longrightarrow \text{citrulline} + NH_4^+$$

Figure 2. Biochemical reactions of L-arginine metabolism (Morris, 2004)

Arginine is involved in multiple metabolic processes that play important roles in a very wide range of physiological and pathophysiological conditions. Poly-amines have been described as modulators of ion channels and neurotransmitter receptors (Williams, 1997). Therefore arginine can be considered as a precursor of several signaling molecules including agmatine, polyamines and NO (Wu and Morris, 1998).

2.1. Nitric oxide

L-arginine is a substrate of all the isoforms of NO synthase which generate from L-arginine and molecular oxygen NO and L-citrulline in a five-electron transfer reaction. L-arginine is the only substrate of all the isoforms of NOS and in the absence of arginine NOS is unable to generate NO. (Xia *et al.*, 1998). It should be emphasized that, due to the diffusible nature of the NO radical, synthesis is already the major control point for its action and is regulated at the transcriptional level of enzyme as well as by cofactors (Xie and Nathan, 1994).

2.1.1. The isoforms of nitric oxide synthases

NO is synthesized from L-arginine by a family of three NO synthases. The first NOS isoform to be purified was constitutively expressed neuronal or brain NOS (nNOS or NOS-1) (Bredt and Snyder, 1994). Stuehr and others were the first ones who described inducible NOS (iNOS or NOS-2) (Stuehr *et al.*, 1991).

Finally, endothelial NOS (eNOS or NOS-3) was purified (Forstermann *et al.*, 1991a;Forstermann *et al.*, 1991b;Forstermann *et al.*, 1990;Pollock *et al.*, 1986).

Table 2. Nitric oxide synthase isoforms

Isoenzyme	Typical localization
nNOS	Central and peripheral neurons, NANC neurons, islets, endometrium, skeletal muscles, etc
iNOS	Macrophage, liver, smooth muscle, endothelium, heart, etc
eNOS	Endothelium, brain, heart, etc

The major difference between inducible and constitutive isoforms is that the activity of nNOS and eNOS is largely regulated by the cytosolic free calcium concentration. In contrast, iNOS activity is mostly dependent on the expression of the molecule in the activated cell (Bredt and Snyder, 1994).

Expression of iNOS protein requires transcriptional activation, which is mediated by specific combinations of cytokines. The NO output by iNOS is further regulated by the availability of L-arginine, depending on the L-arginine transporter activation, and the activity of other L-arginine metabolizing enzymes like arginase (Bredt, 1999).

2.1.2. Targets of nitric oxide

NO is a reactive molecule which has been suggested to have multiple targets. It has been demonstrated that NO is a powerful stimulator of soluble guanylate cyclase (sGC), leading to an increase in levels of 3′–5′-cyclic monophosphate (cGMP) (Schuman and Madison, 1994). The resulting increase in cGMP levels can then modulate the activities of cGMP-dependent protein kinases, phosphodiesterases, and ion channels (Southam *et al.*, 1996;Salter *et al.*, 1996). There are several other possible targets for NO, including cyclooxygenase and tryptophane hydroxylase (Misko *et al.*, 1993;Kuhn and Arthur, 1996). Thus, some physiological effects of NO are independent of the sGC activation, and it has been demonstrated that NO, induced by the NMDA receptor stimulation, activates the p21 (ras) pathway of signal transduction with a cascade involving extracellular signal-regulated kinases and phosphoinositide 3-kinase (Yun *et al.*, 1998;Dawson *et al.*, 1998). Other enzymes that constitute cellular targets for NO are cyclooxygenases, ribonucleotide reductase, some mitochondrial enzymes and NOS itself (Garthwaite and Boulton, 1995).

There are a number of mechanisms through which the effects of NO are mediated, but the reaction of NO with cysteine residues in proteins, a process known as nitrosylation, is emerging as one of the most important mechanisms. Nitrosylation is a physiologically important post-translational modification that affects a wide variety of proteins involved in a number of cellular processes

(Dash *et al.*, 2007)**.** Proteins that can be nitrosylated are, for instance, H-Ras, Dexras 1, NMDA receptor, eNOS (Nakamura and Lipton, 2008;Chung and David, 2010;Stamler *et al.*, 2001).

2.1.3. Nitric oxide in the nervous system

Synthesis of NO occurs in neurons throughout the CNS including spinal cord and retina (Dun *et al.*, 1992), in the endothelial cells lining the capillaries (Seidel and Bicker, 1997) and in most cell types of the parenchyma. NO is quoted to be an unconventional neurotransmitter with potential role also as a retrograde messenger (Esplugues, 2002). Several *in vivo* studies have demonstrated that NO modulates the extracellular levels of various neurotransmitters in the CNS, e.g serotonin, dopamine, GABA and glutamate (Kaehler *et al.*, 1999;Wegener *et al.*, 2000;Segovia *et al.*, 1994). Synthesis of NO in the nervous system has been shown to be connected with the activity of the NMDA receptor. The NMDA receptor is a ligand-gated, voltage-sensitive ionophore which gates Ca^{2+} and, to a lesser extent, Na^+ and K^+ (Meguro *et al.*, 1992). Stimulation of the receptor and opening of the ionophore results in Ca^{2+} entry into the receptive neuron. The Ca^{2+} binds to and stimulates a calcium–calmodulin complex which, in turn, stimulates nitric oxide synthase (Southam *et al.*, 1996).

The role of NO in the CNS is highly multifunctional and it is involved in many physiological and pathological processes, such as neurotransmission, neurodifferentiation, and neurodegeneration (Peunova and Enikolopov, 1995; Gatto *et al.*, 2000;Prast and Philippu, 2001).

2.1.4. Possible role of nitric oxide in depression

Nitric oxide has been implicated in the regulation of multiple processes in the CNS like learning and memory, pain perception, depression, aggression, feeding behaviour etc. (Esplugues, 2002;Harkin *et al.*, 1999;Guimaraes *et al.*, 2005). Most of the evidence linking NO to the mechanisms of depression derives from preclinical studies. Thus, Jefferys and Funder in 1996 and Harkin et al. in 1999 showed that the NOS inhibitors possess antidepressant-like properties in rat and mouse model, respectively (Jefferys and Funder, 1996; Harkin *et al*. 1999). The antidepressant-like effect of the NOS inhibitors is related to the inhibition of neuronal isoform of NOS (Volke *et al.*, 2003) and is mediated by the serotonergic mechanisms (Harkin *et al.*, 2003). In line with that, it has been demonstrated by *in vivo* microdialysis that the NOS inhibitors increase and L-arginine supresses the 5-HT level in the hippocampus of freely moving rats (Wegener *et al.*, 2000). Thus, at least in the ventral hippocampus the serotonergic neurotransmission may be under tonic negative control of NO. Likewise, the NOS inhibitors as well as the invalidation of nNOS gene had an antidepressant effect in the chronic mild stress model of depression (Zhou *et al.*,

2007). Moreover, our group has previously demonstrated the possible indirect effect of some clinically used antidepressants in relevant clinical concentrations on the NOS activity in the hippocampus by means of *in vivo* microdialysis (Wegener *et al.*, 2003). Interestingly, it has also been shown, in the first study describing the antidepressant-like effect of the NOS inhibitor L-nitro-arginine in mice, that the precursor of NO, L-arginine, was able to counteract the antidepressant-like effect of imipramine (Harkin *et al.*, 1999). Recently, the same phenomenon was described using antidepressant drugs bupropion and venlafaxine (Dhir and Kulkarni, 2007a;Dhir and Kulkarni, 2007b) as well as in case of some putative antidepressant drugs, namely memantine and folic acid (Almeida *et al.*, 2006b;Brocardo *et al.*, 2008).

There are also a few supportive clinical studies linking NO to depression. Thus, in a small clinical study, depressed patients had increased plasma levels of nitrate, the end-product of NO metabolism (Suzuki *et al.*, 2001). Even more convincing is the fact that depression as a side-effect of interferon-alpha therapy was accompanied by a significant rise in plasma nitrate levels (Suzuki *et al.*, 2003). However, opposing evidence also exists demonstrating decreased platelet NOS activity and plasma nitrate levels in major depression (Chrapko *et al.*, 2004).

2.2. Arginase

Arginase is the final enzyme in the urea cycle and hydrolyzes arginine to urea and ornithine. Two isoforms of arginase exist in mammals. Arginase I (liver arginase) is located in the cytosol and is most abundant in liver. Arginase II is located primarily in the mitochondrial matrix and is expressed more widely, though at highest levels in kidney and prostate (Jackson *et al.*, 1986;Spector *et al.*, 1994;Gotoh *et al.*, 1996;Gotoh and Mori, 1999;Vockley *et al.*, 1996).

Arginase has potential roles as a regulator of the synthesis of glutamate, polyamines, proline and nitric oxide (Wu and Morris, 1998). Arginase is expressed in many regions of the motor system, sensory system, in the memory-related structures, hippocampus and amygdale (Yu *et al.*, 2001). There is possibility that arginase might function in the sensory and motor system through the arginine-ornithine-glutamate-GABA pathway (Yu *et al.*, 2001). Arginase has a potential role as a regulator of the synthesis of nitric oxide since the regulation of arginine availability could determine cellular rates of NO production (Wu and Morris, 1998). Overall, the functional aspects of arginase in the CNS are not yet well characterised. As this part of the L-arginine pathway is not the main focus of this work, it is not expanded upon.

2.3. L-arginine: glycine amidinotransferase (AGAT)

Another important enzyme in the L-arginine pathway is AGAT. AGAT catalyzes the transfer of a guanidino group from arginine to glycine to form ornithine and guanidinoacetate (GAA). This is believed to be the regulated step of creatine biosynthesis. High activities of AGAT are found in the kidneys and in the pancreas (Walker, 1979). The next step in the pathway is the synthesis of creatine from GAA by guanidinoacetate methyltransferase (GAMT). Interestingly, AGAT and GAMT have been suggested to be involved in the synthesis of creatine in the brain (Beard and Braissant, 2010). Moreover, it is possible that creatine can act as a neuromodulator or even neurotransmitter. Thus, creatine is able to affect GABAA receptors as a partial agonist or antagonist (De Deyn *et al.*, 1991;Neu *et al.*, 2002). Likewise, in the organotypic neuronal cultures creatine is released after electrical stimulation like classical neurotransmitters leading to the suggestion that creatine may act as a neuromodulator or transmitter (Almeida *et al.*, 2006a). Taken together, this possibility adds further complexity to the potential role of L-arginine pathways as from the same substrate potentially 3 neurotransmitters could be synthesised. However, as this part of L-arginine pathway is not in the main focus on this work, it has not been expanded upon.

2.4. Agmatine

In 1994, agmatine was identified in the mammalian brain (Li *et al.*, 1994). It has been postulated that agmatine is synthesized in the brain, stored in synaptic vesicles in a large number of neurons with selective distribution in the CNS, released by depolarization, and inactivated by selective re-uptake (Reis and Regunathan, 2000). As depicted in Figure 3 agmatine can be degraded by agamtinase in the brain and by diamine oxidase in the peripheral tissues (Holt and Baker, 1995;Sastre *et al.*, 1998).
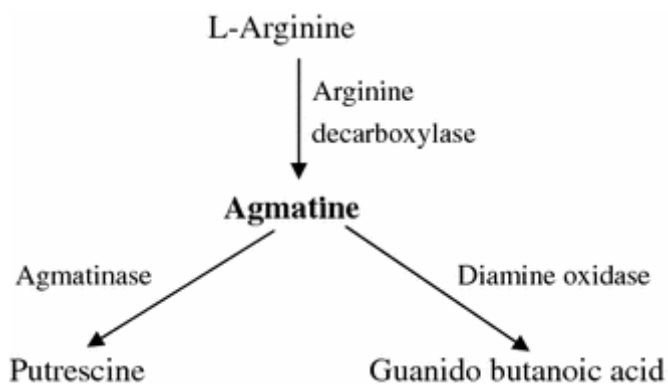
Figure 3: Metabolism of agmatine (Raasch *et al.*, 2001)

2.4.1. Targets of agmatine

Agmatine is able to interact with multiple targets in the CNS. Agmatine is an endogenous ligand at imidazoline 1 and 2 receptors, binding to these receptors with high affinity (Reis and Regunathan, 2000;Li *et al.*, 1994). Initially, agmatine was conceptualised as an endogenous clonidine-displacing substance of imidazoline receptors; however, it has now been established to have affinity for several transmembrane receptors, such as alpha(2)-adrenergic, imidazoline 1 and 2 and glutamatergic NMDA receptors. In addition to activity at these receptors, agmatine may interact with many non-receptor targets. Thus, it irreversibly inhibits neuronal nitric oxide synthase and downregulates inducible nitric oxide synthase (Reis and Regunathan, 2000). Moreover, in preclinical experiments agmatine has been shown to interact with multiple molecular targets important for the nervous system function: blockade of key ionic channels (e.g., ATP-sensitive K+ channels and voltage-gated Ca++ channels); the inhibition of protein ADP-ribosylation and thus, interference with cell signalling; the inhibition of matrix metalloproteases enzymes implicated in nerve cell death (Kawasaki *et al.*, 1985;Yang *et al.*, 2007;Shepherd *et al.*, 1996; Murayama *et al.*, 1993).

2.4.2. Agmatine in nervous system

The fact that agmatine has no single specific receptor in the nervous system has made the studies looking at the possible role of endogenous agmatine a bit elusive. It has been argued that as the concentration of agmatine in brain is comparable to that of classical neurotransmitters it may serve as a neuro-transmitter (Reis and Regunathan, 2000). Most of the evidence linking agmatine to distinct functions in nervous system comes from the studies which have

primarily used the administration of agmatine for the proof of concept. The administration of agmatine has analgesic effect, enhances morphine analgesia but mitigates development of morphine tolerance, dependence and relapse (Wei *et al.*, 2007;Wu *et al.*, 2008).

It has been shown that agmatine may modulate memory, since it produces an amnesic effect when administered before a contextual fear conditioning (Lavinsky *et al.*, 2003), however its post training injection facilitated memory consolidation in an inhibitory avoidance task (Arteni *et al.*, 2002).

Agmatine has been widely studied and is known to have neuroprotective effects against various experimental neuronal injuries. Agmatine reduces the neuronal loss after cerebral ischemia and spinal cord injury, the neurotoxity induced by NMDA/glutamate or glucocorticoid administration (Gilad and Gilad, 2000;Yang and Reis, 1999;Feng *et al.*, 2002). Abe et al reported that agmatine decreased the production of nitric oxide in lipopolysaccharide-treated microglia *in vitro* (Abe *et al.*, 2000). Ahn et al showed a similar finding in oxidative stressed microglia and they also assessed agmatine's effects on the cell viability and apoptosis of cultured microglia and the activity of iNOS in ischaemic penumbra in vivo (Ahn *et al.*, 2011).

2.4.3. Possible role of agmatine in depression

Animal studies have linked agmatine to the stress-related mechanisms in the brain. Zhu et al. demonstrated that the administration of exogenous agmatine protects the hippocampus and medial prefrontal cortex against neuronal insults caused by repeated immobilization (Zhu *et al.*, 2008). The parallel increase in endogenous brain agmatine and arginine decarboxylase protein levels triggered by repeated immobilization indicates that the endogenous agmatine system may play an important role in adaptation to stress as a potential neuronal self-protection mechanism (Zhu *et al.*, 2008).

Several reports have demonstrated that agmatine given systemically (i.p.) or centrally (i.c.v.), is producing significant antidepressant-like effects in the FST in mice and rats and the magnitude of the effect is comparable to those of the classical antidepressant drugs (Zomkowski *et al.*, 2002;Zomkowski *et al.*, 2004;Li *et al.*, 2003;Aricioglu and Altunbas, 2003). The antidepressant-like action of agmatine is also evident in the mouse tail suspension test (Zomkowski *et al.*, 2002). The antidepressant-like effect of agmatine has been shown to depend on serotonergic system (Zomkowski *et al.*, 2004). It has been suggested that NMDA receptors (Li *et al.*, 2003), or imidazoline I1 and I2 receptors are mediating the antidepressant effect (Zeidan *et al.*, 2007;Taksande *et al.*, 2009). The role of agmatine as a modulator of depression has also been suggested in clinical studies. The first study linking agmatine and depression demonstrated that plasma agmatine concentration was significantly elevated in depressed patients compared with healthy controls (Halaris *et al.*, 1999). Moreover,

agmatine concentrations were normalised by antidepressant therapy with bupropion.

Of interest is the very recent clinical trial showing that oral agmatine treatment had analgesic effect in lumbar radiculopathy patients (Keynan *et al.*, 2010). Thus, one can optimistically assume that preclinical effects of agmatine translate into clinical benefit.

AIMS OF THE STUDY

The general aim of the study was to further clarify the possible involvement of L-arginine pathways in the regulation of depression.

The specific aims of the study were as follows:
1. To characterize the behavioural effects of agmatine in animal models predictive of antidepressant- and anxiolytic-like activity, and to evaluate the involvement of serotonin in these effects.
2. To evaluate whether pretreatment with L-arginine can counteract the antidepressant-like effects of different classes of antidepressants in the mouse forced swimming test, and weather these antidepressants modulate the nitrite plus nitrate level in brain.
3. To elucidate the possible role of NO and agmatine in the regulation of marble-burying behaviour.

MATERIALS AND METHODS

1. Animals

Male C57Bl/6J mice (Scanbur AB, Solletuna, Sweden) weighing 20–25 g were used. Mice were kept 10 per cage in an animal house at 20ºC in a 12h light/dark cycle (light on at 7.00 a.m.). Tap water and food pellets were available *ad libitum*. The animals were kept for at least two weeks in the animal colony before entering experiments. All animal procedures were accepted by the National Committee for Ethics in Animal Experimentation and complied with "Principles of laboratory animal care" (NIH publication 25–28, 1996).

2. Behavioural methods

2.1. Forced swimming test

The forced swimming test is a method to estimate behavioural despair in a stressful and inescapable situation. The forced swimming test was performed as described by Porsolt *et al.* (Porsolt *et al.*, 1977). Briefly, a glass cylinder, 12 cm in diameter and 24 cm in height, was filled with 8 cm water at 25 °C. The animal was gently put in the water, and all behaviour videotaped during 6 min. Subsequently, the immobility time was counted by an observer blind to the treatment protocol during the last 4 min of the 6-min test.

2.2. Locomotor activity

Locomotor activity was measured using an automated system with 6 chambers (45x45x45 cm) made from transparent acrylic (MOTI, Technical & Scientific Equipment GMBH, Germany). The apparatus-naïve mice were put into the chamber and the vertical and horizontal activity was counted during a 10-min test period. Time in locomotion, distance travelled (m), number of rearings were registered. In Paper III the locomotion was measured during 20 min.

2.3. Mouse light-dark compartment test

The exploratory model first described by Crawley and Goodwin (Crawley and Goodwin, 1980) was used. The apparatus consisted of two compartments (20x15x20 and 20x30x20 cm) connected by a 7.5x7.5 cm opening in the wall. The smaller compartment was painted black and covered with a roof. The other

compartment had no roof and was brightly illuminated by a 60 W bulb located 25 cm above the box. An animal was placed into the dark compartment, the latency of the first transition, the number of transitions and the time spent in the light compartment was recorded during 5 minutes.

2.4. Marble-burying test

The marble-burying test was performed as described previously (Ichimaru *et al.*, 1995;Joel, 2006). All experiments were conducted between 10:00 and 17:00 h. 24 glass marbles (1.5 cm in diameter) were placed on 5 cm of sawdust bedding along the perimeter of a clear plastic box (44 cm x22 cm x 20 cm). The mice were placed in the box individually for 30 minutes, and the number of marbles buried at least two-thirds deep were counted. The antidepressant drug, agmatine, or NOS inhibitor were administered 30 min prior to testing. In a separate experiment, L-arginine or saline was injected 10 min prior to the antidepressant.

3. Depletion of brain serotonergic system

To test the possible involvement of 5-HT in the behavioural effects of agmatine, animals were pretreated with *para*-Chlorophenylalanine methyl ester HCl (PCPA; 100mg/kg, i.p) or saline, once a day, for four consecutive days. Animals were treated with agmatine 2 hours after the last injection of PCPA or saline and tested 30 minutes later.

4. Measurement of tissue 5-hydroxytryptophane levels

In all the groups brains were rapidly removed on ice, the frontal corticis were dissected, weighed and frozen at $-80°$ until the analysis. On the day of analysis, brain pieces were homogenized (1:10 w/v) in an ice-cold TRIS-HCl buffer containing EDTA. After centrifugation the supernatants were removed. In order to remove protein impurities before the chromatographic analysis, the supernatant was added 5µl 0.05 mM $HClO_4$/50 µl, and centrifuged at 15000g for 15 min. High pressure liquid chromatography (HPLC) with electrochemical detection was used for the sample analysis (ESA Coulochem II with 5014B coulometric analytic cell; ESA Inc. Mass. USA). The mobile phase was composed of 50 mM NaH_2PO_4, 740 µM 1-octanesulfonic acid, 108 µM Na-EDTA, 80 ml/l acetonitrile and 100 µl/l triethylamine with pH adjusted to 3.5 using H_3PO_4.

5. Nitric oxide measurement

The brain nitrite plus nitrate (NOx) concentration was measured by Saville-Griess assay 60 minutes after the drug treatment. The brain tissue (excluding cerebellum and pons) was homogenized 1:10 w/v in ice-cold 20 mM Tris-HCl buffer (pH 7.4) and centrifuged (300 xg, 15 min). For deproteination 30% $ZnSo_4$ was added to the supernatant (10:190), and samples were incubated at room temperature for 15 minutes. The resulting supernatants were subsequently transferred to the microcentrifuge tubes containing 0,5g Cd and incubated at room temperature overnight with agitation, in order to convert the nitrate to nitrite. The NOx was measured the following day with modified Griess reagent (sulfanilamide, *N*-(1-naphthyl)-ethylenediammonium dichloride) (Schmidt, 1995). Absorbance values were read at wavelength 540nm in microtiter plate reader. The NOS inhibitor 7-nitroindazole (7-NI) was used as positive control. The dose (50 mg/kg) and timing of 7-NI was based on the studies showing near maximum effect of that dose on brain NOS activity and on behaviour (Volke *et al.*, 1997;Volke *et al.*, 2003).

6. Drugs

All chemicals were purchased from Sigma (St. Louis, USA). Imipramine hydrochloride, fluoxetine hydrocloride, venlafaxine hydrochloride, bupropion, agmatine hydrochloride and L-arginine hydrochloride were dissolved in saline. PCPA (*p*-Chlorophenylalanine methyl ester) and 7-nitroindazole were dissolved in saline using a few drops of Tween-80. All drugs were freshly prepared and given intraperitoneally (i.p.) in the volume of 0.1 ml per 10 g body weight of mice. Doses of drugs were chosen according to the previous studies (Lavinsky *et al.*, 2003;Zomkowski *et al.*, 2002;Zomkowski *et al.*, 2004;Zomkowski *et al.*, 2002;Harkin *et al.*, 1999;Dhir and Kulkarni, 2007a;Dhir and Kulkarni, 2007b). In case of multiple injections, e.g., L-Arg or saline and antidepressant or saline, the drugs were injected into the opposite sides of the peritoneal cavity.

7. Experimental design

In Paper I the light-dark compartment test, the measurement of locomotor activity, and the forced swimming test were carried out consecutively 30, 40, and 50 min after the treatment with agmatine, respectively. Each group comprised eight animals.

In Paper II the measurement of locomotor activity and FST were carried out consecutively 40 and 50 min after the treatment with antidepressant drug,

respectively. L-Arg or saline was injected 10 min prior to the antidepressant. The number of animals per groups is given in the figure legends.

8. Statistics

Data were statistically treated using one way or two-way analysis of variance (ANOVA). Post hoc comparisons between individual groups were performed by Duncan's multiple range test or Newman-Keuls test. In the experiment where the brain nitrate was measured, t-test for independent samples was used to compare the groups.

The data is expressed as the mean values ± S.E.M. Differences were considered statistically significant when P was less than 0.05.

RESULTS

1. Behavioural effects of Agmatine

1.1. Effect of agmatine in the forced swimming test

Treatment had a significant effect upon the immobility time in FST as indicated by one-way ANOVA ($P< 0.005$). The standard antidepressant imipramine (15 mg/kg) significantly decreased the immobility time. Only the highest dose of agmatine (50 mg/kg) had a significant effect on the immobility time of mice (Figure 4).
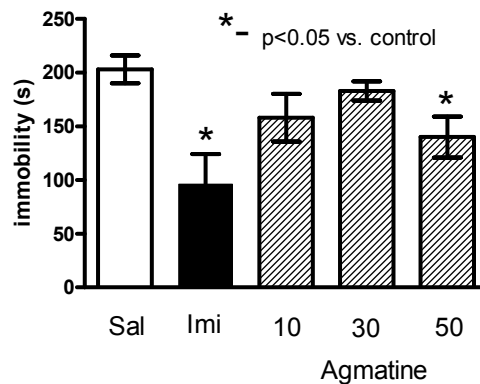
Figure 4. Effect of imipramine (15 mg/kg) and agmatine in the forced swimming test (n= 8 per group). Drugs were injected 50 min prior to testing. Results are expressed as mean ± S.E.M. * $P< 0.05$ versus saline, Duncan's test.

1.2. Effect of agmatine in light-dark compartment test

Post hoc analysis revealed that agmatine did not change the animal behaviour in the light-dark compartment test. The treatment with imipramine (15 mg/kg) increased the time spent in the light compartment. Similar effect has been shown previously in CD1 mice in the same paradigm (de Angelis L., 1996).

1.3. Effect of agmatine on locomotion

One-way ANOVA did not indicate a significant treatment effect on distance ($P= 0.36$). Agmatine had no effect on locomotion.

2. Effect of PCPA on cortical 5-HT and 5-HIIA concentrations

Results are shown in Figure 5. PCPA treatment had a significant effect on both 5-HT ($P< 0.001$) and 5-HIIA ($P< 0.0001$) concentrations. *Post hoc* comparisions revealed that PCPA depleted 5-HT concentrations more than 70% and 5-HIIA concentrations more than 80%.
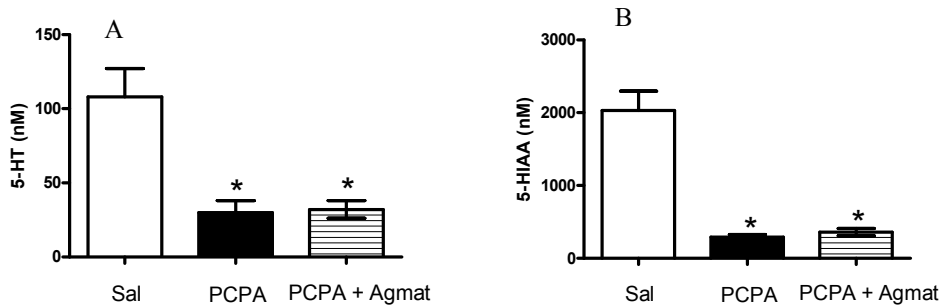

Figure 5. Effect of pre-treatment with PCPA on cortical 5-HT (A) and 5-HIIA (B) + concentrations. Animals were treated with PCPA (100 mg/kg, i.p.) or saline, once a day, for four consecutive days (n= 7–8 per group). Results are expressed as mean ± S.E.M. * $P< 0.001$ vs. saline, Duncan's test.

2.1. Treatment with PCPA did not block the anti-immobility effect of agmatine

The results are shown in Figure 6 A. The treatment had a significant effect on the immobility time ($P< 0.001$) as indicated by one-way ANOVA.

Agmatine significantly decreased the immobility time compared to the animals treated with PCPA alone. Interestingly, the treatment with PCPA itself decreased the immobility compared theto control animals.

The pretreatment with PCPA alone or in combination with agmatine did not change the behaviour of animals in the light-dark compartment test or locomotion ($P= 0.49$; Figure 6B).
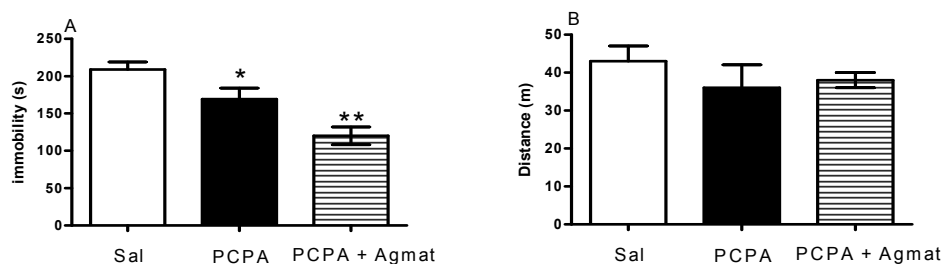
Figure 6. Pretreatment with PCPA did not block the anti-immobility effect of agamatine in the forced swimming test (A) and did not modify the behaviour of animals in the open field (B; n= 8–9 per group). The results are expressed as mean ± S.E.M. * $P< 0.05$ vs. saline, ** $P< 0.05$ vs PCPA group, Duncans's test.

3. Effects of L-arginine pretreatment on the action of antidepressants

3.1. L-arginine pretreatment counteracts the effect of imipramine, venlafaxine and bupropion in the FST

The treatment with L-Arg alone (500 mg/kg) did not modify the behaviour of animals in the forced swimming test (Figure 7). In the experiment with imipramine (Figure 7A) two-way ANOVA indicated a significant effect of the pretreatment ($P< 0.01$), the treatment ($P< 0.0001$) and a pretreatment x treatment interaction ($P< 0.005$). Imipramine (15 mg/kg) significantly decreased the immobility time and the pretreatment with L-Arg counteracted the antidepressant effect of imipramine. In the experiment with venlafaxine (Figure 7B) two-way ANOVA indicated a significant effect of pretreatment ($P< 0.05$), treatment ($P< 0.0001$) and the interaction of pretreatment x treatment ($P< 0.05$). Venlafaxine (6 mg/kg) significantly decreased the immobility time and the pretreatment with L-Arg counteracted the antidepressant effect of venlafaxine. L-arg (500 mg/kg) did not modify the effect of bupropion in the FST.

Similarly, L-Arg pretreatment (500 mg/kg) did not alter the fluoxetine antidepressive effect in the FST.

We next tested whether a higher dose of L-Arg (1000 mg/kg) could reverse the effects of bupropion and fluoxetine. In the experiment with bupropion (Figure 7E) two-way ANOVA indicated a significant effect of pretreatment ($P< 0.05$), treatment ($P< 0.0001$), but not of pretreatment x treatment interaction. Bupropion (20 mg/kg) significantly decreased the immobility time and pretreatment with L-Arg (1000 mg/kg) counteracted the antidepressant effect of bupropion.

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In the experiment with fluoxetine (Figure 7F) two-way ANOVA indicated that fluoxetine significantly decreased the immobility time ($P< 0.001$ vs. saline group) and the pretreatment with L-Arg (1000 mg/kg) did not modify this effect.
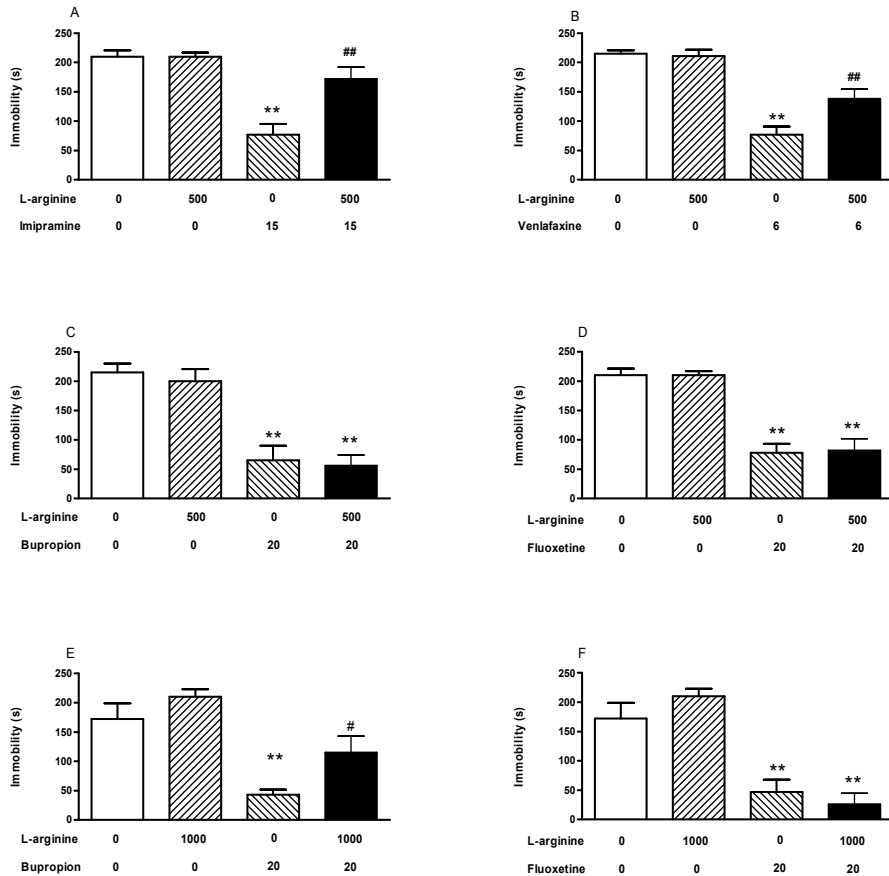
Figure 7. Effects of L-Arg, antidepressants, and their combination on behaviour in the forced swim test. The results are expressed as mean ± S.E.M. ** $P< 0.001$ vs. saline; # $P< 0.05$ vs. antidepressant only; ## $P< 0.005$ vs. antidepressant only, Newman-Keuls test. A. Imipramine 15mg/kg (n= 9–10). B. Venlafaxine 6mg/kg (n= 10). C. Bupropion 20mg/kg (n= 9–10). D. Fluoxetine 20 mg/kg (n= 9–10). E. Bupropion 20 mg/kg, L-Arg 1000 mg/kg (n= 9). F. Fluoxetine 20 mg/kg, L-Arg 100 mg/kg (n= 8–9). Concentration marked 0 is a vehicle injection only.

4. L-arginine pretreatment has no effect on locomotion

The treatment with L-Arg alone did not change any of the parameters measured (Figure 8).

In the experiment with imipramine (Figure 8A) two-way ANOVA indicated significant effects of treatment on the distance travelled ($P< 0.01$); and the number of rearings ($P< 0.0001$). The pretreatment x treatment interaction did not reach significance. Imipramine significantly decreased only the number of rearings but combined the treatment with L-Arg and imipramine decreased the distance travelled and the number of rearings.

In the experiment with venlafaxine (Figure 8B) the treatment with venlafaxine alone or in combination with L-Arg did not induce any change compared with the saline treatment.

In the experiment with bupropion (Figure 8C) two-way ANOVA indicated a significant effect of treatment on the distance travelled ($P< 0.0001$). The *Post hoc* analysis revealed that bupropion increased the distance travelled. The pretreatment with L-Arg did not antagonize the hyperlocomotion induced by bupropion. In the experiment with a higher dose of L-Arg (1000 mg/kg, Fig 8E) the results were identical. Two-way ANOVA indicated a significant effect of treatment on the distance travelled ($P< 0.001$) and the number of rearings ($P< 0.05$). The *Post hoc* analysis revealed that bupropion increased the distance travelled but did not affect the number of rearings. The pretreatment with L-Arg did not antagonize the hyperlocomotion induced by bupropion.

In the experiment with fluoxetine (Figure 8D and 8F) the treatment with fluoxetine alone or in combination with L-Arg did not induce any statistically significant change in the distance travelled but decreased the number of rearings in the experiment with a higher dose of L-Arg ($P< 0.005$ for treatment). The *Post hoc* analysis revealed that the effect was significant both in case of fluoxetine and L-Arg + fluoxetine.
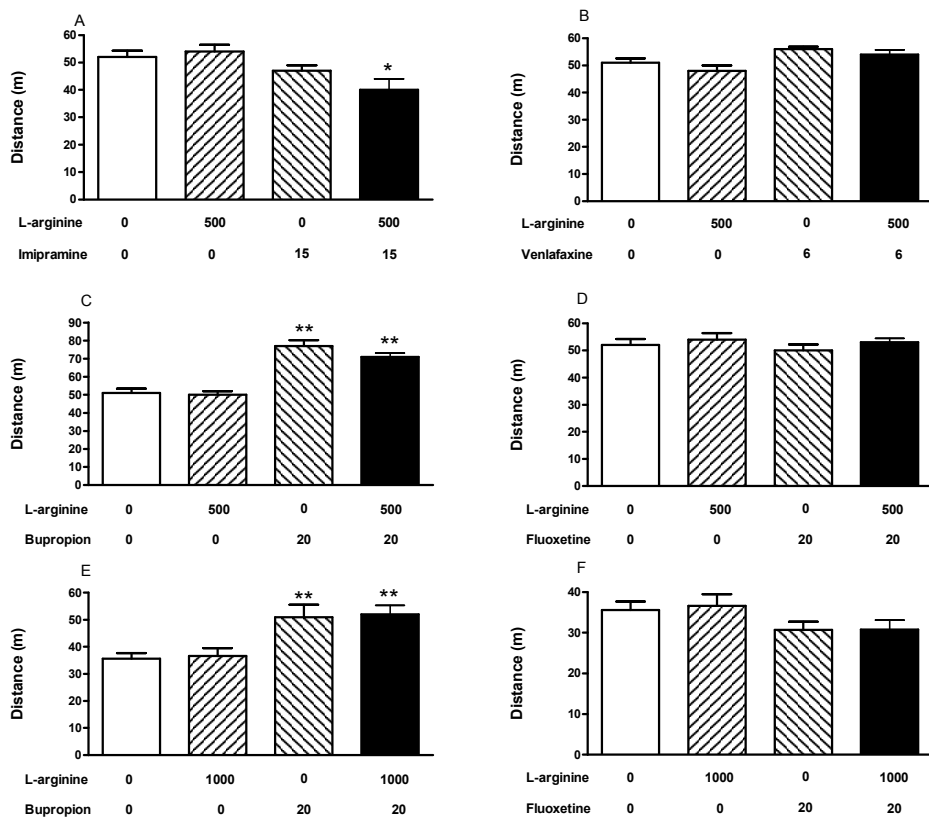
Figure 8. Effects of L-Arg, antidepressants, and their combination on locomotion. Results are expressed as mean ± S.E.M. * *P*< 0.05 vs. saline; ** *P*< 0.001 vs. saline, , Newman-Keuls test. A. Imipramine 15 mg/kg (n=9–10) B. Venlafaxine 6 mg/kg (n=10). C. Bupropion 20 mg/kg, L-Arg 500 mg/kg (n=9–10). D. Fluoxetine 20 mg/kg, L-Arg 500 mg/kg (n=9–*10)*. E. Bupropion 20 mg/kg, L-Arg 1000 mg/kg (n=9). F. Fluoxetine 20 mg/kg, L-Arg 1000 mg/kg (n=8–9). Concentration marked 0 is a vehicle injection only.

5. Effects of the nitric oxide synthase inhibitor, antidepressants and L-arginine on nitric oxide synthesis

The results are shown in Figure 9. In the experiment with 7-nitroindazole and imipramine (Figure 9A) one-way ANOVA indicated a significant treatment effect on the NOx levels (*P*< 0.05). The *Post hoc* comparisons revealed that both 7-NI and imipramine significantly decreased NOx levels. The administration of venlafaxine (figure 9B) decreased NOx levels significantly.

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Bupropion had no significant effect on nitrate levels (Figure 9D), fluoxetine tended to decrease nitrate levels. The treatment with L-Arg (500 mg/kg; figure 9E) increased NOx levels significantly.
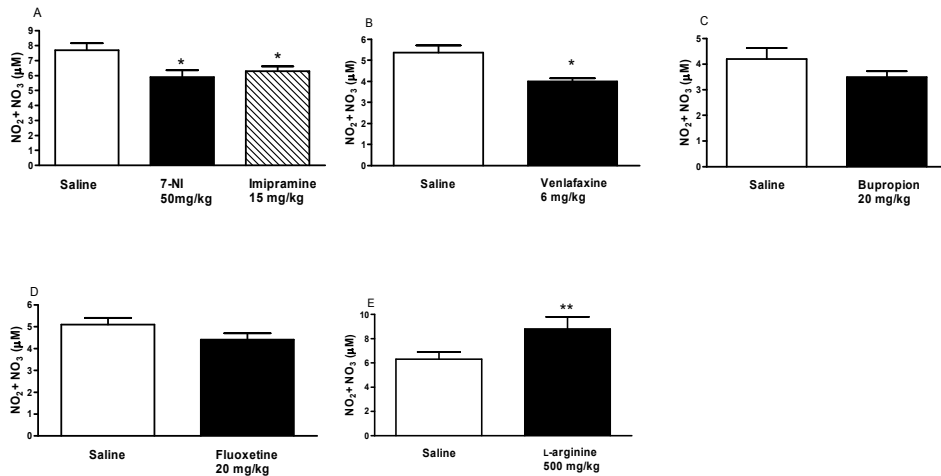
**Figure 9.** Effects of imipramine, 7-NI (A), venlafaxine (B), bupropion (C), and L-Arg (E) on brain nitrite+nitrate (NO2 + NO3) levels. Results are expressed as mean ± S.E.M., * $P< 0.05$ vs. Saline; ** $P< 0.001$ vs. saline, Newman-Keuls or t-test. All the drugs were injected 60 min prior testing. The number of animals in the groups was 12–13 in the experiment with imipramine, 12 in the experiments with venlafaxine and fluoxetine, 11 in the experiment with bupropion, and 10 with L-Arg.

6. Involvement of nitric oxide in the regulation of marble-burying behaviour

6.1. Effects of the NOS inhibitors and agmatine on marble-burying behaviour

The treatment with 7-NI had a significant effect on marble-burying indicated by one-way ANOVA ($P< 0.05$). 7-NI (20 and 50 mg/kg) dose-dependently de-creased the number of marbles buried (Figure 10A). This effect was statistically significant only when the high dose was used. The low dose of the NOS inhibitor also tended to inhibit the marble-burying, but the effect did not reach statistical significance ($P= 0.064$).

The more selective NOS inhibitor, TRIM, suppressed the marble-burying behaviour ($P< 0.01$) in both doses tested (Figure 10A). The treatment with agmatine had a significant effect on marble-burying behaviour ($P< 0.05$) as indicated by one-way ANOVA. The *Post hoc* analysis revealed that agmatine

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(10 and 50 mg/kg) decreased the number of marbles buried (Figure 10C) in both doses used.

The administration of agmatine in a dose of 50 mg/kg had no significant effect on nitrite +nitrate levels (Figure 10D).
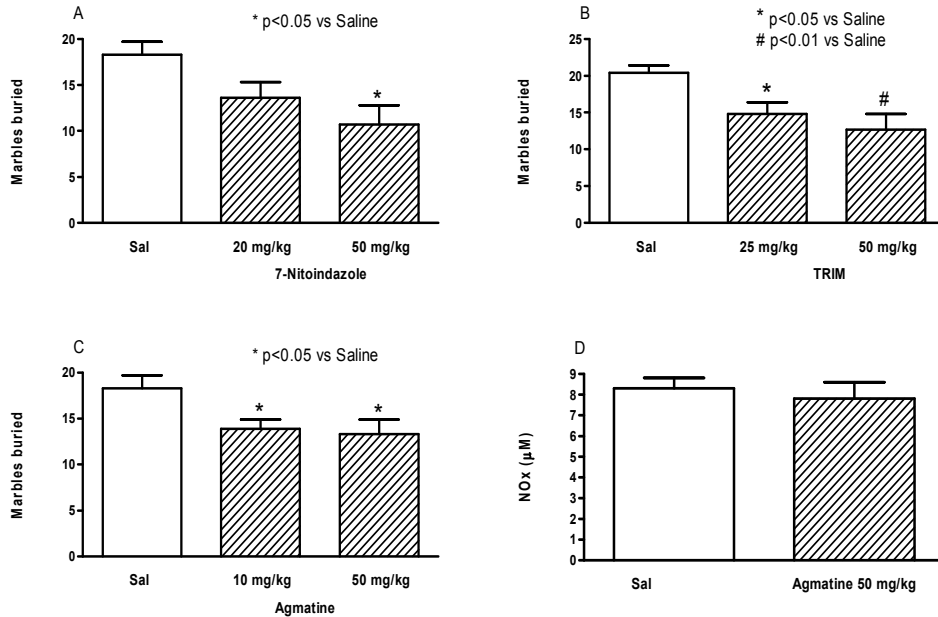
Figure 10. Effects of NOS inhibitors and agmatine on marble-burying behaviour.
A. 7-nitroindazole (n= 9). B. TRIM (n= 9–10). C. Agmatine (n= 9–10).
D. Effect of agmatine (50 mg/kg) on brain NOx level (n= 11). Results are expressed as mean ± S.E.M., *P* values Newman-Keuls test.

6.2. Effects of L-arginine, antidepressants, and their combination on marble-burying behaviour

The treatment with L-arginine alone (500 mg/kg) did not affect the number of marbles buried (Figure 11). In the experiment with paroxetine, two-way ANOVA indicated a significant effect of the treatment ($P< 0.005$), and a pretreatment x treatment interaction ($P< 0.05$). Paroxetine (10 mg/kg) significantly decreased the number of marbles buried and the pretreatment with L-arginine counteracted the effect of paroxetine.

In the experiment with citalopram, two-way ANOVA indicated a significant effect of the treatment ($P< 0.0001$) and the interaction of the pretreatment x treatment ($P< 0.01$). Citalopram (10 mg/kg) significantly suppressed the marble-burying behaviour and pretreatment with L-arginine counteracted the effect.
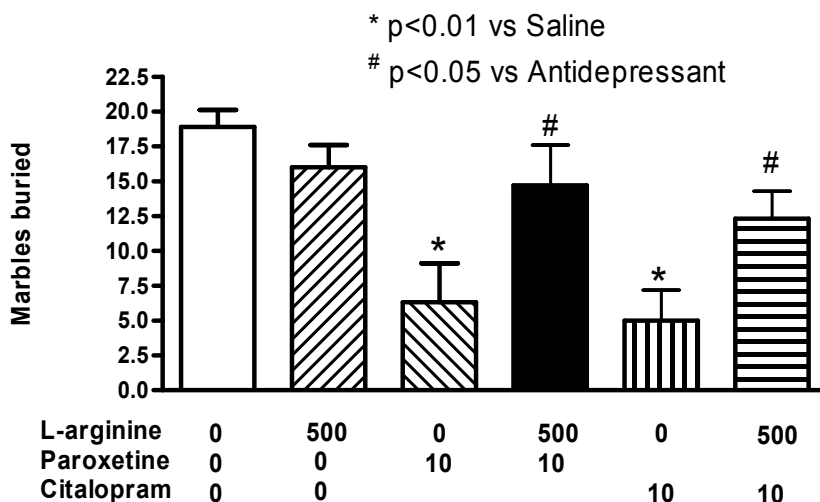
Figure 11. Effects of L-Arg, antidepressants, and their combination on marble-burying behaviour. The results are expressed as mean ± S.E.M. (n= 9–10), *P* values, Newman-Keuls test.

6.3. Effects of NOS inhibitors, agmatine, L-arginine and antidepressants on the locomotion

From the drugs tested only 7-NI decreased the locomotion of animals (Table 3).

Table 3. Effects of agmatine, antidepressants, and NOS inhibitors on the locomotor activity

Drug	Distance (m)
Saline	85±8
Agmatine (50 mg/kg)	69±9
Citalopram (10 mg/kg)	80±11
Paroxetine (10 mg/kg)	76±5
7-NI (20 mg/kg)	66±3
7-NI (50 mg/kg)	45±10*
TRIM (25 mg/kg)	84±12
TRIM (50 mg/kg)	79±7

* *P*< 0.05 compared to saline, Newman-Keuls test. Results are expressed as mean ± S.E.M. (n= 7–8).

DISCUSSION

1. Antidepressant-like effect of agamatine is not mediated by serotonin

The main finding of Study I was that agmatine had an antidepressant-like effect which seems to be independent of the brain serotonergic system.

The magnitude of the antidepressant-like effect of agmatine was slightly weaker compared with that of the standard antidepressant imipramine (15 mg/kg) (Figure 4). Previously, the antidepressant-like effect of agmatine has been reported even after a dose of 0.01 mg/kg in the mouse forced swimming test (Zomkowski *et al.*, 2002). In our hands it took a much higher dose of the drug to be effective. Our data are more consistent with the report of Li et al. and Taksande et al. where the antidepressant-like effect appeared in the dose of 20 mg/kg when administered intraperitoneally or subcutaneously (Li *et al.*, 2003; Taksande *et al.*, 2009). As seen from the Table 4, studies have reported the anti-depressant-like effect of agmatine to appear in rather different doses. The reason for such differences is obscure, but different mouse strains used may account for some variations.

As reported previously (Li *et al.*, 2003;Zomkowski *et al.*, 2002), agmatine did not change the ambulation of animals, excluding the possibility that the drug effect in the FST was of non-specific origin.

Surprisingly, the pretreatment with tryptophane hydroxylase inhibitor PCPA did not antagonize the antidepressant-like effect of agmatine. Up to date, one study has reported the loss of effect of agmatine after the treatment with PCPA (Zomkowski *et al.*, 2004). They used the same pretreatment scheme but did not measure the effect of PCPA on 5-HT level. In our experiment, the pretreatment with PCPA resulted in more than 70% and 80% drop of the tissue levels of 5-HT and 5-HIIA, respectively (Figure 5). However, one cannot totally exclude the possibility that a more complete inhibition of 5-HT synthesis is needed to antagonize the antidepressant-like effect of agmatine. The other difference between these two studies was that the lower dose of agmatine (10 mg/kg) was used in the previous study, making it probably easier to counteract. Likewise, Redrobe et al. have demonstrated that the pretreatment with PCPA attenuated the antidepressant effect of lower doses of venlafaxine, but the drug remained effective at the dose of 32 mg/kg (Redrobe *et al.*, 2005). Thus, it seems likely that 5-HT does not play a major role in the antidepressant-like effect of agmatine. Agmatine did not induce an anxiolytic-like effect in our study. Few studies have reported agmatine to possess an anxiolytic-like activity in mice and rats (Lavinsky *et al.*, 2003;Aricioglu and Altunbas, 2003;Gong *et al.*, 2006). It is noteworthy that agmatine has displayed bell-shaped dose-effects after acute dosing in the animal models of anxiety. Thus, in the mouse light-dark compartment test the only dose of agmatine affecting the number of transitions

was 80 mg/kg (Gong *et al.*, 2006). Moreover, while the classical anxiolyic drug diazepam increased both the number of transitions as well as the time spent in the light compartment, agmatine did change only the latter. Taken together, the anxiolytic-like effect of agmatine seems to be weak.

Table 4. Antidepressant effects of agmatine in different animal models

Animal	Tested doses	Antidepressant doses	Model	Reference
Kunming mice	40 mg/kg, i.p. 10 mg/kg x 2, i.p. Chronic treatment for 3 consecutive weeks	40 mg/kg i.p. 10 mg/kg x 2, i.p.	TST TST	(Jiang *et al.*, 2009)
Swiss mice	5–20 mg/kg, i.p.	20 mg/kg, i.p.	FST	(Taksande *et al.*, 2009)
C57B1/6J mice	10, 30; 50 mg/kg, i.p.	50 mg/kg, i.p.	FST	(Krass *et al.*, 2011;Krass *et al.*, 2008)
Swiss mice	10 mg/kg, i.p.	10 mg/kg, i.p.	FST	(Zomkowski *et al.*, 2005)
Sprague-Dawley rats	10–100 mg/kg, i.p.	10–100 mg/kg, i.p.	FST	(Aricioglu and Altunbas, 2003)
Kunming mice	10–160 mg/kg p.o. 10–80 mg/kg p.o. 5–40 mg/kg s.c. for 3 consecutive days	40 and 80 mg/kg p.o. 40 and 80 mg/kg p.o. 20 mg/kg s.c.	TST FST FST FST	(Li *et al.*, 2003)
Wistar rats	2.5–20 mg/kg, p.o. 1.25–10 mg/kg, s.c.	10 mg/kg, p.o. 1.25; 2,5; 5 mg/kg, s.c.		
Swiss mice	0.01–50 mg/kg, i.p. 0.01–50 mg/kg, i.p. 0.1–100 nmol/site, i.c.v.	0.01–50 mg/kg, i.p. 0.01–50 mg/kg, i.p. 0.1–100 nmol/site, i.c.v.	FST TST FST	(Zomkowski *et al.*, 2002)

Agmatine has been claimed to act as a neurotransmitter or modulator in the central nervous system (Reis and Regunathan, 2000). However, some studies have questioned wheather any meaningful agmatine synthesis occurs under physiological conditions (Coleman *et al.*, 2004;Horyn *et al.*, 2005;Volke *et al.*, 2006). The tissue level of agmatine has been estimated to be 0.1–6 µM (Raasch *et al.*, 1995;Feng *et al.*, 2002;Yang and Reis, 1999). The recent study has determined the concentrations of various L-arginine metabolites in the cerebellum of rats (Liu *et al.*, 2010). The approximate values of arginine meta-bolites and GABA are shown in Table 5.

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Table 5. Concentrations of various L-arginine metabolites and GABA in the cerebellum (Liu *et al.*, 2010).

Substance	Concentration (μg/g wet tissue)
L-arginine	60
L-citrulline	100
L-ornithine	160
agmatine	0.8
GABA	300

It is apparent from the data above that agmatine has a very low concentration compared to other L-arginine metabolites or neurotransmitters in the cerebellum. On the other hand, the agmatine concentration has been shown to increase in the hippocampal synaptic terminals after learning (Leitch *et al.*, 2011). To put these findings into the context, it is intresting to note that the treatment with 100 mg/kg of agmatine resulted in more than 55 times increase in the cortical agmatine concentration measured 3 hours after dosing (Feng *et al.*, 2002). Thus, one can argue that at least the antidepressant-like effect of agmatine is more likely to be a pharmacological effect and agmatine is not functioning as the endogenous regulator of depression. Taken together, the antidepressant-like effect of agmatine seems not to be mediated by the serotonergic system. We failed to confirm the reported anxiolytic-like activity of agmatine.

2. The antidepressant action of imipramine and venlafaxine involves suppression of nitric oxide synthesis

In Study II all the antidepressants tested induced robust and statistically significant antidepressant-like effects in the mouse FST (Figure 7). This finding was not unexpected, as our selection criteria for including these antidepressant drugs in the present work were based on previous efficacy in the mouse FST (Petit-Demouliere *et al.*, 2005). Moreover, we observed that the magnitude of the antidepressant-like effect, as evaluated by the decreased immobility time, was very similar between different treatments (the immobility time 65–80 sec). L-Arginine, given in the dose of 500 or 1000 mg/kg (Figure 7), had no effect on immobility of mice which is also in good accordance with the previous studies (Dhir and Kulkarni, 2007;Volke *et al.*, 2003;Harkin *et al.*, 1999). From the antidepressants tested here, only bupropion increased the locomotion of animals in the open field test (Figure 8), which is in agreement with earlier studies, where the motor stimulant effect of bupropion has been described, and which is

hypothesized to be caused by an increase in the synaptic dopamine level (Dhir and Kulkarni, 2007b).

The pretreatment with L-arginine (500 mg/kg) opposed the antidepressant-like effect of imipramine and venlafaxine, but not that of bupropion or fluoxetine (Figure 7). These results are in good agreement with the previous reports describing attenuating effect of L-arginine in the FST with simultaneous administration of imipramine (Harkin *et al.*, 1999) and venlafaxine (Dhir and Kulkarni, 2007a;Kumar *et al.*, 2010). Moreover, increasing the dose of L-arginine to 1000 mg/kg, attenuated the antidepressant-like effects of bupropion, which is in line with the previous report showing that L-arginine (750 mg/kg) attenuates the effect of bupropion (Dhir and Kulkarni, 2007b). To our knowledge, there are no previous studies describing the effect of pretreatment with L-arginine and the subsequent administration of fluoxetine. However, the selective nNOS inhibitor TRIM has been shown to augment the antidepressant-like effect of fluoxetine (Ulak *et al.*, 2008). The current study did not observe even a tendency in the attenuation of the fluoxetine effect with the L-arginine pretreatment in the FST.

This selective interaction with some antidepressants cannot be explained by L-arginine effect on locomotion. Only bupropion changed the distance travelled and that effect was not reversed by the L-arginine pretreatment. Imipramine and fluoxetine decreased the number of rearings and again L-arginine did not modify the effect. The combined treatment with L-arginine and imipramine decreased the locomotion of animals compared with the saline treated group, but not with the imipramine treated group. Thus, one can conclude that there was no correlation between the ability of L-arginine to counteract the antidepressant effect and its effects on locomotion. The current evidence concerning the ability of drugs activating the NMDA/NO/cGMP pathway to antagonize the antidepressant effect of the classical antidepressant has been compiled in Table 6.

Table 6. Effect of NMDA/NO/cGMP activators on antidepressants
+ antagonizes antidepressant effect

Antidepressant	NMDA agonist	L-arginine	PDE-5 inhibitor
TCA Imipramine		+ (Harkin *et al.*, 2003) + (Krass *et al.*, 2011)	
SSRI s-Citalopram	+	+ (Zomkowski *et al.*, 2010)	+ (Zomkowski *et al.*, 2010)
SNRI Venlafaxine		+ (Dhir and Kulkarni, 2007a) + (Krass *et al.*, 2011)	+ (Dhir and Kulkarni, 2007a)
NDRI Bupropion		+ (Dhir and Kulkarni, 2007b) + (Krass *et al.*, 2011)	

While it is clear from the previous reports and the current study that L-arginine can reverse the antidepressant-like effects of many, but not all antidepressants, the mechanistic basis of the effect remains undefined. Theoretically, there are two possibilities. I The classical antidepressant drugs and the NO system may work in parallel in the regulation of the neurobiological pathologies of depression, or II the antidepressant drugs may directly affect the activity of nitric oxide synthesis. To address that question in the current work, we also measured the brain nitrite + nitrate content after the antidepressant treatment. Interestingly, the effect of the antidepressants on the NO metabolism paralleled the behavioural action except in case of bupropion. Thus, imipramine and venlafaxine decreased the NOx levels, whereas bupropion and fluoxetine did not have any effect, however, the effect of fluoxetine was close to statistical significance. As the effect of imipramine and venlafaxine was already evident after 60 minutes, we presume that the most likely NOS isoenzyme to be inhibited was neuronal NOS.

Collectively, our data support the hypothesis that imipramine and venlafaxine decrease the NO synthesis (Figure 9) and this mechanism is important for the antidepressant action. However, we should stress that there are clear limitations of the NOx assay used in the study. First, the method may not be sensitive enough to measure the NOx levels in different brain regions, and second, since NOx is the end-product of all NO metabolism a high background level exists. Thus, it cannot be ruled out, that bupropion and/or fluoxetine may decrease the NOx levels in some brain regions, e.g., hippocampus. For example, by using a voltammetry method, fluoxetine has been shown to block the NMDA evoked increase in the NO level in the striatum (Crespi, 2010). Unfortunately, while the classical assay executing the conversion of L-arginine to L-citrulline has much better sensitivity, it is difficult to apply it for ex vivo. The main problem would be that to see a drug effect *ex vivo*, the drug must tightly bind to the NOS enzyme. We have previously shown that antidepressants do not inhibit the NOS activity *in vitro* in pharmacologically relevant concentrations but decrease it *in vivo* (Wegener *et al.,* 2003).

On the basis of the present findings it is difficult to explain directly the possible site of interaction between the antidepressant action, and the glutamate/NMDA/NO system. Considering the fact that in our study some antidepressants were able to inhibit the NO synthesis in the brain, the site of interaction seems to be at the level of nitric oxide synthase or upstream. We speculate that some antidepressants may modulate the availability of calcium or some other NOS co-factor. Interestingly, the current behavioural and neurochemical evidence indicates that drugs within the same class of antidepressants may have different relation to nitrergic mechanisms. Thus, paroxetine inhibits the NO synthesis and L-arginine can reverse its effect in the marble-burying paradigm (Wegener *et al.*, 2003;Krass *et al.*, 2010) whereas the effect of fluoxetine was resistant to the L-arginine pretreatment in the current study.

Further studies are needed to dissect the interplay between antidepressants and NO.

All in all, our results support the idea that some antidepressants are able to inhibit the nitric oxide synthesis in the brain, and this effect mechanistically may underlie the ability of L-arginine to counteract the antidepressant-like effect in the FST of antidepressants.

3. Nitric oxide is involved in the regulation of marble-burying behaviour

Study III showed that all the antidepressants tested had statistically significant effects in the marble-burying test (Figure 11). Moreover, both of the NOS inhibitors decreased significantly the number of marbles buried (Figure 10). The efficacy of 7-NI in the marble-burying test was shown in a very recent paper by Umathe et al. in the similar dose range (Umathe *et al.*, 2009). As TRIM and 7-NI have different selectivity towards distinct NOS isoforms (for review see Wegener and Volke 2010), but very similar effect in the marble-burying test, it seems likely that the behavioural effect is mediated by the neuronal isoform of NOS. This is similar to the effect of NOS inhibitors in the forced swimming test (Volke *et al.*, 2003). The effect of NOS inhibitors on marble-burying seems not to be related to the suppression of locomotion. In the current study only 7-NI decreased the locomotion of animals (Table 3), whereas TRIM had no effect. The data concerning the effect of the NOS inhibitors on locomotion are contradictory. Our group has demonstrated that 7-NI decreases locomotion (Volke *et al.*, 2003), but the data presented by Umathe et al. do not support this finding (Umathe *et al.*, 2009). Agmatine is an L-arginine metabolite assumed to serve as a putative neurotransmitter (Reis and Regunathan, 2000). The treatment with agmatine induces antidepressant-like and, at least in some cases, anxiolytic-like effects (Zomkowski *et al.*, 2002;Lavinsky *et al.*, 2003;Krass *et al.*, 2008). Our study was first to demonstrate that agmatine was also effective in the marble-burying paradigm where both doses tested (10 and 50 mg/kg) decreased the number of marbles buried. As in the marble-burying paradigm both antidepressants and anxiolytic drugs are active, we cannot state which of the potential actions of agmatine underlies its effect in the test. The nature of the exact mechanism mediating the effect of agmatine in anxiety and depression models is currently unclear. As agmatine has been shown to inhibit the NOS activity in vitro (Galea *et al.*, 1996), we tested whether the brain NOx level was changed by the agmatine treatment. Since agmatine in the dose of 50 mg/kg did not modify the NOx level in the brain, the NOS inhibition seems unlikely to underlie the effect of the drug.

The treatment with L-arginine did not modify the behaviour of the mice. This is not unexpected, as the majority of studies with L-arginine show a neutral effect in anxiety and depression tests. However, the pretreatment with

L-arginine clearly counteracted the effects of two SSRI antidepressants, i.e., citalopram and paroxetine, in the marble burying test. Several studies have demonstrated that L-arginine may attenuate the antidepressant-like effect of many antidepressants in the forced swimming test (Harkin *et al.*, 1999;Dhir and Kulkarni, 2007a;Dhir and Kulkarni, 2007b). However, some SSRIs, including citalopram, do not affect swimming behaviour in the FST when administered acutely (Borsini, 1995). Thus, we have shown in Study III that the ability of L-arginine to antagonize the effects of antidepressant drugs also include SSRIs, confirming and supporting the recently published data describing the action of the L-arginine pretreatment on the anti-compulsive effect of paroxetine (Umathe *et al.*, 2009). The current findings are in line with our previous study demonstrating that the local administration of paroxetine and citalopram suppressed the NOS activity in the hippocampus *in vivo* (Wegener *et al.*, 2003).

We conclude that both NOS inhibitors and agmatine dose-dependently inhibit the marble-burying behaviour. The inhibition of nNOS seems to play the key role in the behavioural effects of the NOS inhibitors, whereas agmatine seems to have a different mechanism of action. Moreover, enhancement of the NO synthesis by L-arginine can reverse the effect of SSRI antidepressants, further demonstrating the role of NO in regulating the marble-burying behaviour.

4. Concluding remarks

Is there a link between the behavioral effects of agmatine and drugs modifying nitric oxide levels
As both agmatine and NO are derived from L-arginine, it is tempting to speculate that some of the similarities in the behavioral action of agmatine and the drugs modifying NO level are due to some common mechanism. Both agmatine and NOS inhibitors elicit antidepressant-like effect in animal models. Similarly, anxiolytic-like effects have been described in both cases. In the current study, both agmatine and two NOS inhibitors (TRIM and 7-NI) were active in the marble burying test.

Does agmatine act as a nitric oxide synthase inhibitor
A possible explanation would be that agmatine acts as NOS inhibitor to induce behavioural effects. In line with the hypothesis, agmatine has been shown to inhibit NOS activity (Galea *et al.*, 1996). Moreover, in our hands, agmatine doses needed to be quite high to possess the antidepressant activity (50 mg/kg) or suppress marble burying (10 and 50 mg/kg). However, several lines of evidence contradict the hypothesis. First, we were not able to demonstrate the anxiolytic-like effect of agmatine while with the NOS inhibitors the anxiolytic effect appears in a similar dose-range as the antidepressant effect. Second, 5-HT depletion was not able to counteract the antidepressant-like effect of agmatine,

yet the antidepressant-like effect of the NOS inhibitors has been shown to depend on 5-HT (Harkin *et al.*, 2003;O'Leary *et al.*, 2007;Kiss, 2000;Page *et al.*, 1999). Last but not least, in Study III we measured the effect of agmatine (50 mg/kg) on the brain NOx levels and did not find any suppression of that. Thus, one can conclude that it is rather unlikely that the mechanism underlying the behavioral effects of agmatine is the inhibition of NOS.

Does nitric oxide synthase inhibition increase agmatine levels

Another possible mechanism linking the actions of agmatine and NOS inhibitors would be the increased synthesis of agmatine due to the NOS inhibition. That would be mechanistically plausible as similar effects have been described with NOS and arginase (Wu and Morris, 1998). However, no such data exist for increased agmatine synthesis after the NOS inhibition. Again, as mentioned above, different dependence on 5-HT systems of agmatine and NOS inhibitors argues against the hypothesis. Moreover, the ability of L-arginine to counteract the antidepressant effect of different drugs would be impossible to explain in light of agmatine as a mediator of effects of the NOS inhibitors.

Collectively, it seems unlikely that the effects of agmatine and drugs affecting the NO synthesis are interdependent.

CONCLUSIONS

1. A high dose of agmatine possessed an antidepressant-like effect which was not mediated by the serotonergic system. Thus, agmatine may act pharmacologically as an antidepressant but its involvement in the pathophysiological mechanisms of depression is doubtful.

2. Some antidepressants are able to inhibit the nitric oxide synthesis in the brain, and this effect may mechanistically underlie the ability of L-arginine to counteract the effect of antidepressants in the FST.

3. Our results demonstrate that NO is involved in the regulation of marble-burying behaviour as the NOS inhibitors dose-dependently inhibited the marble-burying and L-arginine counteracted the effect of SSRI antidepressants in this model. In line with the known antidepressant-like effect of agmatine the drug was also effective in the marble-burying model.

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SUMMARY IN ESTONIAN

L-arginiini rajad ja antidepressiivne toime

Depressioon on sage ja palju-uuritud haigus, ent selle täpsed neurokeemilised mehhanismid on tänini ebaselged. Praegu kasutusel olevad antidepressandid ei ole piisava efektiivsusega- nii on probleemiks aeglane depressioonivastase toime algus ning suhteliselt suure protsendi haigete ravile mittereageerimine. Seega on vajalik depressioonimehhanismide parem mõistmine ning uute märklaudade leidmine selle raske haiguse raviks.

Lämmastikmonooksiid (NO) on tavatute omadustega virgatsaine närvisüsteemis. NO-d sünteesib L-arginiinist NO süntaas (NOS). NO võimalikust osast depressiooni kujunemisel ja antidepressantide toimes on andmeid nii loomkatsetes kui ka inimesel. Kuigi NO on peamiseks ja enimuuritud L-arginiinist sünteesitavaks mediaatoriks, pööratakse üha suuremat tähelepanu ka teistele arginiinist lähtuvatele metaboolsetele radadele ajus. Nii tekib arginiini dekarboksülaasi toimel L-arginiinist agmatiin, mis ilmselt toimib samuti mediaatorina nii närvisüsteemis kui ka veresoonkonnas.

Käesoleva uuringu eesmärkideks oli täpsustada NO osalemist depressiooni mehhanismides ning teise arginiinist lähtuva võimaliku mediaatormolekuli, agmatiini, rolli selgitamine.

Meie uuringutes omas agmatiin küll antidepressiivset toimet Porsolt' testis, kuid see toime ilmnes märgatavalt kõrgemas annuses (50 mg/kg) kui varasemates töödes. Agmatiinil puudus toime ärevuse tasemele hele-tume puuris ning loomade liikumisaktiivsusele. Kuna on esitatud hüpotees, et agmatiini toime depressioonimudeleis on vahendatud serotoniinergilise süsteemi poolt, uurisime ka serotoniini sünteesi pärssiva ravimi manustamise toimet agmatiini efektidele. PCPA manustamine 4 päeva jooksul vähendas ajus frontaalkoores 5-HT kontsentratsiooni 5 korda. Serotoniini sünteesi pärssimine ei mõjutanud agmatiini antidepressiivset efekti sundujumise katses, mis näitab, et agmatiini toime ei sõltu serotoniinergilisest süsteemist. Arvestades agmatiini kõrget annust meie katses võrreldes selle füsioloogilise nivooga ajukoes, on tegemist selgelt farmakoloogilise toimega.

Varem on leitud, et NO eellasmolekuli L-arginiini manustamine kõrvaldab mõningate antidepressantide efekti Porsolt' testis. On viidatud ka võimalusele, et mõned antidepressandid võivad toimida *in vitro* ja *in vivo* kui NOS-i inhibiitorid. Seetõttu uurisime, kas L-arginiin on võimeline blokeerima erinevat tüüpi antidepressantide toimet Porsolt testis, ja kas uuritavad antidepressandid mõjutavad NO sünteesi hiire ajukoes. NO prekursori L-arginiini 500mg/kg manustamine blokeeris antidepressantide imipramiini ja venlafaksiini antidepressiivse toime, ent ei mõjutanud bupropiooni ja fluoksetiini efekti sundujumise katses. L-arginiin annuses 1000 mg/kg blokeeris bupropiooni antidepressiivse efekti, ent ei mõjutanud fluoksetiini toimet. L-arginiini sellis toime seletamiseks on kaks võimalust: 1) klassikalised antidepressandid ja NO

süsteem mõjutavad paralleelselt depressiooni patofüsioloogilisi mehhanisme, või 2) antidepressandid võivad otseselt mõjutada NO sünteesi aktiivsust. Selle selgitamiseks mõõdeti antud töös ka nitritite ja nitraatide (NOx) taset ajus antidepressandi manustamise järgselt. Imipramiini, venlafaksiini ja fluoksetiini korral oli mõju NO tasemele paralleelne käitumiskatsetes leituga: imipramiin ja venlafaksiin vähendasid NOx taset, fluoksetiin ei mõjutanud NO sünteesi. Bupropiooni korral oli L-arginiin küll võimeline antidepressiivset efekti blokeerima, kuid bupropioon ei mõjutanud NO sünteesi ajus. Seega võib järeldada, et mõned antidepressandid on võimelised inhibeerima NO sünteesi ajus ja see mehhanism võib seletada L-arginiini võimet kõrvaldada nende antidepressiivset toimet.

Lisaks uurisime NO ja agmatiini võimalikku osalemist obsessiiv-kompulsiivse käitumise regulatsioonis nn kuulide matmise mudelis. Kuna mõnedel antidepressantidel ei ole akuutselt manustatuna toimet sundujumise testis, ent need on efektiivsed kuulide matmise testis, siis uurisime lisaks, kas L-arginiini eelnev manustamine kõrvaldab ka selliste antidepressantide toimet. Mõlemad testitud NO süntaasi inhibiitorid (7-NI ja TRIM) vähendasid oluliselt maetud marmorkuulikeste arvu. Sarnaselt teistele ärevuse ja depressioonimudelitele, ei omanud L-arginiin ka selles testis eraldi manustatuna mingit toimet. L-arginiini eelnev manustamine vähendas paroksetiini ja tsitalopraami toimet kuulide matmise testis. Seega osaleb NO ka obsessiiv-kompulsiivse käitumise regulatsioonis, kusjuures sarnaselt ärevuse ja depressioonimehhanismidele on neuronaalse isoensüümi roll juhtiv. Ka agmatiin oli antud mudelis efektiivne, kuid selle toime ei olnud seotud NO sünteesi mõjutamisega.

Kokkuvõtteks võib öelda, et agmatiinil on antidepressiivne efekt sundujumise katses ja see on efektiivne ka kuulide matmise testis. Agmatiini toimeid vahendavad mehhanismid pole täpselt teada, kuid need ei ole vahendatud serotoniinergilise süsteemi ega NO sünteesi mõjutamise kaudu. Meie tulemused andsid kinnitust väitele, et mõned antidepressandid on võimelised inhibeerima NO sünteesi ajus ning see mehhanism võib olla seotud NO eellasmolekuli L-arginiini võimega vähendada antidepressantide toimet.

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PUBLICATIONS

CURRICULUM VITAE

Maarja Krass

Date and Place of Birth:	March 9th 1980, Tartu, Estonia
Citizenship:	Estonia
Address:	Department of Physiology, University of Tartu, Ravila 19 50411 Tartu, Estonia
Phone:	+372 731 4338
E-mail:	Maarja.Krass@ut.ee

Education

2004–2010 University of Tartu, Faculty of Medicine, Department of Physiology, PhD student
1998–2003 University of Tartu, Faculty of Medicine, Department of Pharmacy
1987–1998 Tartu Kivilinna Gymnasium

Professional employment

2010– University of Tartu, Faculty of Medicine, Department of Physiology, assistant
2003– Boehringer-Ingelheim Pharma GmbH Estonia Filial, hospital products specialist

Scientific work

2004– University of Tartu, Faculty of Medicine, Department of Physiology.
Main field of investigation:
L-arginine pathways and antidepressant action. 3 scientific publications, 1 poster- and 1 oral presentation at scientific conference.

Teaching experience

2010– University of Tartu, Faculty of Medicine, Department of Physiology, assistant, (160 h) practical works

Publications

Scientific articles in peer-reviewed journals

I. **Krass M**, Wegener G, Vasar E, Volke V.(2008). Antidepressant-like effect of agmatine is not mediated by serotonine. Behavioural Brain Research., 188: 324–328.

II. **Krass M**, Wegener G, Vasar E, Volke V.(2011). The antidepressant action of imipramine and venlafaxine involves suppression of nitric oxide synthesis. Behavioural Brain Research., 218: 57–63.

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Conference Abstracts

IV. Volke V, **Krass M**.(2005). Do arginase inhibitors have antidepressant action? Abstracts of Annual Conference of Medical Faculty of Tartu Univesity, Tartu.

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Patented invention

VI. VolkeV, **Krass M**, Volke A, Vasar E.(2008). Arginase inhibitors for the treatment of depression. PC/EE2008/000027.

CURRICULUM VITAE

Maarja Krass

Sünniaeg ja -koht: 09.03.1980, Tartu, Eesti
Kodakondsus: Eesti
Aadress: TÜ Füsioloogia instituut, Ravila 19, 50411 Tartu
Telefon: +372 737 4338
E-post: Maarja.Krass@ut.ee

Haridus

2004–2010   Tartu Ülikool arstiteaduskond, doktoriõpe
1998–2003   Tartu Ülikool arstiteaduskond, proviisoriõpe
1987–1998   Tartu Kivilinna Gümnaasium

Teenistuskäik

2010–   Tartu Ülikool, arstiteaduskond, füsioloogia instituut, assistent
2003–   Boehringer-Ingelheim Pharma GmbH Eesti Filiaal, haigla-
             toodete spetsialist

Teadustegevus

2004–   Tartu Ülikool, arstiteaduskond, füsioloogia instituut
             Peamine uurimisvaldkond:
             L-arginiini rajad ja antidepressiivne toime. 3 teaduslikku publi-
             katsiooni, 1 stendi- ja 1 suuline ettekanne teaduskonverentsidel.

Õppetöö

2010–   Tartu Ülikool, arstiteaduskond, füsioloogia instituut, assistent
             (160 tundi) praktikumid

Publikatsioonid

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I. **Krass M**, Wegener G, Vasar E, Volke V.(2008). Antidepressant-like effect of agmatine is not mediated by serotonine. Behavioural Brain Research., 188: 324–328.

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Patentsed leiutised

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DISSERTATIONES MEDICINAE
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