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Behavioural, pharmacological and neurochemical characterisation of limbic system-associated membrane protein (LSAMP) deficient mice

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Supervisors: Eero Vasar, MD, PhD, Professor, Department of Physiology, University of Tartu

Sulev Kõks, MD, PhD, Professor, Department of Physiology, University of Tartu

Reviewers: Anti Kalda, MD, PhD, Senior Research Fellow, Department of Pharmacology, University of Tartu

Vallo Volke, MD, PhD, Leading Research Fellow, Department of Physiology, University of Tartu

Opponent: Heikki Tanila, MD, PhD, Professor of Molecular Neurobiology, University of Eastern Finland

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I **Innos J**, Philips MA, Leidmaa E, Heinla I, Raud S, Reemann P, Plaas M, Nurk K, Kurrikoff K, Matto V, Visnapuu T, Mardi P, Kõks S, Vasar E. (2011). Lower anxiety and a decrease in agonistic behaviour in *Lsamp*-deficient mice. Behavioural Brain Research, 217: 21–31.

II **Innos J**, Philips MA, Raud S, Lilleväli K, Kõks S, Vasar E. (2012). Deletion of the *Lsamp* gene lowers sensitivity to stressful environmental manipulations in mice. Behavioural Brain Research, 228: 74–81.

III **Innos J**, Leidmaa E, Philips MA, Sütt S, Alttoa A, Harro J, Kõks S, Vasar E. (2013). *Lsamp*$^{-/-}$ mice display lower sensitivity to amphetamine and have elevated 5-HT turnover. Biochemical and Biophysical Research Communications, 430: 413–418.

Contribution of the author:

I The author participated in designing the study, performed some of the behavioural and gene expression experiments, carried out most statistical analyses, wrote most parts of the manuscript and handled correspondence.

II The author designed the study, took care of housing conditions, performed most of the behavioural experiments, carried out statistical analysis, wrote the manuscript and handled correspondence.

III The author designed the study, performed all the behavioural and pharmacological experiments (jointly with Este Leidmaa), carried out statistical analysis, wrote the manuscript and handled correspondence.

ABBREVIATIONS

129 – 129S6/SvEv strain or, more generally, all 129 strains
3-MT – 3-methoxytyramine
5-HIAA – 5-hydroxyindoleacetic acid
5-HT – 5-hydroxytryptamine or serotonin
B6 – C57BL/6 (strain; a substrain to C57)
C57 – C57BL strains
CA1 – cornu ammonis 1 of hippocampus
CAM – cell adhesion molecule
cDNA – complementary DNA
CNS – central nervous system
DA – dopamine
DAT – dopamine transporter
D_2 – dopamine type 2 (receptor)
DNA – deoxyribonucleic acid
DOPAC – 3,4-dihydroxyphenylacetic acid
EE – environmental enrichment
ES cells – embryonic stem cells
GABA – gamma-aminobutyric acid
$GABA_A$ – gamma-aminobutyric acid-A (receptor)
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
GPI – glycosylphosphatidylinositol
HPLC – high performance liquid chromatography
Hprt1 – hypoxanthine phosphor-ribosyl-transferase 1
HVA – homovanillic acid
IgLON – name of a protein family (**i**mmuno**g**lobulin **L**samp, **O**BCAM, **n**eurotrimin)
LacZNeo – beta-galactosidase/neomycin fusion gene
Lsamp – limbic system associated membrane protein gene
LSAMP – limbic system associated membrane protein
$Lsamp^{-/-}$ – homozygous mutants lacking both functional alleles of the Lsamp gene
$Lsamp^{+/-}$ – heterozygous mutants having only one functional allele of the Lsamp gene
$Lsamp^{+/+}$ – wild-type littermates of Lsamp-deficient mice
MDD – major depressive disorder
mRNA – messenger ribonucleic acid
NA – noradrenaline
NMN – normetanephrine

OBCAM	–	opioid-binding cell adhesion molecule
qRT-PCR	–	quantitative real-time polymerase chain reaction
SEM	–	standard error of the mean
siRNA	–	small interfering RNA
SNP	–	single nucleotide polymorphism
VEGF	–	vascular endothelial growth factor
VMAT2	–	brain vesicular monoamine transporter

INTRODUCTION

The human genome contains approximately 20,000 protein-coding genes (Pennisi, 2012). Several thousands of these are still of unknown function and many more have been only superficially characterised. However, it should be kept in mind that studying a single gene and its protein is an endless task. We have to find out the sequence of the gene, the localisation and interaction partners of its protein, relevant molecular pathway(s) and possible role(s) in diseases, developmental expression patterns, possible alternative splicing variants, single nucleotide polymorphisms (SNPs) and the existence and role of different promoters. Furthermore, its function and structure (or even existence) in different species have to be elucidated. And last, factors like epigenetic mechanisms, *de novo* mutations, endless individual variability, numerous substrains of species and amazing plasticity of a developing organism in coping with genetic abnormalities make scientists' task even more challenging. So, characterising a gene is a daunting, but not hopeless task that takes tens of years and requires concerted efforts of tens or even hundreds of scientists.

The Lsamp gene codes limbic system-associated membrane protein (LSAMP), which is a cell adhesion molecule belonging to the small family of IgLON proteins. It is known that the main function of the LSAMP protein is axon targeting and neurite outgrowth. This gene has been studied for almost 30 years and search with its name in the PubMed database returns approximately 100 articles. Sounds like a lot? It depends on the perspective. For example, the most famous cancer gene "p53" returns over 40,000 results and another hot name in the cancer field "BRCA" that has been studied only 20 years, returns over 1800 results. Compared to these numbers, Lsamp is a relatively little studied gene. However, considering how often the name of this gene keeps popping up in recent studies of psychiatric disorders and cancer, it is certainly a key player which needs to be studied in much more detail, especially at the organism level.

Some important terminological remarks. First, in this thesis, in the interests of clarity, I have not strictly followed the HUGO Gene Nomenclature Committee guidelines, but instead I have used everywhere the term "Lsamp" for denoting the gene and "LSAMP" for denoting the protein. Sometimes italic is used to differentiate between human (Lsamp) and mouse (*Lsamp*) genes, however, it tends to create more confusion than clarity, especially when more species are involved, so I have avoided that. In this text, it should be clear from the context, which one is meant. Second, in literature protein names LSAMP and LAMP (and sometimes IGLON3) are used interchangeably. I prefer the newer variant LSAMP; however, in the headings of some reference articles, for example, and in everywhere else the name LAMP means exactly the same thing as LSAMP.

This thesis – "Behavioural, pharmacological and biochemical characterisation of limbic system-associated membrane protein (LSAMP) deficient

mice" – contributes three original research articles to the slowly, but steadily growing body of knowledge on the Lsamp gene. In all of these, mice lacking the Lsamp gene were used to study the possible functions of the LSAMP protein. In the first article, a general behavioural profile of Lsamp-deficient mice is provided. Also, it contains some preliminary pharmacological and gene expression experiments demonstrating alterations in the GABAergic system in Lsamp-deficient mice. In the second article, the impact of different housing conditions is studied on the behavioural profile of Lsamp-deficient mice. It turns out that some aspects of the phenotype of Lsamp-deficient mice do not depend on the rearing conditions, but the existence of other behavioural alterations depends on whether mice are raised in isolation, standard housing conditions or enriched environment. This study shows that some phenotypic changes in Lsamp-deficient mice are much more stable than others and therefore probably more relevant to the function of the Lsamp gene. The third article studies the sensitivity of Lsamp-deficient mice to the activating and rewarding effects of amphetamine. Furthermore, the levels of major monoamines and their metabolites in response to saline or amphetamine administration are measured. Also, the expression levels of dopaminergic system-related genes are studied. This study shows that extensive changes in major monoamine systems in the brain take place in response to the genetic invalidation of the Lsamp gene.

I would like to add a few words of caution to readers who are not very familiar with the type of studies performed for this thesis and who are tempted to jump at conclusions based on the results presented here. First, the sequence of the Lsamp gene is very similar in mice and humans, but mice and humans are very different organisms. No conclusions about the behavioural outcome of the lack of a functional Lsamp gene in humans can be done based on mice studies, only guesses. Furthermore, "mice" in the context of the work presented here means a highly variable genetic mixture of BL6 and SV mice. There are tens of different mouse lines and the deletion of the Lsamp gene in all these, not to speak of other species, almost surely yields different results behaviourally, pharmacologically and neurochemically. Second, there is a huge individual variability in both human and mouse populations. The effects described here are purely statistical, not absolute; it means that even if Lsamp-deficient mice score as an average, say, 6 points and wild-type mice 10 points in test A, we can almost always find some Lsamp-deficient mice that score for example 15 points, i.e. similarly to or even more than an average wild-type mouse. We can usually refrain from abusing stereotypes in human relations, but all too often transform our research subjects into rigid stereotypes, this should be avoided. Third, the multiplicity of methods used in this work is deceptive. There is really only one method – deleting a gene in one certain model organism and observing the consequences of this manipulation by different "sub-methods". Suppressing the Lsamp gene by means of siRNAs or yet-to-be-developed drugs in an animal would probably reveal quite a different story than studying an animal that has

started its life without the Lsamp gene. Fourth, the function of the Lsamp gene and LSAMP protein cannot be exhaustively elaborated without studying the other members of the IgLON protein family in parallel. The four (maybe five) proteins in the IgLON family form heterodimers (i.e. a conglomerate consisting of two proteins) with each other and according to the present knowledge, only these protein complexes, not single IgLON family proteins, perform functions in a living organism. By deleting one gene of the IgLON family – in this case Lsamp – almost certainly results in extensive compensatory processes in the developing organism. Brain is amazingly plastic and flexible and by the time we start to make experiments with our 2-months-old Lsamp-deficient mice, we are studying animals that have adapted to their genetic shortcoming. For example, the production of other proteins of the IgLON family may increase in response to the lack of the Lsamp gene and this effect may compensate for and mask the lack of the LSAMP protein. The ability of the members of the IgLON family to substitute each other is a crucial question yet to be studied at the organism level.

These cautious remarks are in no way meant to lessen or criticise my own results. On the contrary, the years working with Lsamp-deficient mice have been full of pleasant surprises, nice AHA moments and sense of wonder at how different an animal without only a single gene can become compared to its "normal" littermates. Many a time the results have been encouragingly in line with previous work done by other research teams which adds credibility to the data and injects new energy to continue. But still, scientific work is a humbling experience. You start with many hopes and illusions that start to crumble, one by one, year after year. The more experienced you become, the more clearly you see the minuteness of your discoveries, the roughness of recognized modern methods, the diabolic deceptiveness of statistics, the simplicity of the most elaborate hypotheses and the endlessness of scientific endeavours. But then at one point you either quit (the subject or the field) or start to see the beauty of play which is the key to success in all human activities. These little animals, missing the Lsamp gene, have made me a hard-headed skeptic I never imagined I would become, but have also taught me the beauty of play, little by little. Paraphrasing Neil Armstrong, this thesis is one small step for mankind, one giant leap for me.

REVIEW OF LITERATURE

1. General characterisation of the Lsamp gene and LSAMP protein

The Lsamp gene is located on chromosome 3 in humans (Figure 1). Its product, the limbic system-associated membrane protein (LSAMP) is a cell adhesion molecule (CAM) of the IgLON family expressed in cortical and sub-cortical limbic-associated regions of the developing and adult brain (Cote et al, 1995; Cote et al, 1996; Horton and Levitt, 1988; Levitt, 1984; Pimenta et al, 1996b; Reinoso et al, 1996; Zacco et al, 1990). LSAMP is a 64- to 68-kDa heavily glycosylated protein, structurally characterised by three immunoglobulin (Ig) domains (Pimenta et al, 1996a). LSAMP protein is expressed on the surface of somata and proximal dendrites of neurons (Zacco et al, 1990) where it integrates via glycosylphosphatidylinositol (GPI) anchor (Pimenta et al, 1995). The amino acid sequence of LSAMP is highly conserved among species. The protein exhibits 99% homology between rodent and human (Pimenta et al, 1996a) and there is a close correlation between *Lsamp* mRNA and protein distribution patterns in rat (Levitt, 1984; Pimenta et al, 1996b; Reinoso et al, 1996; Zacco et al, 1990), monkey (Cote et al, 1995; Cote et al, 1996), and human (Prensa et al, 1999; Prensa et al, 2003), indicating strong phylogenetic conservation of protein structure and associated functional properties. LSAMP immunoreactivity in mice is developmentally present within 24–36 hours after neurons undergo their final mitosis on embryonic days E15–E19 (Horton and Levitt, 1988). Functional and biochemical studies have revealed that LSAMP can promote or inhibit neurite outgrowth depending on counter partners (Mann et al, 1998, Gil et al, 2002), more specifically, experimental manipulations of LSAMP in vitro result in altered axon targeting and neurite growth (Eagleson et al, 2003; Keller et al, 1989; Mann et al, 1998; Pimenta et al, 1995; Zhukareva et al, 1997). No observable anatomical alterations have been identified in the brain of the Lsamp-deficient mouse line (Catania et al, 2008). Therefore, LSAMP protein is probably not necessary for general development of anatomical brain structures, but probably needed for more specific connections between neurons.

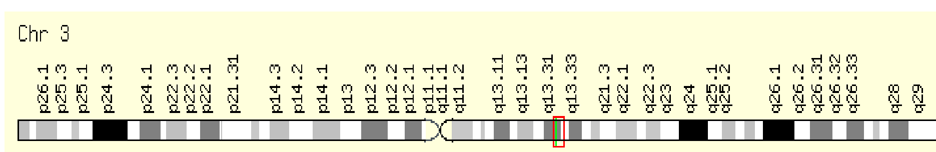

Figure 1. The Lsamp gene in humans is located on chromosome 3 (3q13.2–q21). http://www.genecards.org/cgi-bin/carddisp.pl?gene=LSAMP

2. Neuroanatomical distribution of LSAMP

LSAMP distribution in whole adult mammalian brain was originally described in rat, with immunohistochemical (Levitt, 1984) and *in situ* hybridization (Reinoso et al, 1996) labeling. Other publications have explored embryonal expression of LSAMP (Horton and Levitt, 1988; Pimenta et al, 1996b) or concentrated to a limited anatomical area in mammalian brain. Chesselet et al (1990) mapped LSAMP in the caudate nucleus and substantia nigra of the cat. In primates, the basal ganglia (Cote et al, 1995), hippocampus and amygdaloid area (Cote el al, 1996) have been precisely explored. In post-mortem human brain tissue the analysis has been concentrated to striatum and adjacent basal forebrain structures (Prensa et al, 1999; Prensa et al, 2003). Several studies have mapped LSAMP anatomical distribution in birds: Brummendorf et al (1997) characterised chick LSAMP (chLAMP) expression in embryonal (E5 and E7) and adult chick brain and spinal cord using polyclonal chick antibody; Kimura et al (2001) used *in situ* hybridization to map LSAMP in developing chick brain. LSAMP expression has been explored in amygdaloid nuclei in pigeon and chick (Yamamoto et al, 2005) and in pigeon forebrain and midbrain (Yamamoto and Reiner, 2005).

Several early works have proposed that LSAMP protein is limbic system specific expressing in cortical and subcortical regions of the limbic system (Levitt, 1984; Reinoso et al, 1996). However, it has been reported that LSAMP expression is not restricted to limbic areas, but it is also present in sensory nuclei such as sensory thalamic nuclei, including lateral posterior nucleus of thalamus (Yamamoto et al, 2003) and both medial and lateral geniculate nucleus (Pimenta et al, 1995; Reinoso et al, 1996), superior and inferior colliculus, dorsal and ventral cochlear nuclei, nucleus of lateral lemniscus, superior olive and spinal trigeminal nucleus (Reinoso et al, 1996). The LSAMP ortholog in chick is expressed in the retina and sensory ganglia (Brummendorf et al, 1997; Lodge et al, 2000) and also in axons of the optic nerve and in the retinotectal system (Brummendorf et al, 1997). Yamamoto and Reiner (2005) have proposed that the high levels of LSAMP in some interconnected visual nuclei of midbrain and thalamus suggest that LSAMP may contribute to axon guidance in some nonlimbic regions as well. LSAMP clearly is not an absolute marker of limbic regions.

3. IgLON family

LSAMP is a member of IgLON family of proteins that consists of four (or five, see below) members: 1) LSAMP, 2) neurotrimin (Ntm)/CEPU-1 (rat and chick orthologues, respectively) (Struyk et al, 1995), 3) OBCAM (opioid-binding cell adhesion molecule) (Schofield et al, 1989) or OPCML (opioid-binding protein/cell adhesion molecule) (Panichareon et al, 2012), and 4) NEGR1 (neuronal growth regulator 1) (human) or kilon/neurotractin (rat and chick

orthologues, respectively) (Funatsu et al, 1999). All these molecules are highly glycosylated membrane proteines, characterised by three Ig domains and GPI anchor. Reed et al (2004) have proposed that IgLONs function predominantly as subunits of heterodimeric proteins (Diglons). Thus, the four IgLONs can form six Diglons, and studies about the function of LSAMP protein need to take into account other IgLON family members. It is suprising that LSAMP deficiency does not induce severe disturbances in the brain anatomy as could be predicted from previous body of data about LSAMP. It is possible that other members of IgLON family (neurotrimin, OBCAM or kilon) can partly take over the functions of LSAMP. Reed et al (2004) propose that, based on their relative affinities in the chick, CEPU-1 (neurotrimin in rat) might be both a homo- and a heterophilic cell adhesion molecule, whereas LSAMP and OBCAM act only as heterophilic cell adhesion molecules. Recently, a fifth member of the IgLON family, IGLON5, was proposed, based on sequence similarity (Diez-Roux et al, 2011), however, it is the most distant member of the five and presently it is not clear, whether it is a protein-encoding gene or a pseudogene.

4. Lsamp and other IgLONs in psychiatric disorders

The first evidence for a role of the Lsamp gene in the regulation of emotional behaviour came from a study where male Wistar rats were selected according to their exploratory behaviour in the elevated plus-maze model of anxiety. Animals with lower exploratory activity (increased anxiety) had elevated levels of the Lsamp transcript in the periaqueductal gray (Nelovkov et al, 2003). In the same rats, an increase in Lsamp gene expression was also noticed in the amygdala, but not in the frontal cortex (Nelovkov et al, 2006). Exposure of rats to cat odour, another model of anxiety in rodents, also increased the expression of Lsamp transcript in the amygdala (Kõks et al, 2004). These findings were extended by Alttoa et al (2010) demonstrating that the transcript for Lsamp was more expressed in the raphe, hippocampus and frontal cortex of rats displaying reduced exploratory activity in the motility box. Lamprecht et al (2009) established that fear conditioning that leads to auditory fear conditioning memory formation, increased the expression level of Lsamp in the amygdala of rats. Altogether, rodent studies indicate that increased level of the Lsamp transcript in several brain areas is related with increased trait anxiety (Alttoa et al, 2009; Nelovkov et al, 2003; Nelovkov et al, 2006), acute fear reaction (Kõks et al, 2004) and fear conditioning (Lamprecht et al, 2009).

The first Lsamp gene knockout mouse line, generated at the Vanderbilt University (USA) by means of deleting exon 2 in the gene and by backcrossing the mice to C57/BL6 background, displayed no changes in sensory and motor development, was slightly hyperactive in novel environments, and performed more open arm entries and headdips and spent more time on open arms in the elevated plus maze (Catania et al, 2008). Furthermore, it exhibited a pronounced

deficit in spatial memory acquisition in the water maze and poorly sustained CA1 long-term potentiation (Qiu et al, 2010).

Human data link the Lsamp gene not only with anxiety, but also with a wider spectrum of psychiatric disorders: polymorphisms in the human Lsamp gene have been associated with panic disorder (Koido et al, 2006) and male completed suicide (Must et al, 2008). Also, a relation between gene polymorphisms of the Lsamp gene and major depressive disorder (MDD) has been found (Koido et al, 2012). Furthermore, the levels of LSAMP protein have been found to be approximately 20% increased in postmortem frontal cortex both in patients with schizophrenia and bipolar disorder (Behan et al, 2009). Furthermore, links between other members of the IgLON family and psychiatric disorders have been found. For example, OBCAM (OPCML) turned out to be a susceptibility gene for schizophrenia in both European (O'Donovan et al, 2008) and Thai population (Panichareon et al, 2012). In this light, a recent finding that NEGR1 (kilon) is a candidate gene for body weight control (Lee et al, 2012) is especially interesting as there is a clear link between metabolic abnormalities and psychiatric disorders; for example, patients with schizophrenia may be at greater risk for metabolic disorders such as insulin resistance, lipid abnormalities, and weight gain. In addition, the use of atypical antipsychotics in the treatment of schizophrenia appears to be associated with varying degrees of comorbid metabolic disorders, such as metabolic syndrome (Henderson 2005).

5. Lsamp as a tumour suppressor gene and a possible role in other diseases

Interestingly, recent publications have revealed LSAMP as a putative tumor-suppressor, being associated with clear cell renal cell carcinomas (Chen et al, 2003), myeloid leukemia (Kühn et al, 2012), and osteosarcomas (Kresse et al, 2009; Yen et al, 2009; Pasic et al, 2010); for example, Pasic et al (2010) found ubiquitous changes in the Lsamp gene in osteosarcoma, usually involving loss of expression. Depleting LSAMP promoted proliferation of normal osteoblasts by regulation of apoptotic and cell-cycle transcripts and also VEGF receptor 1. Also, the whole IgLON family has been found to be implicated in epithelial ovarian cancer (Ntougkos et al, 2005).

Association mapping on chromosome 3q13–21 detected evidence for association at the Lsamp gene in individuals with late-onset coronary artery disease (CAD). The risk conferred by the Lsamp haplotype appears to be mediated by Lsamp down-regulation, which may promote smooth muscle cell proliferation in the arterial wall and progression of atherosclerosis (Wang et al, 2008).

6. Concluding remarks

The general function of the Lsamp gene is known: its protein LSAMP forms heterodimers with other members of the IgLON family and these protein complexes are responsible for axon targeting and the regulation of neurite outgrowth. Association studies have revealed links between the IgLON proteins, including Lsamp, and psychiatric disorders. Several animal studies with both rats and mice have revealed links between the Lsamp gene and anxiety. The Lsamp gene is therefore a promising target for neuropsychiatric disorders. Therefore, the crucial question is: what are the molecular mechanisms behind these links? In other words, how is the genetics of the IgLON family translated into behavioural outcomes? Studying Lsamp gene deficient mice helps to bring some light into this question.

AIMS OF THE STUDY

1. To provide a general behavioural characterisation of Lsamp gene deficient mice, including sensory, locomotor, anxiety-related and social behaviour-related aspects.
2. To study the effect of different housing conditions (standard housing, isolation and environmental enrichment) on the behavioural phenotype and a biochemical stress marker (corticosterone level) in Lsamp-deficient mice. To study the stability of phenotypic deviations of Lsamp-deficient mice seen in standard housing in other housing conditions.
3. To study the activating and rewarding effect of amphetamine on Lsamp-deficient mice and corresponding changes in the level of major monoamines and their metabolites in five different brain regions; to measure the expression levels of dopaminergic system-related genes in Lsamp-deficient mice.

MATERIALS AND METHODS

1. Animals

Lsamp knockout targeting construct was created by amplifying both genomic arms from wild-type 129/SvEvTACfBr mouse genomic DNA. The 5' genomic arm (1.8 kb) included a Lsamp 1b promoter sequence, and the 3' genomic arm (4.6 kb) included a sequence from Lsamp intron 1. During targeted homologous recombination a LacZNeo cassette completely replaced Lsamp exon 1b (according to exon-intron structure of the Lsamp gene described by Pimenta et al (2004)). pGEM-3Zf(+) cloning plasmid (Promega) was used as a backbone during cloning and a pgk-TK negative selection cassette was cloned upstream of the 5' genomic arm and pGEM-3Zf(+) sequence (Figure 2a). BamHI-linearized targeting construct was electroporated into W4/129S6 embryonic stem (ES) cells (Taconic) which were selected for resistance to Neomycin and Gancyclovir. ES cell colonies were tested for homologous recombination by PCR using recombination-specific primer pair LacZRev 5'-GTGCTGCAAGGCGAT TAAGTTG and Lsamp_F_-2.25 kb 5'- GCACGTGTCTGTAGCTAACCA. 2.3 kb PCR-product was sequenced to verify the integration site. ES cell clone 6F1 was injected into C57BL/6 blastocysts, and Lsamp F1 (+/–) founder animals were produced by mating male chimeras with C57BL/6 female mice. F2 generation Lsamp wild-type (Lsamp$^{+/+}$) and homozygous (Lsamp$^{-/-}$) and heterozygous (Lsamp$^{+/-}$) littermates were obtained by mating heterozygous (Lsamp$^{+/-}$) founder animals. All studies were performed in male F2 hybrids [(129S6/SvEvTac×C57BL/6)×(129S6/SvEvTac×C57BL/6)]. Primers: Lsamp+706bpRev (5'- CCTATGATGTCAATTCAGAGATC); Lsamp-290bpF 5'-ATTGACAGTCGCCTCCTCATC) and LacZRev were used for multiplex genotyping reaction for all three genotypes (+/+, +/–, –/–) (Figure 2b).

Successful deletion of the LSAMP protein after targeted disruption of Lsamp 1b exon (Figure 2c) was confirmed with Western blotting. Brain samples from wild-type mice exhibited a single LSAMP antibody specific band of approximately 68 kDa, whereas samples harvested from Lsamp$^{-/-}$ mice were negative for this band. Non-neural tissues of all mice were negative for LSAMP immunoreactivity that confirms brain-specificity of the LSAMP protein. All samples were positive for GAPDH antibody that was used as a housekeeping control.

Male wild-type (Lsamp$^{+/+}$) mice and their homozygous *Lsamp*-deficient littermates (Lsamp$^{-/-}$) mice were used in the present study. In some experiments, heterozygous (Lsamp$^{+/-}$) animals were also included. Mice were group-housed in standard laboratory cages (42.5 × 26.6 × 15.5 cm) 8 (in some instances 7 or 9) animals per cage in the animal colony at 22±1°C under a 12:12 h light/dark cycle (lights off at 19:00 h). 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia) was used in each cage and changed every week. No other enrichment was used besides nesting

material (except as described in Paper II). Tap water and food pellets (R70, Lactamin AB, Sweden) were available *ad libitum*. Unless noted otherwise, all experiments were performed with mice aged 2–3 months.

All animal procedures in this study were performed in accordance with the European Communities Directive (86/609/EEC) and permit (No. 59, September 5, 2006) from the Estonian National Board of Animal Experiments.

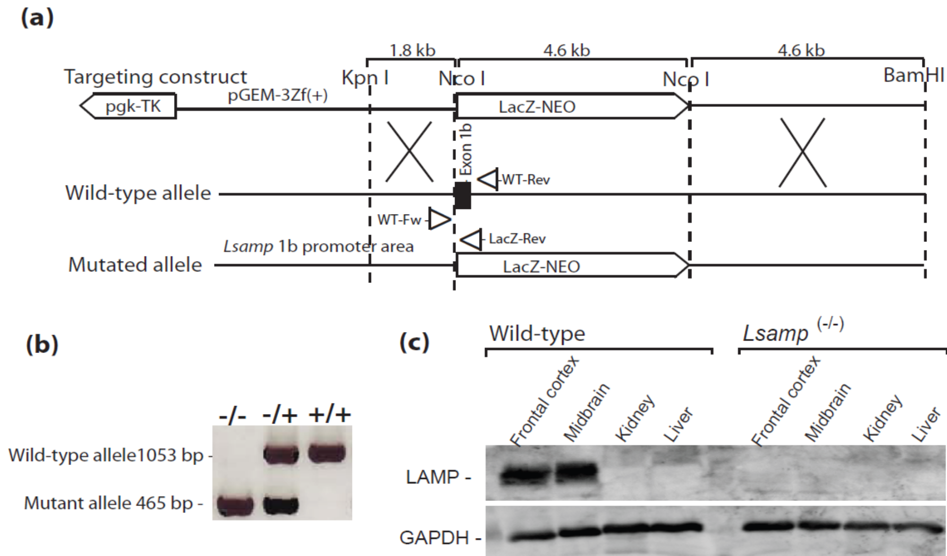

Figure 2. Targeted disruption of the Lsamp gene. (a) A knockout construct was made by replacing 4.6 kb of the Lsamp gene (exon 1b through part of intron 1) with a 4.6 kb NLS-LacZ-NEO cassette. Arrowheads represent locations and directions of genotyping primers (WT-Rev, WT-Fw and LacZ-Rev). (b) A multiplex-PCR-based genotyping assay amplifies 1053 bp fragment from the endogenous allele and a 465 bp fragment from the targeted allele. (c) Western blot of wild-type and Lsamp$^{-/-}$ mouse tissue extracts from the frontal cortex, midbrain, kidney and liver. Brain samples from wild-type mice exhibit a a single band of approximately 68 kDa, whereas samples harvested from Lsamp$^{-/-}$ mouse samples are negative for this band. Non-neural tissues of both wild-type and Lsamp$^{-/-}$ mouse are negative for LSAMP immunoreactivity. GAPDH antibody (38 kDa) was used as a housekeeping control.

2. Environmental manipulations (Paper II)

Mice used in Papers I and III lived in standard housing as described in section „Animals". However, in Paper II, three batches of mice were reared in three different housing conditions. At 5 weeks of age, batch A was assigned to standard housing conditions for 5 weeks, batch B was assigned to standard housing conditions for 4 weeks which was followed by a 1-week social isolation, and batch C was assigned to environmentally enriched conditions for 5 weeks.

Behavioural testing began at 10 weeks of age. Standard housing conditions consisted of standard laboratory cages (42.5 cm × 26.6 cm × 15.5 cm), 8 mice per cage. Mice in the environmentally enriched conditions were housed 8 mice per cage in larger cages (59.5 cm × 38.0 cm × 20.0 cm) containing stainless steel wheels and aspen houses, igloos, ladders and tubes, which were changed and repositioned once a week as described in Abramov et al (2008). For individual housing, smaller cages (33 cm × 12 cm × 13 cm) were used. In all conditions, aspen bedding (Tapvei, Estonia) and aspen nesting material (Tapvei, Estonia) were used in each cage and changed every week. All mice were housed in the animal colony at 22±1 °C under a 12:12 h light/dark cycle (lights off at 19:00 h). In all groups, tap water and food pellets (R70, Lactamin AB, Sweden) were available ad libitum. The body weight data was collected weekly from 5 weeks of age until 14 weeks of age.

3. Behavioural experiments (Papers I, II, III)

Testing was carried out between 11:00 and 19:00 of the light phase. Before each experiment, mice were let to habituate to the experimental room and the lighting conditions therein for one hour.

3.1. Sensory testing (Paper I)

Sensory testing was performed to rule out robust deficits in vision, hearing, mechanical sensitivity and olfaction. Forepaw reach test (also called "visual placing test"), estimating vision, and ear twitch test, estimating hearing, were performed generally in the same way as described in Philips et al (2008). In short, in the reach test, a mouse was held by its tail at a height of 15 cm from a table surface. As the mouse was gradually lowered, extension of its forepaws for a "soft landing" was observed. In the ear twitch test, ear twitching reflex in response to a pen click was observed. Von Frey test for determining mechanical sensitivity was conducted as described by Kurrikoff et al (2004) by means of TouchTest® (North Coast Medical, Inc) monofilaments (bending forces 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g). Mice were placed into individual transparent (16 × 23 × 14 cm) chambers positioned on a metal mesh floor. Each filament was applied to the hind paw four times for 0.5–1 s with an inter-stimulus interval of approximately 5 s. When the hind paw was withdrawn from a particular hair two or more times out of four applications, it was considered as a positive response, in which case a next weaker filament was used. Otherwise, a next stronger filament was used until the threshold of sensitivity was crossed. Buried food-finding test, measuring olfactory abilities, was carried out as described by Radyushkin et al (2009). Starting 4 days prior to testing, mice received each day several pieces of chocolate cookies (1.2 g per mouse) with water *ad libitum*. All groups consumed all cookies within 24 h. Then, mice were

deprived of food for 12 h before testing, with water *ad libitum*. For testing, mice were placed individually into clear cages (29.5 × 18.5 × 13 cm), in which a piece of a chocolate cookie was hidden under 1.5 cm standard bedding at the end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food-finding time, i.e. the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing, was recorded. A fresh cage and bedding was used for each trial.

3.2 Ink test (Paper I)

Ink test was used to analyze the gait of animals. Mice were trained to run through a lighted 50 cm wooden canal into a dark cardboard box. The floor of the canal was covered with white paper. Before each trial the forefeet of the animals were marked with red and hind limbs with blue nontoxic paint. Stride length (distance between two ipsilateral prints) was measured.

3.3 Beam walk test (Paper I)

Four 100 cm beams with different diameters (Ø 20, 17.5, 13 and 9 mm) mounted 50 cm above floor height were used. Mice were trained to walk on a 20 mm training beam for four consecutive days (two beam crossings per day) and on the last day, about 1 h after training, test was performed with 17.5, 13 and 9 mm beams (inter-trial interval 30 min). Traversing time in seconds and the number of slips were measured for each beam (the sum of two consecutive beam crossings for each mouse).

3.4. Dynamometer test (Paper I)

Dynamometer was adapted from Smith et al (1995) measuring mouse forelimb muscle strength and endurance. A self-designed dynamometer was used that exploits a mouse's tendency to grasp a horizontal metal bar while suspended by its tail. The magnitude of force that the mouse could exert was obtained by first allowing the animal to grasp the bar and then steadily increasing the downward force of the cable to which the bar was attached. Each mouse was tested five times with an inter-trial period of ca 10 min. The best result was counted.

3.5. Locomotor activity test (Papers I, II, III)

Locomotor activity of individual mice was measured for 30 min in sound-proof photoelectric motility boxes (44.8 × 44.8 × 45 cm) connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse. Computer registered the distance travelled, the number of rearings, corner visits and time spent and distance covered in the central part of the box.

3.6. Morris water maze (Papers I, II)

The water maze consisted of a circular pool (Ø 150 cm), escape platform (Ø 16 cm in diameter), video camera and computer with software (TSE, Technical & Scientific Equipment GmbH, Germany). The pool (depth 50 cm) was filled with tap water (22 °C, to a depth of 40 cm) that was made opaque by adding a small amount of non-toxic white putty. The escape platform was positioned in the centre of the Southwest quadrant (Q2), 20 cm from the wall. The water level was 1 cm above the platform, making it invisible. Each trial, the animals were put into the water, facing the wall, at pseudo-randomly assigned starting positions (East, North, South or West). The acquisition phase of the experiment consisted of a series of 20 training trials (five trials per day for four consecutive days, inter-trial interval 1 h). Mice were allowed to search for the platform for a maximum of 60 s at which time the mice were gently guided to the platform by means of a metal sieve. Mice remained on the platform for 15 s. Posters and furniture around the maze served as visual cues. During testing, the room was dimly lit with diffuse white light (20 lx). Distance travelled during the trial, latency to find the submerged platform and swim velocity were registered. We used average value per day, which was obtained by collapsing data on five trials for each animal. On Day 4, 1 h after the last training trial, the platform was removed for a probe trial. Mice were put into the water in the Northeast position (Q4) and were allowed to swim for 60 s. Time spent in all four quadrants (Q1, Q2, Q3, Q4) was measured, with time spent in the target quadrant (Q2) where the platform had been located serving as indicator of spatial memory. In Paper II the method was used only for measuring swim speed and the mice were let to swim for 1 min without the hidden platform.

3.7. Active avoidance test (Paper I)

Active avoidance learning was carried out in a rectangular two-way automated shuttle-box (TSE, Technical & Scientific Equipment GmbH, Germany), consisting of two identical chambers (14 × 11 × 16 cm) connected by an arched opening (4 × 4 cm). The box was surrounded by a soundproof chamber. The apparatus was located in a quiet, very dimly (5 lx) illuminated room. The shuttle-boxes had a cover with a light-bulb (10 W) attached above each compartment. Foot-shocks could be administered through stainless steel rod floor (Ø 3 mm, spaced 5 mm). Mice were placed in the dark compartment facing the wall of the chamber and submitted to an active avoidance test for five consecutive days, 30 consecutive trials a day. The test started with a habituation time of 10 s. The conditioned stimulus (CS) was a 10 kHz tone with a maximum duration of 20 s accompanied by lighting up of the target compartment (light and sound signal). The unconditioned stimulus (US; 0.3 mA electrical foot-shock for 5 s) was switched on 5 s after CS and was followed by a stronger US (by a 0.6 mA foot-shock for a maximum of 10 s) in case the mouse failed to

move to the target compartment. Intertrial interval was 10 s. The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse.

3.8. Novel object recognition test (Paper I)

Two identical glass blocks (1 × 4 × 5 cm) were placed upright on the floor of the open field box equidistant from the walls at either end. Mice were placed in the dimly lit (5 lx) arena and allowed to explore for 5 minutes, after which they were transferred to their home cage. 150 minutes later the two glass blocks were replaced with one identical clean glass block and one novel object (5 cm high glass bottle with a white plastic cap) and the mouse was returned to the arena for 5 minutes. Distance travelled, rearings and the number of explorations of each object were recorded from both trials. The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse.

3.9. Elevated plus maze (Papers I, II)

The apparatus consisted of two opposite open (17.5 × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. The entire plus-maze apparatus was elevated to a height of 30 cm and placed in a dim room (10 lx in open arms). Testing began by placing the animal on the central platform of the maze facing an open arm. An arm entry was counted only when all four limbs were within a given arm. The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse. Standard 5 min test duration was employed and the sessions were videotaped. The following parameters were observed by an experienced observer: (1) latency to enter an open arm; (2) number of entries in open arms; (3) number of entries in closed arms; (4) time spent in open arms; (5) total number of head-dippings and (6) number of unprotected head-dippings defined as head-dippings made in open arms.

3.10. Scoring barbering behaviour (Papers I, II)

In Paper I, after weaning at 21 days of age, the mice were group-housed by genotypes. At that point in time a difference was noted in the appearance of wild-type and Lsamp$^{-/-}$ mice: while most wild-type mice had trimmed whiskers and facial hair, most Lsamp$^{-/-}$ mice had full sets of whiskers. Barbering behaviour was consequently estimated in group-housed male mice (7–9 animals per cage) on a three-point scale: (1) no whiskers, (2) partially trimmed whiskers and (3) full whiskers (see „Results" section, Figure 7d). As there were clear age-dependent differences in whisker trimming, we differentiated between three different age groups: (1) 4–5 weeks; (2) 6–15 weeks; (3) 16–30 weeks. The whisker-trimming data was collected in two sessions conducted in (1) April-

May and (2) October-November, but no seasonal fluctuations in trimming patterns could be observed, thus the results were pooled. In Paper II, whiskers were evaluated at the start and at the end of the experiment by using the same three-point scale.

3.11. Social dominance tube test (Paper I)

The test apparatus was adapted from Lijam et al (1997) and Koh et al (2008) with some modifications in the experimental design. Two waiting chambers, sized 10 × 10 × 10 cm, were connected by a 30 cm clear plexiglas tube (Ø 3 cm). One wild-type mouse and one Lsamp$^{-/-}$ mouse were placed in the waiting chambers at the opposite ends of the tube and were thereafter simultaneously released into the tube. The mouse that remained in the tube, while its opponent completely backed out from the tube, was declared "winner". The winner was given a score "1" and the loser a score "0". Each trial lasted a maximum of 10 min and an even score "0.5" was counted when both opponents remained into the tube. During testing, the room was dimly lit with diffuse white light (25 lx). The results are presented as % of wins for each genotype (see "Results" section, Figure 7e). Each mouse was tested six times with six different weight-matched mice of the opposite genotype.

3.12. Social interaction between male mice (Papers I, II)

In Paper I, social interaction test was carried out as described by Philips et al (2008) with some modifications in the scoring system. Two unfamiliar mice of the same sex and genotype were simultaneously placed in an empty housing cage (22 × 16 × 14 cm) with a cover made of transparent Plexiglas. One week later the test was repeated with a new partner. Illumination level of the testing arena was 25 lx. Mice were matched for body weight, and their behaviour was videotaped for 10 min. The videotapes were later scored by a trained observer. The following measures were registered for each mouse: (1) sniffing the body of the other mouse, (2) anogenital sniffing of the other mouse, (3) self-grooming, (4) digging, (5) rearing, (6) aggressive attacks, and (7) passive contact.

In Paper II, two male mice (one mice from the control, EE or isolation group and one wild-type age- and weight-matched partner) were simultaneously placed in an empty housing cage (22 cm × 16 cm × 14 cm) with a cover made of transparent Plexiglas. Illumination level of the testing arena was 25 lx. Mice were videotaped for 10 min. The videotapes were later scored by a trained observer. The following measures were registered for each mouse: (1) sniffing the body of the other mouse, (2) anogenital sniffing of the other mouse, (3) self-grooming, (4) digging and (5) aggressive attacks.

3.13. Social interaction between male and ovariectomized female mice (Paper I)

Twenty four female C57BL/6J mice were bilaterally ovariectomized under general anesthesia three weeks before the interaction test at 10 weeks of age. Oestrus in ovariectomized females (average weight 25±2.4 g) was induced by 5 µg of estradiol (E8875-1G, Sigma, St Louis, MO, USA) and 0.5 mg of progesterone (P0130-25G, Sigma, St Louis, MO, USA) dissolved in 0.2 ml of sesame oil and subcutaneously injected 24 h and 4–5 h before the test, respectively. The doses of estradiol and progesterone were derived from Ågmo et al (2008). Twelve wild-type and 12 Lsamp$^{-/-}$ male mice were let to interact with an unfamiliar female wild-type mouse three days before testing to adapt to the testing situation. On the testing day, each male mouse was let to interact with a separate ovariectomized female matched for body weight in an empty housing cage (22 × 16 × 14 cm) with a transparent Plexiglas cover and an illumination level of 25 lx for 10 min. The sessions were videotaped and the following parameters were scored: (1) sniffing the body of the female mouse, (2) anogenital sniffing of the female mouse, and (3) passive contact with the female mouse.

3.14. Marble burying test (Paper II)

Twenty glass marbles (1.5 cm in diameter) were placed on 5 cm of sawdust bedding as a 4 × 5 grid in a clear plastic box (42.5 cm × 26.6 cm × 15.5 cm). The mice were placed in the box individually for 30 min, and the number of marbles buried at least two-thirds deep were counted.

3.15. Conditioned place preference test (Paper III)

Conditioned place preference test was conducted with the amphetamine dose of 2.5 mg/kg in a two-chamber apparatus (TSE, Technical & Scientific Equipment GmbH, Germany) with two equal sized chambers that differed in wall colour and pattern and were separated by a doored wall. This dose was chosen based on a pilot study to avoid the behavioural stereotypies and motor activation, but to induce a measurable preference effect in wild-type mice. During preconditioning, mice were habituated to the apparatus for 30 min in 2 consecutive days (Days 1–2) and the last session was taken as baseline. The less preferred chamber was designated as the conditioning chamber where the animal would receive amphetamine. Each mouse underwent an experiment with a biased non-counterbalanced design including 12 conditioning sessions. On odd-numbered days (Days 3, 5, 7, 9, 11, 13), half of the mice received a saline injection and were placed in the preferred chamber for 30 min and the second half received amphetamine and were placed in the less preferred chamber for 30 min. On even-numbered days (Days 4, 6, 8, 10, 12, 14), the procedure was reversed.

Finally, 48 h after the last conditioning session, mice were placed in the apparatus and given free access to the two chambers for 30 min. The amount of time spent in each chamber was recorded. Testing was carried out between 10:00 and 19:00 of the light phase. Before each experiment, mice were let to habituate with the experimental room for 1 h.

4. Phenotyping batteries used in Paper I

Table 1 gives an overview of the phenotyping batteries and the number of animals used in Paper I.

Table 1. Phenotyping batteries and the number of animals used in the experiments.

	Test battery	**Behavioural test**	**Batch of mice (letter) and testing order (number)**	**Number of animals used**	
				Wild-type	**Lsamp$^{-/-}$**
I	Sensory-motor testing	Locomotor activity test	A1	31	29
		Ink test	B2	21	16
		Beam walking test	B3	21	16
		Dynamometer test	B4	21	16
		Von Frey test	B1	21	16
		Buried food-finding test	C1	16	16
		Forepaw reach test	C2	16	16
		Ear twitch test	C3	16	16
II	Memory and learning	Morris water maze test	A2	18	16
		Active avoidance test	A3	18	16
III	Anxiety	Elevated plus-maze in dim room	D1	16	16
		Elevated plus-maze in illuminated room	E	17	17
		Elevated plus-maze with diazepam/vehicle injection	F	15/17	15/13
IV	Social behaviour	Barbering behaviour		178	174
		Social dominance tube test	G	36	36
		Social interaction test between male mice	H	28	18
		Social interaction between male and female mice	D2	12	12

5. Gene expression analysis by qRT-PCR (Papers I, III)

In Paper I, we analyzed the expression of GABA-ergic system-related genes in the prefrontal cortex (perpendicular cut was made straight before the outer contours of the tuberculum olfactorium), temporal lobe (including amygdala) and mesolimbic area (including nucleus accumbens and olfactory tubercle) of experimentally naïve wild-type (N = 7) and Lsamp$^{-/-}$ mice (N = 9) at 10 weeks of age. Mice were taken from the home-cage and decapitated immediately in a separate room. Brains were quickly dissected into three parts according to the coordinates presented in the mouse brain atlas (Franklin and Paxinos, 1997) and frozen in liquid nitrogen. mRNA levels of alpha1 (Gabra1) and alpha2 (Gabra2) subunits of $GABA_A$ receptors and Gad1 and Gad2 genes (glutamate decarboxylases responsible for the synthesis of GABA) were determined by qRT-PCR as described by Raud et al (2009). Briefly, qRT-PCR was performed using ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, USA) equipment and ABI Prism 7900SDS Software. The experiment for Gabra1 and Gabra2 expression studies was performed using SYBR Green I qPCR Core Kit (Eurogentec, Belgium). For Gad1 and Gad2 gene expression, Taqman assays Mm00725661_s1 and Mm01329282_m1 (Applied Biosystems) were used, respectively. All reactions were performed in four parallel samples to minimize the effect of technical errors and in a final volume of 10 µl, using 50–100 ng of cDNA. The housekeeping gene Hprt1 (hypoxanthine phosphor-ribosyl-transferase 1) was used as an endogenous control in all qRT-PCR reactions. The expression levels of GABA-ergic system related transcripts were determined relative to the housekeeping gene using the comparative ΔC_T method (Livak et al, 2001).

In Paper III, wild-type, Lsamp$^{+/-}$ and Lsamp$^{-/-}$ mice were decapitated and their brains were quickly dissected. The dorsal striatum, ventral striatum (including the nucleus accumbens and olfactory tubercle) and mesencephalon were collected and frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas (Franklin and Paxinos, 1997). The expression level of the dopamine D2 receptor gene was measured in the mesolimbic area and striatum, and the level of the dopamine transporter (DAT) and brain vesicular monoamine transporter (VMAT2) gene were measured in the mesencephalontotal RNA was extracted individually from each brain structure of each mouse using Trizol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. First strand cDNA was synthesized by using poly $(T)_{18}$ oligonucleotides and SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Analyses with wild-type, heterozygous and homozygous animals were conducted in parallel. For quantitative real-time PCR (qRT-PCR) analysis the ABI PRISM 7900HT Fast Real-Time PCR System equipment (PE Applied Biosystems, USA) and the ABI PRISM 7900 SDS 2.2.2 Software were used. Every reaction was made in four parallel samples to minimize possible errors. All reactions were performed in a final volume of 10 µl, using 50–100 ng of cDNA.

6. Biochemical studies (Papers II, III)

6.1. Corticosterone measurement (Paper II)

In Paper II, a separate group of mice, raised analogously to the animals used in the behavioural experiments, was used for measuring corticosterone levels in response to environmental enrichment and isolation. To control for naturally occurring fluctuations in corticosterone levels, the blood samples from both genotypes were collected in parallel. Mice were decapitated and truncal blood (a mixture of arterial and venous blood) was collected into heparinised tubes. Blood samples were centrifuged after collection for 10 min at 1500 × g. Sera were stored at –20 °C. We used Corticosterone HS ELISA kit from Immuno-diagnostic Systems (UK) according to manufacturer's instructions.

6.2. Monoamine content measurements by HPLC (Paper III)

Lsamp$^{-/-}$ and Lsamp$^{+/+}$ mice were randomly divided into groups that received an intraperitoneal injection of either saline or 5 mg/kg of amphetamine. After 30 min in isolation, the mice were decapitated. Brains were quickly dissected into five parts – the frontal cortex, ventral striatum (including the nucleus accumbens and olfactory tubercle), dorsal striatum, mesencephalon and temporal lobe (including the amygdala) – and frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas (Franklin and Paxinos, 1997). Monoamines – noradrenaline (NA), dopamine (DA) and serotonin (5-HT) – and their metabolites – normetanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and 3-methoxytyramine (3-MT) – were assayed by high performance liquid chromatography (HPLC) with electro-chemical detection. The mouse brain tissue samples were homogenized with Bandelin Sonopuls ultrasonic homogenizer (Bandelin Electronic, Berlin, Germany) in ice-cold solution of 0.1 M perchloric acid (10–30 µl/mg) containing 5 mM sodium bisulphite and 0.4 mM EDTA to avoid oxidation. The homogenate was then centrifuged at 17,000×*g* for 10 min at 4°C. Aliquots (10 µl) of the obtained supernatant were chromatographed on a Lichrospher 60 RP Select B column (250 × 3 mm; 5 µm). The separation was done in isocratic elution mode at column temperature of 30 °C using the mobile phase containing 0.05 M sodium citrate buffer at pH 3.7; 0.02 mM EDTA; 1 mM KCl; 1 mM sodium octylsulphonate and 5.6% acetonitrile. The chromatography system consisted of a Hewlett Packard HP 1100 Series isocratic pump, a thermostatted autosampler, a thermostatted column compartment and an HP 1049 electrochemical detector (Agilent, Waldbronn, Germany) with glassy carbon electrode. The measurements were done at an electrode potential of + 0.7 V versus the Ag/AgCl reference electrode.

7. Drugs (Papers I, III)

In Paper I, diazepam (Grindex, Latvia), a $GABA_A$ receptor agonist was used. Diazepam (1 mg/kg) was administered 30 min before the study and the control group received vehicle (a few drops of Tween 80 [Sigma] in physiological saline [0.9% of sodium chloride solution]). Diazepam was suspended in saline with the help of a few drops of Tween 80 (Sigma).

In Paper III, amphetamine (amphetamine sulphate, Sigma-Aldrich, USA) was freshly prepared in sterile, pyrogen free, 0.9% solution of sodium chloride (B. Braun Melsungen AG, Germany).

For saline injections, sterile, pyrogen free, 0.9% solution of sodium chloride (B. Braun Melsungen AG, Germany) was used. All drugs were injected intraperitoneally (i.p.) at a volume of 10 ml/kg.

8. Statistical analysis (Papers I, II, III)

Results are expressed as mean values ± SEM. Statistica for Windows 7.0 (Paper I) or 10.0 (Papers II, III) software was used for statistical analysis.

In Paper I, Mann-Whitney U-test was used to analyze genotype effects in whisker trimming and inter-male and male-female social interactions. Student's t-test was applied for the sensory-motor testing battery and locomotor activity test. Two-way independent-groups ANOVA was used for the following measurements and tests: elevated plus-maze (genotype × light condition or genotype × diazepam/saline injection), probe trial of the water maze (genotype × quadrant) and novel object recognition (genotype × object). Repeated measures ANOVA was used to analyze body weight changes, locomotor activity by 10 min periods and the learning curves in the active avoidance and water maze tests. Comparisons between individual groups were performed by means of Newman-Keuls (behavioural studies) and Tukey HSD (gene expression studies) post hoc tests. Chi-square one-sample analysis was used to analyze the results of the social dominance tube test. P value below 0.05 was considered to be significant.

In Paper II, for the behavioural experiments, two-way independent-groups ANOVA was used (genotype × housing). Repeated measures ANOVA was used to analyze body weight changes. Comparisons between individual groups were performed by means of Newman-Keuls post hoc test. P value below 0.05 was considered to be significant.

In Paper III, the results of the amphetamine experiment in the motility box (genotype × dose) and monoamine measurements (genotype × treatment) were analysed by means of two-way ANOVA. Gene expression experiments and the conditioned place preference experiment were analysed by means of one-way ANOVA. Newman-Keuls post hoc test was used. In all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

1. Paper I

1.1 Initial characterisation of Lsamp-deficient mice

F2 hybrid Lsamp-deficient (Lsamp$^{-/-}$) mice were vital and fertile. In general, there were no obvious deviations in Lsamp$^{-/-}$ mice in the development or gross-anatomy of the brain similarily to Lsamp knockout mice created at the University of Vanderbilt (Catania et al, 2008). However, our Lsamp$^{-/-}$ mice had approximately 10% lower body weight than both their wild-type littermates and Lsamp$^{+/-}$ mice; repeated measures ANOVA revealed a significant genotype effect ($F_{2,57}$ = 15.86, P < 0.000) and age effect ($F_{9,513}$ = 534.07, P < 0.000), but no genotype x age effect for body weight (Figure 3a). The only visible deviation that could be observed in our Lsamp$^{-/-}$ mice was lack or remarkable decrease of barbering behaviour that was common in their wild-type littermates. A group of Lsamp$^{-/-}$ male mice (N = 7) was preserved for a protracted follow-up until 22 months of age without any notable health manifestations.

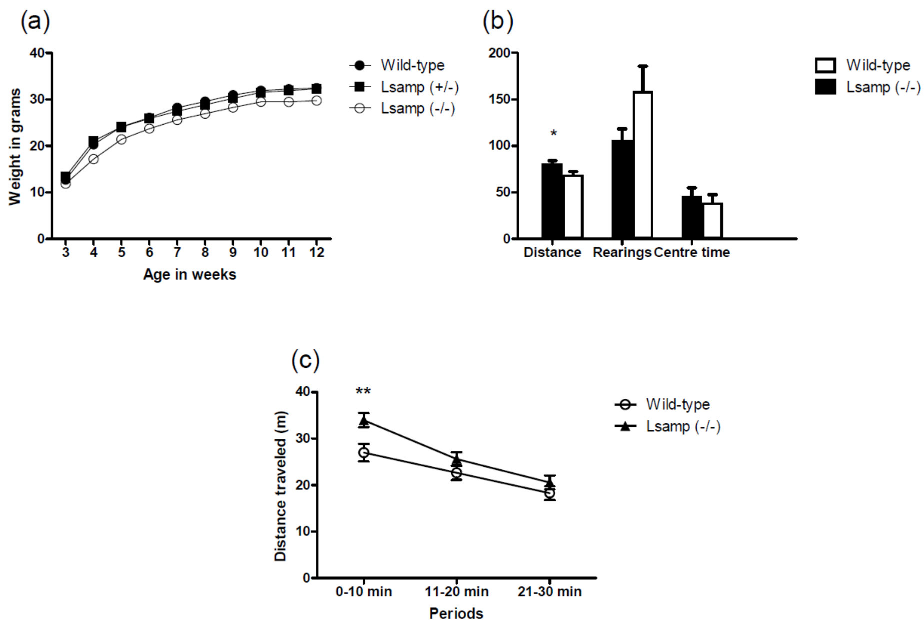

Figure 3. Body weight and locomotor activity. (a) Body weight, (b) distance traveled, rearings and centre time in the motility box, (c) distance traveled by 10 min periods in the motility box. * P < 0.05; ** P < 0.01: Lsamp$^{-/-}$ mice compared to wild-type mice.

1.2. Sensory-motor testing

Lsamp$^{-/-}$ mice showed results similar to those of wild-type mice in the forepaw reach, ear twitch, and buried food-finding tests, which indicated that Lsamp$^{-/-}$ mice had no gross hearing, vision or olfaction deficiencies. There were no differences between the two genotypes in the Von Frey test that measures mechanical sensitivity. Also, Lsamp$^{-/-}$ mice did not differ from wild-type mice in the ink test, beam walk test and dynamometer test that measure gait, motor abilities and muscle power, respectively. The average values for wild-type and Lsamp$^{-/-}$ mice are presented in Table 2.

Table 2. Statistically non-significant differences between wild-type and Lsamp$^{-/-}$ mice in the sensory-motor testing battery. Abbreviations: (s) seconds, (mm) millimeters, (g) grams.

Test	**Parameter**	**Wild-type**	**Lsamp$^{-/-}$**
		Average (SEM)	**Average (SEM)**
Beam walk test	Traversing time, 17,5 mm (s)	10.5 ± 1.0	10.8 ± 1.3
	Traversing time, 14 mm (s)	12.7 ± 1.3	13.0 ± 1.4
	Traversing time, 9 mm (s)	15.0 ± 1.5	15.3 ± 1.4
Ink test	Stride length (mm)	68.0 ± 1.2	67.5 ± 1.5
Dynamometer test	Grasping power (g)	78.0 ± 2.1	79.0 ± 2.3
Forepaw reach	Responce to visual placing (%)	100	100
Ear twitch	Response to auditory stimuli (%)	100	100
Buried food-finding test	Latency to find buried cookie (s)	52.6 ± 17.7	39.0 ± 18.5
	Latency to find visible cookie (s)	20.1 ± 4.2	18.2 ± 6.4
Von Frey test	Mechanical sensitivity (g)	1.2 ± 0.1	1.4 ± 0.1

1.3 Locomotor activity test

Experimentally naïve Lsamp$^{-/-}$ mice were more active in the motility box in terms of distance traveled than wild-type mice ($F_{1,58} = 4.11$, $P < 0.05$). Lsamp$^{-/-}$ mice performed somewhat less rearings, but the difference was not significant ($F_{1,58} = 0.27$, $P = 0.09$). There was no difference in time spent in the central region (Figure 3b). To elucidate the question of increased motor activity, we performed repeated measures ANOVA by 10 min periods that revealed significant effects for genotype ($F_{1,58} = 4.02$, $P < 0.05$), period ($F_{2,116} = 106.1$, $P < 0.0001$) and period × genotype ($F_{2,116} = 5.56$, $P < 0.01$). Post hoc analysis showed that the difference in activity was caused by initial hyperactivity in Lsamp$^{-/-}$ mice as the difference between the two genotypes in distance traveled during the first 10 min was highly significant ($P < 0.01$), but did not differ during

the second and third 10 min period (Figure 3c). No significant differences in other parameters were observed when the results were analyzed by 10 min periods.

1.4 Morris water maze

Significant effect of genotype ($F_{1,29} = 30.552$, $P < 0.001$) was observed in swim velocity, $Lsamp^{-/-}$ mice being slower swimmers (Figure 4a). Hence the distance covered in finding the escape platform was a more appropriate measure of the learning effect than escape latency. Swimming distance (Figure 4b) was affected by trial ($F_{3,87} = 60.32$, $P < 0.001$) and genotype × trial interaction ($F_{3,87} = 2.87$, $P < 0.05$), but not genotype. On the first day, the distance covered by wild-type mice was significantly ($P < 0.001$) longer than in $Lsamp^{-/-}$ mice, however, as in ca 90% of the trials on the first day both genotypes failed to find the platform and swam freely for 60 s, this difference reflects just the difference in swim velocity, rather than differences in learning speed and/or search strategies. On Days 2, 3 and 4, no differences in distance covered were observed. In the probe trial, there was a significant quadrant effect ($F_{3,116} = 69.9$, $P < 0.001$), but no genotype or genotype × quadrant effect. Both wild-type and $Lsamp^{-/-}$ mice spent significantly ($P < 0.001$) more time in the target quadrant Q2 as compared to other quadrants (Figure 4c).

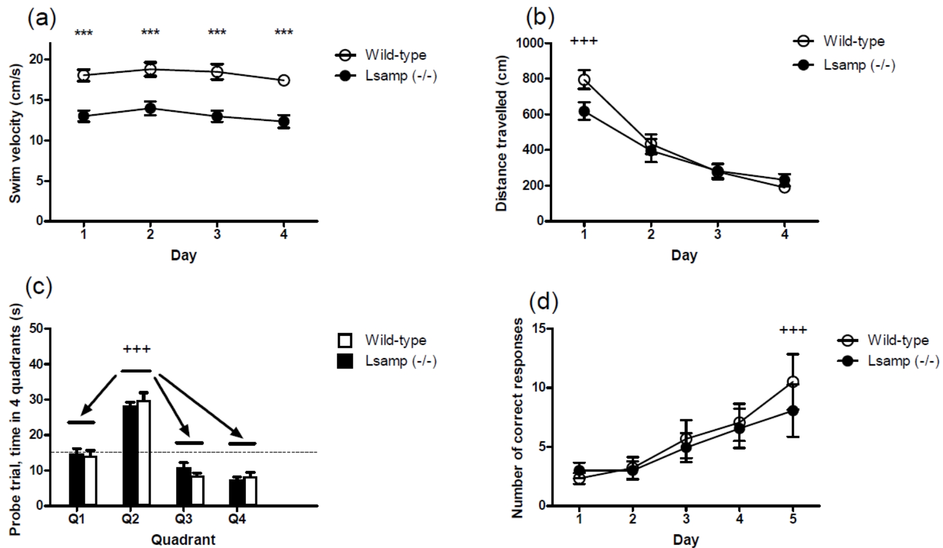

Figure 4. Morris water maze and active avoidance test. (a) Swim velocity, (b) distance travelled, (c) time in 4 quadrants in the probe trial in the water maze test, and (d) the number of correct responses in the active avoidance test. Dotted line in (c) denotes chance performance level (15 s). +++ $P < 0.001$: day 1 compared to day 4 (b), time in target quadrant Q2 compared to time in any other quadrant (c), day 5 compared to day 1 (d) of respective genotype. *** $P < 0.001$: $Lsamp^{-/-}$ mice compared to wild-type mice.

1.5. Active avoidance test

Both wild-type and Lsamp$^{-/-}$ mice learned the task without significant differences. The number of correct responses was affected by trial (within-subjects) ($F_{4,128} = 14.22$, $P < 0.001$), but not genotype or genotype × trial. The learning curve is presented in Figure 4d.

1.6. Novel object recognition test

Both genotypes displayed preference for the new object without significant differences. In the first session with two similar objects, there was no genotype, object or genotype × object effect. In the second session with one familiar and one new object, there was a significant object effect ($F_{1,60} = 13.203$, $P < 0.001$), but no genotype or genotype × object effect.

1.7. Elevated plus maze test in dim and illuminated room

The effect of illumination on the exploratory behaviour in the plus maze is shown in Figure 5. Number of open arm entries was significantly affected by illumination ($F_{1,53} = 5.01$, $P < 0.05$) and genotype ($F_{1,53} = 7.58$, $P < 0.01$) (Figure 5b). In the number of closed arm entries, a significant genotype ($F_{1,53} = 6.12$, $P < 0.05$) and an almost significant illumination ($F_{1,53} = 3.73$, $P = 0.06$) effect was observed (Figure 5c). Time in open arm was affected by genotype ($F_{1,53} = 5.02$, $P < 0.05$) (Figure 5d). Ratio between open and total arm entries was affected by illumination ($F_{1,53} = 12.6$, $P < 0.001$) (Figure 5e). There was a main effect of genotype ($F_{1,53} = 12.1$, $P < 0.001$) on the number of unprotected head-dippings (Figure 5f). In a dim room the exploratory behaviour of wild-type and Lsamp$^{-/-}$ mice did not differ. Illuminating the room did not affect the behaviour of Lsamp$^{-/-}$ mice, but reduced the exploratory activity of wild-type mice: increased the latency to enter open arm by more than two times (Figure 5a) and decreased the number of open arm entries by five times (Figure 5b), time spent in open arms by three times (Figure 5c) and the number of unprotected head-dippings by three times (Figure 5f). However, as the interactions (plus maze parameter × illumination) did not reach the level of statistical significance, these differences cannot be reported as significant.

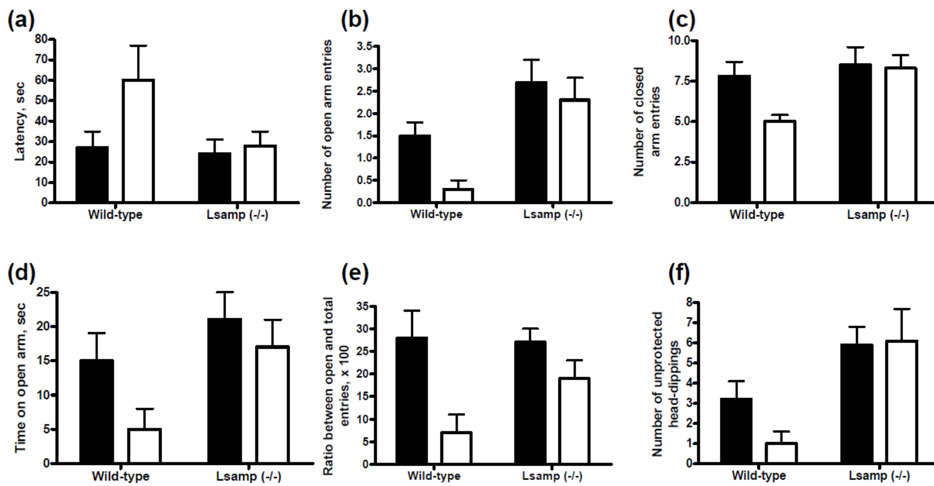

Figure 5. Effect of different illumination conditions on the exploratory behaviour of Lsamp$^{-/-}$ mice in the elevated plus-maze. (a) Latency to enter an open arm, (b) number of open arm entries, (c) number of closed arm entries, (d) time in open arm, (e) ratio between open and total arm entries, and (f) number of unprotected head-dippings. Black columns: dark room; white columns: illuminated room.

1.8. Effect of diazepam in illuminated elevated plus maze

The effect of diazepam on the exploratory behaviour in the plus maze is shown in Figure 6. There was a main effect of genotype on the latency to enter an open arm ($F_{1,58} = 4.24$, $P < 0.05$), number of open arm entries ($F_{1,58} = 7.67$, $P < 0.01$), time in open arm ($F_{1,58} = 5.49$, $P < 0.05$), and number of unprotected head-dippings ($F_{1,58} = 11.4$, $P < 0.001$). The number of total head-dippings was affected by treatment ($F_{1,58} = 5.02$, $P < 0.05$). As established already in the first plus maze experiment, Lsamp$^{-/-}$ mice displayed higher exploratory activity in an illuminated room. The number of open arm entries (Figure 6b) as well as the number of unprotected head-dippings (Figure 6e) were higher in Lsamp$^{-/-}$ mice compared to wild-type mice and treatment with diazepam (1 mg/kg) increased the number of open arm entries by more than four times (Figure 6b) and the number of unprotected head-dippings (Figure 6e) by ten times in wild-type mice. In contrast, in Lsamp$^{-/-}$ mice diazepam increased time spent in open arms by almost three times. However, these differences did not reach the level of statistical significance, and should be regarded as preliminary.

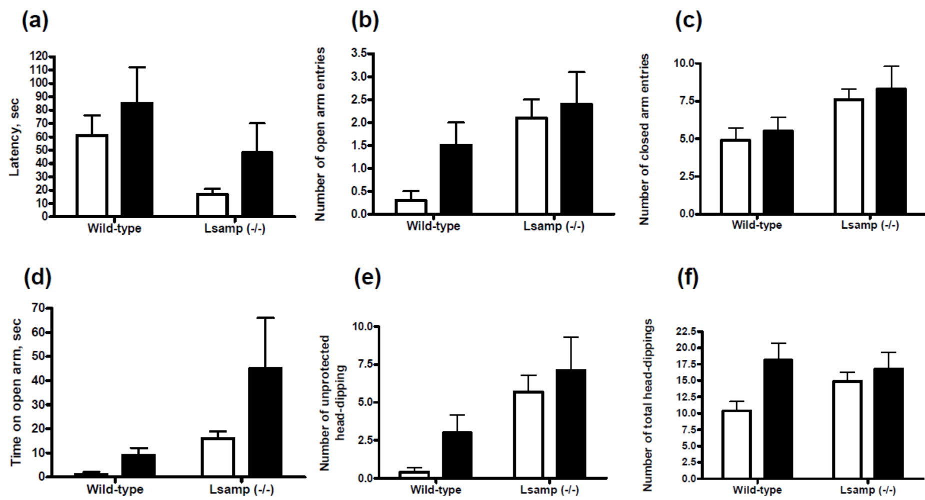

Figure 6. Effect of diazepam on exploratory behaviour of Lsamp$^{-/-}$ mice in the elevated plus-maze. (a) Latency to enter an open arm, (b) number of open arm entries, (c) number of closed arm entries, (d) time in open arm, (e) number of unprotected head-dippings, and (f) number of total head-dippings. Black columns: diazepam; white columns: vehicle.

1.9. Barbering behaviour

Lsamp$^{-/-}$ mice showed a remarkable decrease in barbering behaviour and this effect was strongly dependent on age: in both genotypes no whisker-trimming was observed until four weeks of age (data not shown). At age 4–5 weeks the first manifestations of barbering behaviour could be noted in wild-type mice (Figure 7a). Strong barbering tendency was present in wild-type mice older than six weeks: there was typically only one mouse in every cage that had whiskers. The genotype effect in barbering behaviour was the most obvious in mice aged 6–15 weeks (Figure 7b). In Lsamp$^{-/-}$ mice barbering behaviour was hardly present before 16 weeks of age, but started to manifest after that (Figure 7c). The whisker trimming behaviour of Lsamp$^{+/-}$ mice was similar to wild-type mice (data not shown). Whisker-trimming was not triggered by social learning, as Lsamp$^{-/-}$ mice, separated from a housing cage with mixed genotypes, did not start trimming and their whiskers were quickly restored (data not shown). Environmental enrichment for 5 weeks also failed to modify the barbering behaviour of Lsamp$^{-/-}$ mice (data not shown).

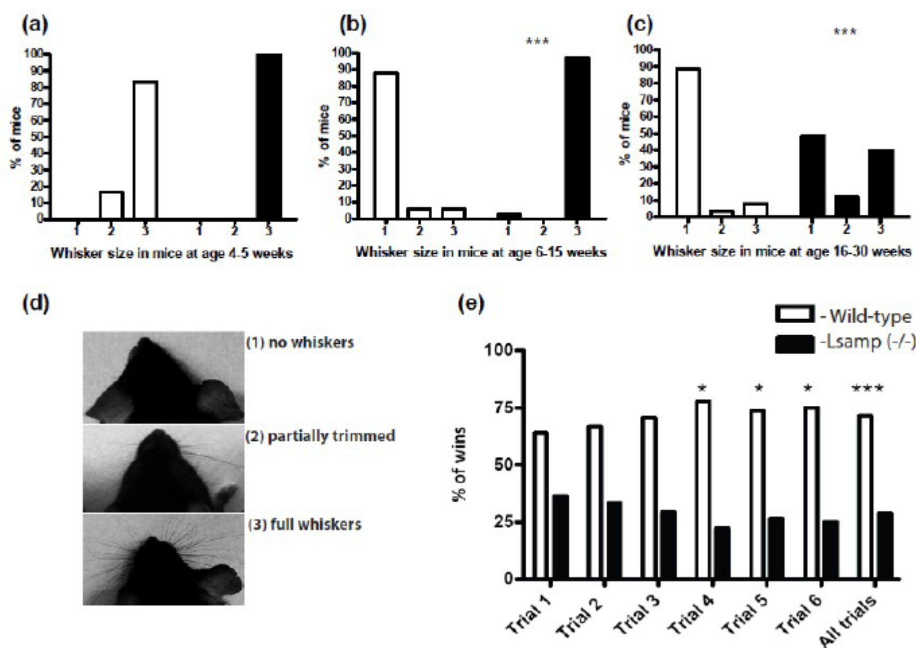

Figure 7. Barbering and social dominance tube test. Whisker size in mice (a) at 4–5 weeks, (b) at 6–15 weeks, and (c) at 16–30 weeks of age. (d) Illustrative examples used for scoring whisker size. (e) Social dominance tube test. * P < 0.05; *** P < 0.001: Lsamp$^{-/-}$ mice compared to wild-type mice.

1.10. Social dominance tube test

After summarizing the results from all six trials the difference between the two genotypes was strongly significant: wild-type mice won 154 fights and Lsamp$^{-/-}$ mice won 62 fights out of 216 fights ($\chi^2 = 20.5$, $P < 0.001$). It shows that wild-type mice are more dominant than Lsamp$^{-/-}$ mice when confronting with each other. However, taken separately, in the first three trials, wild-type mice displayed only a tendency to win over Lsamp$^{-/-}$ mice, but in the last three trials their prevalence became statistically significant. Initial hyperactivity of Lsamp$^{-/-}$ mice that was noticable in several other behavioural tests probably compensated for their subordinate behaviour in the first trials and only after habituation to the testing arena the dominance of wild-type mice became significant (Figure 7e). During the first, second and third trial, wild-type mice won 23, 24 and 25.5 fights out of 36, respectively. During the fourth trial, 28 out of 36 wild-type mice tested won over their Lsamp$^{-/-}$ opponent, which was significantly more than expected by chance ($\chi^2 = 6.02$, $P < 0.05$). In the fifth and sixth trial, wild-type mice won 26.5 ($\chi^2 = 4.8$, $P < 0.05$) and 27 ($\chi^2 = 4.8$, $P < 0.05$) fights out of 36, respectively.

1.11. Social interaction between male mice

As no differences were noted in the results of week 1 and week 2, the results were pooled. There were no significant differences between different genotypes in the general time spent in non-aggressive contact. However, Lsamp$^{-/-}$ mice spent substantially less time sniffing the anogenital area of other male mice ($P < 0.01$; Table 3). There were also no aggressive attacks registered during interactions between Lsamp$^{-/-}$ males. Aggressive attacks were present in 43% of interactions of wild-type mice that corresponds to an average of 23.6 seconds of aggressive attacking during each 10 min testing session. Lsamp$^{-/-}$ and wild-type males did not differ in time spent in passive contact, sniffing other body parts of the partner and in non-social activities such as digging, rearing and self-grooming during a 10 min testing session.

1.12. Social interaction with ovariectomized female

No behavioural difference was detected between Lsamp$^{-/-}$ and wild-type males in the social interaction test with ovariectomized female wild-type mice (Table 3). No direct sexual behaviour (mounting, intromission, ejaculation) was evident between male and female mice in this study.

Table 3. Social interaction scores in inter-male social interactions and in male mice interacting with ovariectomized females. Abbreviation: (s) seconds. * $P < 0.05$; ** $P < 0.01$

		Wild-type	**Lsamp$^{-/-}$**	
Test	**Parameter**	**Average (SEM)**	**Average (SEM)**	
Inter-male social interaction	Sum of non-aggressive contact (s)	138.5 ± 9.2	144.7 ± 9.7	
	Passive contact (s)	71.7 ± 9	103.3 ± 11.3	
	Anogenital sniffing (s)	33.8 ± 5.7	11.8 ± 3.7	**
	Sniffing of other body parts (s)	32.9 ± 2.6	29.6 ± 3	
	Duration of aggressive attacks (s)	23.6 ± 6.9	0.1 ± 0.1	*
	Digging (s)	54.0 ± 6.9	85.3 ± 10.9	
	Self-grooming (s)	11.3 ± 2.9	13.5 ± 2.7	
	Rearings (count)	18.7 ± 1.6	12.3 ± 1.7	
Interaction with female	Sum of non-aggressive contact (s)	129.3 ± 8.3	136.9 ± 10.8	
	Passive contact (s)	63.0 ± 8.9	52.6 ± 6.8	
	Anogenital sniffing (s)	20.3 ± 5.7	23.2 ± 6	
	Sniffing of other body parts (s)	46.0 ± 6.2	61.1 ± 8.5	

1.13. GABA-ergic gene expression in Lsamp-deficient mice

There was a main effect of genotype on the Gabra1 expression level ($F_{1,14} = 6.54$, $P < 0.05$) and a main effect of brain structures on the Gabra2 expression level ($F_{2,28} = 40.5$, $P < 0.0001$), on the ratio between Gabra2 and Gabra1 genes ($F_{2,28} = 24.2$, $P < 0.001$), Gad1 expression level ($F_{2,20} = 29.7$, $P < 0.001$), and Gad2 expression level ($F_{2,20} = 125.7$, $P < 0.001$). The expression level of Gabra1 mRNA was significantly reduced in the temporal lobe, but not in the frontal cortex or mesolimbic area of Lsamp$^{-/-}$ mice (Figure 8a). By contrast, the expression level of Gabra2, Gad1 and Gad2 transcripts was not changed in any of the three brain areas (Figures 8b, 8d and 8e) indicating that there is no systemic impact of Lsamp-deficiency on the GABAergic system. Moreover, we found that the expression of the Gabra2 gene was significantly higher in the temporal lobe compared to the other brain regions (Figure 8b). This is in line with findings that the Gabra2 gene is enriched in the central extended amygdala (Becker et al, 2008). Calculation of the ratio between the Gabra2 and Gabra1 transcripts in wild-type and Lsamp$^{-/-}$ mice revealed a significant shift in favor of the Gabra2 gene in the temporal lobe of Lsamp$^{-/-}$ mice (Figure 8c). Calculation of the ratio between the Gad1 and Gad2 genes did not establish any significant differences between the two genotypes. However, there were significant differences between brain regions. For example, the expression level of the Gad1 gene was significantly higher than that of the Gad2 gene in the frontal cortex compared to other brain regions (compare Figures 8d and 8e).

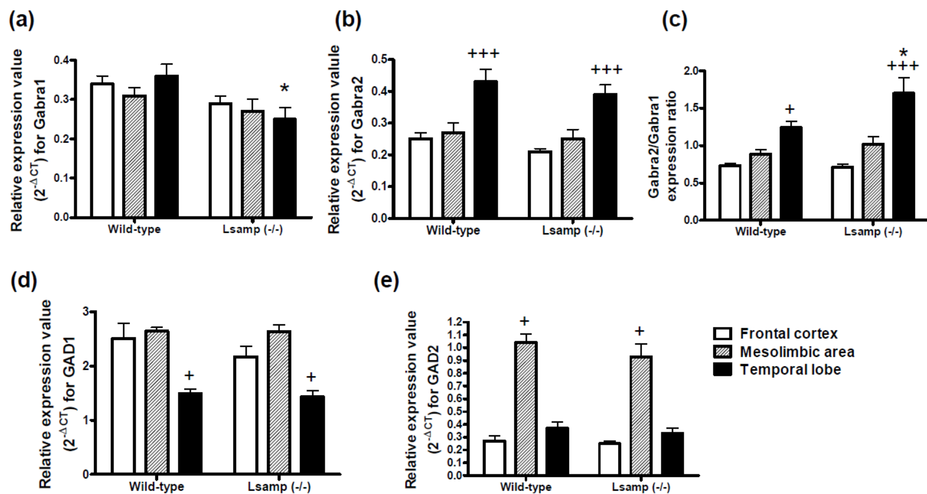

Figure 8. Expression of GABA-related gene transcripts in Lsamp$^{-/-}$ mice. Relative expression values for the Gabra1 (a), Gabra2 (b), Gad1 (d), and Gad2 (e) transcripts, and the expression ratio of the Gabra2 and Gabra1 genes (c). * $P < 0.05$: Lsamp$^{-/-}$ mice compared to wild-type mice; + $P < 0.05$, +++ $P < 0.001$: compared to other brain structures of respective genotype.

2. Paper II

2.1. Body weight measurements and whisker trimming behaviour

EE failed to change whisker trimming behaviour in wild-type and Lsamp$^{-/-}$ mice compared to standard housing. As before (Paper I), wild-type mice had only one or two dominant male(s) per cage that had whiskers and all the other mice were without whiskers, while no whisker trimming behaviour was evident in Lsamp$^{-/-}$ mice, i.e. all mice had whiskers. In standard housing conditions, body weight was significantly dependent on genotype ($F_{1,14}$ = 7.05, P < 0.05), time ($F_{9,126}$ = 146.84, P < 0.001) and genotype × time interaction ($F_{9,126}$ = 2.07, P < 0.05). At 12 and 14 weeks of age, the body weight of Lsamp$^{-/-}$ mice was significantly (P < 0.05) smaller than in wild-type littermates (Figure 9a). In the isolation group, repeated measures ANOVA was applied only to weeks 9–14, i.e. only to the isolation period. Weight was dependent on time ($F_{5,70}$ = 25.14, P < 0.001) and genotype × time interaction ($F_{5,70}$ = 8.42, P < 0.001), but not genotype ($F_{1,14}$ =0.75, P = 0.4). The last measurement before assigning to individual housing at 9 weeks of age showed that the body weight of wild-type mice (28.2 g) was ca 14% bigger than in Lsamp$^{-/-}$ mice (24.7 g). First week in isolation induced an almost significant (P = 0.08) drop in body weight in wild-type mice, however, the body weight of Lsamp$^{-/-}$ mice increased and did so significantly (P < 0.001). After 1 week in isolation, the body weights of both genotypes were identical (Figure 9b). In mice raised in enriched conditions, only time effect was significant ($F_{9,126}$ = 157.04, P < 0.001) and there were no differences between Lsamp$^{-/-}$ and wild-type mice (Figure 9c).

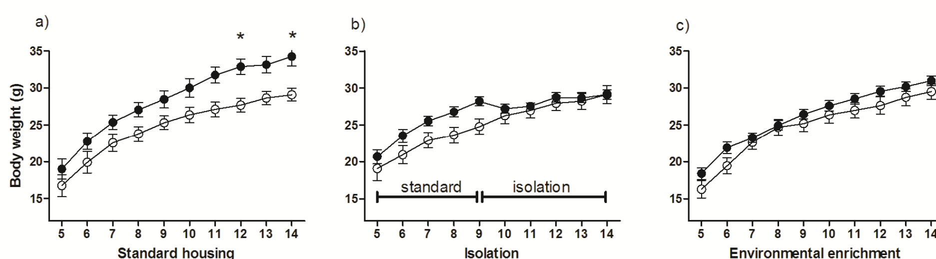

Figure 9. Effect of different housing conditions on body weight. (a) Standard housing, (b) isolation, (c) environmental enrichment. Black circles: wild-type; white circles: Lsamp$^{-/-}$. * P < 0.05: Lsamp$^{-/-}$ mice compared to wild-type mice in the same housing conditions. N = 8 in each genotype × housing group.

2.2 Elevated plus maze test

In the plus maze, the latency to enter open arm was dependent on genotype ($F_{1,42}$ = 7.33, P < 0.01) and genotype × housing interaction ($F_{2,42}$ = 3.25, P < 0.05). The latency of $Lsamp^{-/-}$ mice in the enrichment group was significantly (P < 0.05) shorter than in respective wild-type group (Figure 10a). As for the number of open arm entries, only genotype × housing interaction was significant ($F_{2,42}$ = 4.33, P < 0.05); the main effects for genotype ($F_{1,42}$ = 3.52, P = 0.07) and housing ($F_{2,42}$ = 2.96, P = 0.06) slightly missed significance. In the enrichment group, $Lsamp^{-/-}$ mice performed significantly (P < 0.05) more open arm entries than their wild-type littermates (Figure 10b). The number of closed arm entries was dependent on genotype only ($F_{1,42}$ = 7.81, P < 0.01) as $Lsamp^{-/-}$ mice performed more closed arm entries in the control and enrichment groups compared to wild-type animals, but none of the differences was significant (Figure 10c). Time on open arms was dependent on genotype × housing interaction ($F_{2,42}$ = 3.42, P < 0.05). $Lsamp^{-/-}$ mice tended to stay longer on the open arms than wild-type mice (P = 0.07) (Figure 10d). The total number of headdips was dependent on genotype ($F_{1,42}$ = 6.66, P < 0.05), housing ($F_{2,42}$ = 3.79, P < 0.05) and genotype × housing interaction ($F_{2,42}$ = 3.19, P = 0.05). $Lsamp^{-/-}$ mice raised in enriched conditions performed significantly (P < 0.01) more headdips than any other group (Figure 10e). The number of unprotected headdips was affected by genotype ($F_{1,42}$ = 4.39, P < 0.05), housing ($F_{2,42}$ = 3.35, P < 0.05) and genotype × housing interaction ($F_{2,42}$ = 3.77, P = 0.05). $Lsamp^{-/-}$ mice in enriched conditions performed significantly (P < 0.01) more unprotected headdips than any other group (Figure 10f).

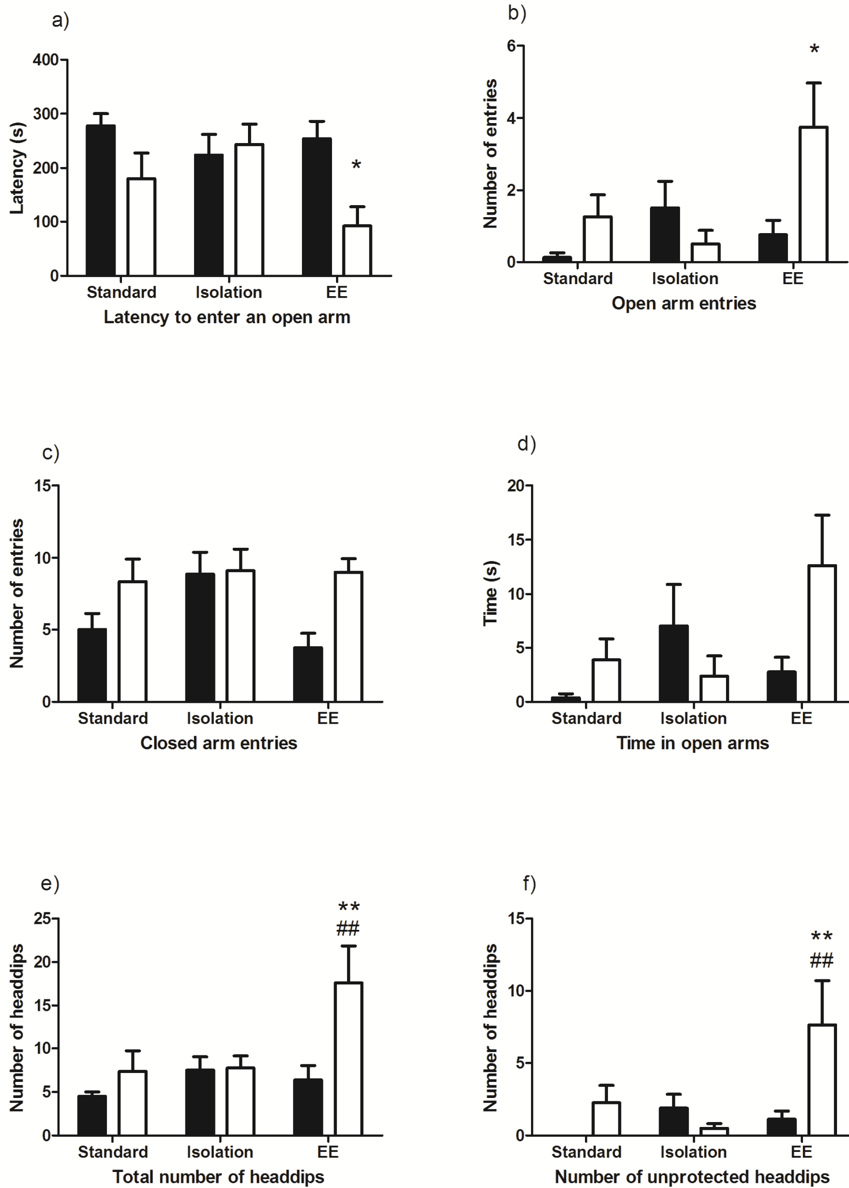

Figure 10. Effect of different housing conditions on the performance in the elevated plus maze. (a) Latency to enter an open arm, (b) number of open arm entries, (c) number of closed arm entries, (d) time in open arms, (e) total number of headdips, (f) number of unprotected headdips. Black columns: wild-type; white columns: Lsamp$^{-/-}$. ** $P < 0.01$, .* $P < 0.05$: Lsamp$^{-/-}$ mice compared to wild-type mice in the same housing conditions. ## $P < 0.01$: Lsamp$^{-/-}$ mice in EE compared to Lsamp$^{-/-}$ mice in standard housing conditions. N = 8 in each genotype × housing group.

2.3. Locomotor activity test

No main effects for distance travelled (Figure 11a) and the number of rearings (Figure 11b) were evident. Time in centre was significantly affected by genotype ($F_{1,42} = 4.23$, $P < 0.05$) and housing ($F_{2,42} = 3.62$, $P < 0.05$). Lsamp$^{-/-}$ mice in the enrichment group spent significantly more time in the central square than Lsamp$^{-/-}$ mice raised in standard housing conditions ($P < 0.01$) (Figure 11c). For centre distance, only the effect of housing was significant ($F_{2,42} = 4.75$, $P < 0.05$) (Figure 11d).

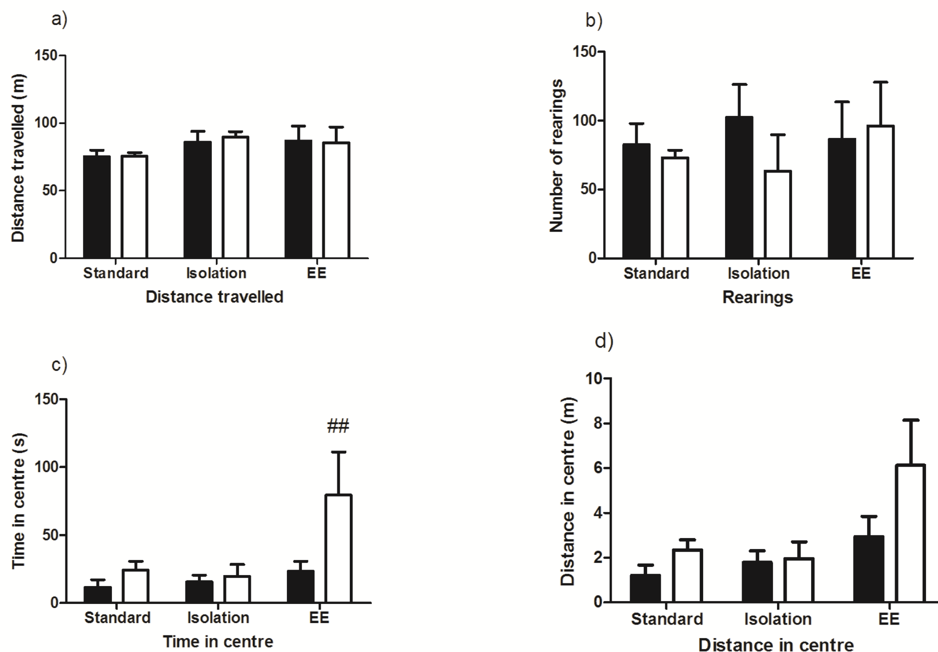

Figure 11. Effect of different housing conditions on the performance in the motility box. (a) Distance travelled, (b) number of rearings, (c) time in centre, (d) distance in centre. Black columns: wild-type; white columns: Lsamp$^{-/-}$. ## $P < 0.01$: Lsamp$^{-/-}$ mice in EE compared to Lsamp$^{-/-}$ mice in standard housing conditions. N = 8 in each genotype × housing group.

2.4. Swimming speed measurement

Significant effect of genotype ($F_{1,42} = 32.56$, $P < 0.001$) and housing ($F_{2,42} = 3.48$, $P < 0.05$) were observed in the swimming speed test, Lsamp$^{-/-}$ mice being slower swimmers in all housing conditions, however, isolation decreased swimming speed in Lsamp$^{-/-}$, but not in wild-type mice compared to standard housing conditions (Figure 12a).

2.5. Marble burying test

There were no significant differences in the number of marbles buried between wild-type and Lsamp$^{-/-}$ mice and between housing conditions (Figure 12b).

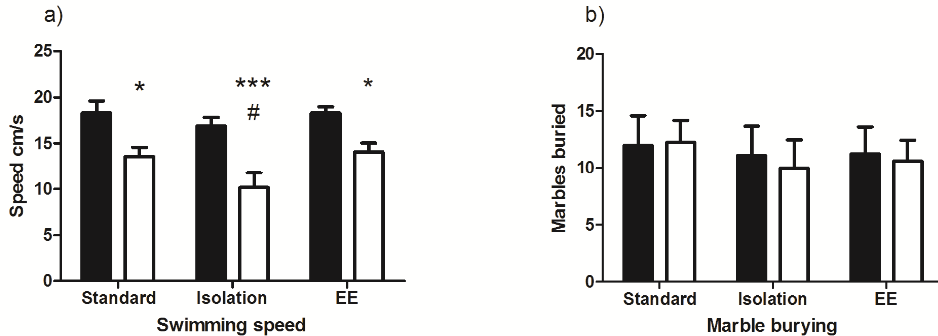

Figure 12. Effect of different housing conditions on swimming speed and marble burying. (a) Swimming speed (cm/s), (b) number of marbles buried. Black columns: wild-type; white columns: Lsamp$^{-/-}$. *** $P < 0.001$, * $P < 0.05$: Lsamp$^{-/-}$ mice compared to wild-type mice in the same housing conditions. # $P < 0.05$: Lsamp$^{-/-}$ mice in isolation compared to Lsamp$^{-/-}$ mice in standard housing conditions. N = 8 in each genotype × housing group.

2.6. Social interaction test

In the social interaction test, sniffing the body of the other mouse was dependent on genotype ($F_{2,42} = 8.15$, $P < 0.001$), and genotype × housing interaction ($F_{2,42} = 3.48$, $P < 0.05$) and the effect of housing approached significance ($F_{1,42} = 3.46$, $P = 0.07$). Isolation increased sniffing in wild-type mice compared to both Lsamp$^{-/-}$ mice in isolation ($P < 0.01$) and wild-type mice in control conditions ($P < 0.01$). No other differences between the genotypes were evident (Figure 13a). Anogenital sniffing of the other mouse was dependent on genotype ($F_{2,42} = 19.44$, $P < 0.001$), housing ($F_{1,42} = 33.25$, $P < 0.001$) and genotype × housing interaction ($F_{2,42} = 7.01$, $P < 0.01$). Again, isolation increased anogenital sniffing in wild-type mice compared to both Lsamp$^{-/-}$ mice in isolation ($P < 0.001$) and wild-type mice in control conditions ($P < 0.001$). Also, in control conditions anogenital sniffing was more frequent in wild-type mice than in their Lsamp$^{-/-}$ littermates ($P < 0.05$); however, in mice raised in an enriched environment, there was no difference between the genotypes ($P = 0.18$) (Figure 13b). As for grooming and digging, no differences between housing conditions or genotypes were evident (data not reported). No aggressive attacks were scored.

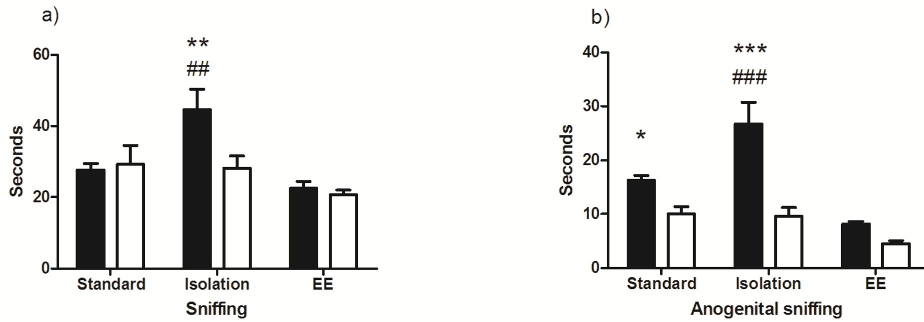

Figure 13. Effect of different housing conditions on the performance in the social interaction test. (a) Duration of sniffing (s), (b) duration of anogenital sniffing (s). Black columns: wild-type; white columns: Lsamp$^{-/-}$. *** P < 0.001, ** P < 0.01, * P < 0.05: wild-type mice compared to Lsamp$^{-/-}$ mice in the same housing conditions. ### P < 0.001, ## P < 0.01: wild-type mice in isolation compared to wild-type mice in standard housing conditions. N = 8 in each genotype × housing group.

2.7. Corticosterone measurements

Genotype ($F_{1,35}$ = 0.37, P = 0.55) and housing ($F_{2,35}$ = 1.27, P = 0.29) had no effect on the levels of corticosterone. However, genotype and housing interacted almost significantly ($F_{2,35}$ = 2.84, P = 0.07). Isolation and enrichment lowered the level of corticosterone approximately two-fold in wild-type mice, but had relatively little effect on the corticosterone level in Lsamp$^{-/-}$ mice compared to mice in standard housing conditions (Figure 14). It should be noted however, that these differences did not reach the level of statistical significance.

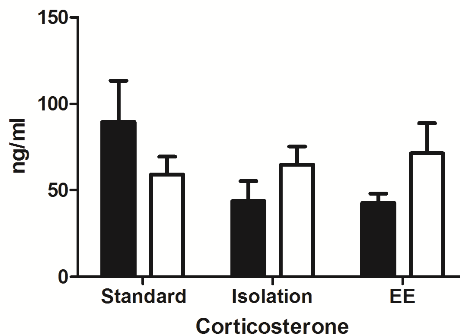

Figure 14. Effect of different housing conditions on the level of plasma corticosterone. Black columns: wild-type; white columns: Lsamp$^{-/-}$. N = 8 in each genotype × housing group.

Paper III

3.1. Locomotor activity test with amphetamine

In the amphetamine dose curve study, distance travelled was significantly influenced by genotype ($F_{(1,40)}$ = 16.25; P < 0.001), dose ($F_{(3,40)}$ = 22.07; P < 0.001), and genotype × dose interaction ($F_{(3,40)}$ = 8.47; P < 0.001), $Lsamp^{-/-}$ mice being significantly less sensitive to the stimulating effect of 5 mg/kg and 7.5 mg/kg of amphetamine (Figure 15). We found no main effects for rearings, and time and distance in the central square. The number of corner entries was, like distance travelled, significantly influenced by genotype ($F_{(1,40)}$ = 15.98; P < 0.001), dose ($F_{(3,40)}$ = 17.13; P < 0.001), and genotype × dose interaction ($F_{(3,40)}$ = 7.31; P < 0.001) and the results almost coincided with those for distance travelled.

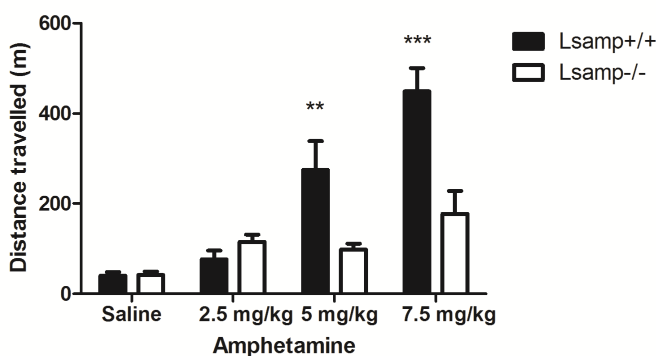

Figure 15. The effect of amphetamine on distance travelled in the motility boxes in $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice. Data are presented as ± SEM, N = 6 for all treatment groups. *** p<0.001; ** p<0.01, $Lsamp^{+/+}$ mice vs. respective $Lsamp^{-/-}$ group.

3.2. Conditioned place preference test

Amphetamine (2.5 mg/kg) shifted preference for the conditioned chamber in $Lsamp^{+/+}$ mice by 388.5 ± 84.8 s and in $Lsamp^{-/-}$ mice by 86.4 ± 70.6 s and the difference between the genotypes was significant ($F_{(1,14)}$ = 7.5; P < 0.05).

3.3. Monoamine content measurements

Dorsal striatum In the dorsal striatum, there was a significant genotype effect on the turnover of 5-HT ($F_{(1,19)}$ = 5.36; P < 0.05), and an almost significant genotype effect on the content of 5-HIAA ($F_{(1,19)}$ = 3.74; P = 0.07). Amphetamine treatment had a significant effect on the content of NMN ($F_{(1,19)}$ = 17.85; P < 0.001), DA ($F_{(1,19)}$ = 5.67; P < 0.05), DOPAC ($F_{(1,19)}$ = 6.07; P < 0.05), 5-HT ($F_{(1,19)}$ = 9.93; P < 0.01) and 3-MT ($F_{(1,19)}$ = 12.52; P < 0.01), and the turnover of NA ($F_{(1,19)}$ = 33.56; P < 0.001), DA ($F_{(1,19)}$ = 55.41; P < 0.001) and 5-HT ($F_{(1,19)}$ = 11.18; P < 0.01). Genotype × treatment interaction had a significant effect on the turnover of 5-HT ($F_{(1,19)}$ = 4.6; P < 0.05). See Table 4 for detailed values and significant post hoc comparisons.

Table 4. Monoamine levels in the dorsal striatum of $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice 30 min after saline or 5 mg/kg of amphetamine administration.

	$Lsamp^{+/+}$ saline	$Lsamp^{-/-}$ saline	$Lsamp^{+/+}$ amphetamine	$Lsamp^{-/-}$ amphetamine
Dorsal striatum				
NA	1.48 ± 0.2	1.4 ± 0.15	1.54 ± 0.13	1.54 ± 0.14
NMN	2.82 ± 0.41	2.64 ± 0.21	1.31 ± 0.21 [aa]	1.66 ± 0.27 [a]
(NMN/NA)	1.91 ± 0.18	1.94 ± 0.15	0.84 ± 0.1 [aaa]	1.12 ± 0.19 [aa]
DA	47.57 ± 8.31	35.79 ± 4.28	55.54 ± 9.41	67.07 ± 9.96 [a]
DOPAC	0.34 ± 0.13	0.78 ± 0.32	0.18 ± 0.01	0.13 ± 0.00
HVA	4.84 ± 0.63	3.92 ± 0.64	3.78 ± 0.44	3.91 ± 0.54
(DOPAC+HVA)/DA	0.11 ± 0.01	0.12 ± 0.01	0.07 ± 0.01 [aaa]	0.06 ± 0.00 [aaa]
5-HT	3.67 ± 0.35	2.96 ± 0.29	4.73 ± 0.45	5.16 ± 0.78 [a]
5-HIAA	1.59 ± 0.13	2.48 ± 0.43	1.53 ± 0.18	1.7 ± 0.21
(5-HIAA/5-HT)	0.44 ± 0.03	0.89 ± 0.18 [bb]	0.33 ± 0.03	0.34 ± 0.03 [aa]
3-MT	2.52 ± 0.34	1.9 ± 0.27	3.95 ± 0.43	3.67 ± 0.66 [a]

The values (mean ± SEM) are expressed as pmol/mg wet weight tissue. N = 6 per group. To increase readability, significant differences are shaded.
[a] P < 0.05; [aa] P < 0.01; [aaa] P < 0.001 vs. respective saline group
[bb] P < 0.01 vs. respective $Lsamp^{+/+}$ group

Ventral striatum In the ventral striatum, there was a significant genotype effect on the content of NMN ($F_{(1,20)} = 5.26$; $P < 0.05$) and an almost significant genotype effect on the content of DA ($F_{(1,19)} = 3.74$; $P = 0.07$) and the turnover of DA ($F_{(1,19)} = 4.01$; $P = 0.06$). Amphetamine treatment significantly affected the content of NMN ($F_{(1,20)} = 46.27$; $P < 0.001$), HVA ($F_{(1,20)} = 10.51$; $P < 0.01$), 5-HT ($F_{(1,19)} = 5.89$; $P < 0.05$), 5-HIAA ($F_{(1,20)} = 6.25$; $P < 0.05$), and 3-MT ($F_{(1,19)} = 8.75$; $P < 0.01$), and the turnover of NA ($F_{(1,20)} = 21.6$; $P < 0.001$), DA ($F_{(1,19)} = 15.69$; $P < 0.001$), and 5-HT ($F_{(1,17)} = 7.1$; $P < 0.05$. See Table 5 for detailed values and significant post hoc comparisons.

Table 5. Monoamine levels in the ventral striatum of $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice 30 min after saline or 5 mg/kg of amphetamine administration.

	$Lsamp^{+/+}$ saline	$Lsamp^{-/-}$ saline	$Lsamp^{+/+}$ amphetamine	$Lsamp^{-/-}$ amphetamine
Ventral striatum				
NA	3.55 ± 0.62	3.6 ± 0.48	4.16 ± 0.54	4.87 ± 0.36
NMN	2.45 ± 0.26	3.49 ± 0.26 [bb]	1.11 ± 0.29 [aa]	1.27 ± 0.24 [aaa]
(NMN/NA)	0.85 ± 0.23	1.05 ± 0.13	0.3 ± 0.1 [a]	0.27 ± 0.06 [aa]
DA	20.4 ± 2	26.68 ± 2.51	23.61 ± 6.03	29.74 ± 1.67
DOPAC	0.17 ± 0.05	0.1 ± 0.02	0.17 ± 0.07	0.19 ± 0.07
HVA	3.18 ± 0.41	3.88 ± 0.42	2.14 ± 0.41	2.52 ± 0.19 [a]
(DOPAC+HVA)/DA	0.16 ± 0.01	0.15 ± 0.01	0.13 ± 0.02	0.09 ± 0.01 [aa]
5-HT	6.46 ± 0.96	5.7 ± 0.7	8.22 ± 1.69	8.64 ± 0.5
5-HIAA	1.95 ± 0.21	2.61 ± 0.23	1.32 ± 0.4	1.7 ± 0.35
(5-HIAA/5-HT)	0.34 ± 0.07	0.51 ± 0.09	0.25 ± 0.03	0.24 ± 0.01 [a]
3-MT	1.41 ± 0.19	1.54 ± 0.13	2.81 ± 0.83 [a]	2.24 ± 0.23

The values (mean ± SEM) are expressed as pmol/mg wet weight tissue. N = 6 per group. To increase readability, significant differences are shaded.
[a] $P < 0.05$; [aa] $P < 0.01$; [aaa] $P < 0.001$ vs. respective saline group
[bb] $P < 0.01$ vs. respective $Lsamp^{+/+}$ group

Mesencephalon In the mesencephalon, there was a significant genotype effect on the content of DA ($F_{(1,19)} = 4.9$; $P < 0.05$), DOPAC ($F_{(1,17)} = 6.73$; $P < 0.05$), and 5-HT ($F_{(1,20)} = 6.91$; $P < 0.05$), and the turnover of 5-HT ($F_{(1,20)} = 9.35$; $P < 0.01$); also, an almost significant genotype effect on the content of HVA ($F_{(1,19)} = 3.84$; $P = 0.06$) was observed. Amphetamine treatment significantly affected the content of NA ($F_{(1,20)} = 22.49$; $P < 0.001$), DOPAC ($F_{(1,17)} = 8.16$; $P < 0.05$), 5-HT ($F_{(1,20)} = 31.73$; $P < 0.001$), 5-HIAA ($F_{(1,20)} = 22.49$; $P < 0.001$), and the turnover of 5-HT ($F_{(1,20)} = 18.24$; $P < 0.001$). See Table 6 for detailed values and significant post hoc comparisons.

Table 6. Monoamine levels in the mesencephalon of $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice 30 min after saline or 5 mg/kg of amphetamine administration.

	$Lsamp^{+/+}$ saline	$Lsamp^{-/-}$ saline	$Lsamp^{+/+}$ amphetamine	$Lsamp^{-/-}$ amphetamine
Mesencephalon				
NA	3.21 ± 0.29	2.91 ± 0.36	4.48 ± 0.43 [a]	4.73 ± 0.18 [aa]
NMN	0.55 ± 0.1	0.5 ± 0.06	0.64 ± 0.21	0.45 ± 0.05
(NMN/NA)	0.18 ± 0.03	0.18 ± 0.03	0.15 ± 0.05	0.1 ± 0.01
DA	1.22 ± 0.2	0.75 ± 0.12	1.09 ± 0.16	0.85 ± 0.16
DOPAC	0.15 ± 0.02	0.09 ± 0.01	0.2 ± 0.03	0.15 ± 0.01
HVA	0.8 ± 0.08	0.59 ± 0.11	1.33 ± 0.43	0.66 ± 0.06
(DOPAC+HVA)/DA	0.76 ± 0.07	0.91 ± 0.15	0.97 ± 0.13	0.93 ± 0.09
5-HT	5.82 ± 0.63	4.22 ± 0.42 [b]	8.48 ± 0.33 [aa]	7.37 ± 0.62 [aaa]
5-HIAA	3.09 ± 0.19	2.93 ± 0.13	3.62 ± 0.37	3.66 ± 0.29
(5-HIAA/5-HT)	0.55 ± 0.04	0.72 ± 0.05 [bb]	0.43 ± 0.04	0.5 ± 0.03 [aa]
3-MT	0.18 ± 0.05	*N.D.*	1.12 ± 0.8	0.15 ± 0.02

The values (mean ± SEM) are expressed as pmol/mg wet weight tissue. N = 6 per group. *N.D.* not detected due to low concentration or technical reasons. To increase readability, significant differences are shaded.
[a] $P < 0.05$; [aa] $P < 0.01$; [aaa] $P < 0.001$ vs. respective saline group
[b] $P < 0.05$; [bb] $P < 0.01$ vs. respective $Lsamp^{+/+}$ group

Prefrontal cortex In the prefrontal cortex, amphetamine treatment significantly affected the content of DA ($F_{(1,20)} = 8.2$; $P < 0.01$), and 5-HT ($F_{(1,20)} = 10.74$; $P < 0.01$), and the turnover of NA ($F_{(1,20)} = 7.13$; $P < 0.05$) and 5-HT ($F_{(1,20)} = 6.33$; $P < 0.05$). See Table 7 for detailed values and significant post hoc comparisons.

Table 7. Monoamine levels in the prefrontal cortex of $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice 30 min after saline or 5 mg/kg of amphetamine administration.

	$Lsamp^{+/+}$ saline	$Lsamp^{-/-}$ saline	$Lsamp^{+/+}$ amphetamine	$Lsamp^{-/-}$ amphetamine
Prefrontal cortex				
NA	1.53 ± 0.18	1.8 ± 0.27	1.85 ± 0.17	2.02 ± 0.15
NMN	0.89 ± 0.15	0.98 ± 0.16	0.55 ± 0.15	0.75 ± 0.16
(NMN/NA)	0.64 ± 0.15	0.57 ± 0.07	0.3 ± 0.08	0.38 ± 0.08
DOPAC	*N.D.*	0.17 ± 0.04	0.17 ± 0.05	0.16 ± 0.03
HVA	1.31 ± 0.19	1.73 ± 0.27	1.26 ± 0.18	1.3 ± 0.26
DA	6.42 ± 1.43	4.03 ± 1.65	10.67 ± 1.53	11.46 ± 3.09
(DOPAC+HVA)/DA	*N.D.*	0.35 ± 0.12	0.13 ± 0.02	0.17 ± 0.05
5-HT	3.56 ± 0.41	3.48 ± 0.48	4.61 ± 0.45	5.02 ± 0.16
5-HIAA	1.19 ± 0.14	1.51 ± 0.17	1.03 ± 0.12	1.18 ± 0.11
(5-HIAA/5-HT)	0.38 ± 0.1	0.5 ± 0.13	0.22 ± 0.01	0.23 ± 0.02
3-MT	1.23 ± 0.5	1.29 ± 0.34	2.93 ± 0.84	2.37 ± 0.68

The values (mean ± SEM) are expressed as pmol/mg wet weight tissue. N = 6 per group. *N.D.* not detected due to low concentration or technical reasons.

Temporal lobe In the temporal lobe, amphetamine treatment significantly affected the content of NMN ($F_{(1,20)} = 8.67$; $P < 0.01$), and 5-HT ($F_{(1,20)} = 8.03$; $P < 0.01$), and the turnover of NA ($F_{(1,20)} = 11.42$; $P < 0.01$). Genotype × treatment interaction had a significant effect on the turnover of 5-HT ($F_{(1,19)} = 5.3$; $P < 0.05$). See Table 8 for detailed values and significant post hoc comparisons.

Table 8. Monoamine levels in the temporal lobe of $Lsamp^{+/+}$ and $Lsamp^{-/-}$ mice 30 min after saline or 5 mg/kg of amphetamine administration.

	$Lsamp^{+/+}$ saline	$Lsamp^{-/-}$ saline	$Lsamp^{+/+}$ amphetamine	$Lsamp^{-/-}$ amphetamine
Temporal lobe				
NA	1.84 ± 0.17	1.71 ± 0.38	1.98 ± 0.11	2.06 ± 0.07
NMN	0.81 ± 0.21	0.75 ± 0.15	0.32 ± 0,04	0.46 ± 0.05
(NMN/NA)	0.47 ± 0.14	0.45 ± 0.06	0.16 ± 0.02	0.22 ± 0.02
DOPAC	*N.D.*	0.19 ± 0.09	0.14 ± 0.02	0.26 ± 0.04
HVA	2.92 ± 0.49	*N.D.*	1.4 ± 0.42	1.95 ± 0.4
DA	6.8 ± 2.39	5.52 ± 1.24	6.39 ± 1.31	6.38 ± 0.89
(DOPAC+HVA)/DA	*N.D.*	*N.D*	0.28 ± 0.09	0.34 ± 0.06
5-HT	4.62 ± 0.43	3.57 ± 0.64	5.21 ± 0.5	5.84 ± 0.4 [a]
5-HIAA	1.61 ± 0.1	1.96 ± 0.5	2.35 ± 0.3	2.23 ± 0.44
(5-HIAA/5-HT)	0.36 ± 0.03	0.59 ± 0.07	0.48 ± 0.07	0.4 ± 0.08
3-MT	0.62 ± 0.14	0.58 ± 0.12	0.82 ± 0.13	0.78 ± 0.05

The values (mean ± SEM) are expressed as pmol/mg wet weight tissue. N = 6 per group. *N.D.* not detected due to low concentration or technical reasons. To increase readability, significant difference is shaded.

[a] $P < 0.05$ vs. respective saline group

In conclusion, most notably, in all five brain regions measured, $Lsamp^{-/-}$ mice had somewhat lower levels of 5-HT in response to a saline injection than wild-type mice (in the mesencephalon the difference was significant). Also, $Lsamp^{-/-}$ mice reacted with a stronger elevation in 5-HT levels than $Lsamp^{+/+}$ mice to 5 mg/kg of amphetamine in all five parts of the brain analysed (in the dorsal striatum, mesencephalon and temporal lobe the difference was significant). Wild-type mice had a significant increase in the level of 5-HT only in the mesencephalon and the magnitude of this increase was lower than in $Lsamp^{-/-}$ mice (Figure 16).

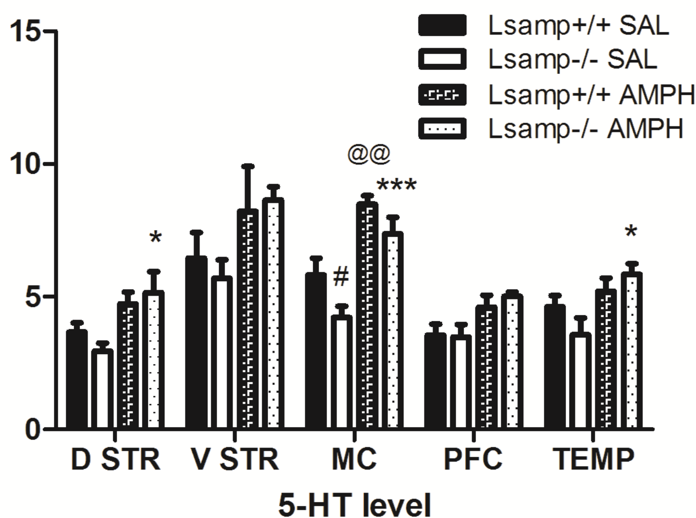

Figure 16. 5-HT levels in the dorsal striatum (D STR), ventral striatum (V STR), mesencephalon (MC), prefrontal cortex (PFC) and temporal lobe (TEMP) in $Lsamp^{-/-}$ mice 30 min after saline (SAL) or 5 mg/kg of amphetamine (AMPH) administration. N = 6 per group. # $P < 0.05$, $Lsamp^{-/-}$ vs. respective $Lsamp^{+/+}$ group; @@ $P < 0.01$, $Lsamp^{+/+}$ amphetamine group vs. $Lsamp^{+/+}$ saline group; *** $P < 0.001$; * $P < 0.05$ $Lsamp^{-/-}$ amphetamine group vs. $Lsamp^{-/-}$ saline group.

Furthermore, Lsamp$^{-/-}$ mice had a significantly higher turnover of 5-HT compared to Lsamp$^{+/+}$ mice in the dorsal striatum and mesencephalon and somewhat higher in the ventral striatum, prefrontal cortex and temporal lobe. It is also remarkable that amphetamine lowered the turnover of 5-HT in Lsamp$^{-/-}$ mice significantly in the dorsal striatum, ventral striatum and mesencephalon, while none of these changes in Lsamp$^{+/+}$ mice were significant (Figure 17). It is noteworthy that in all these brain regions dopamine-mediated neurotransmission plays a prominent role.

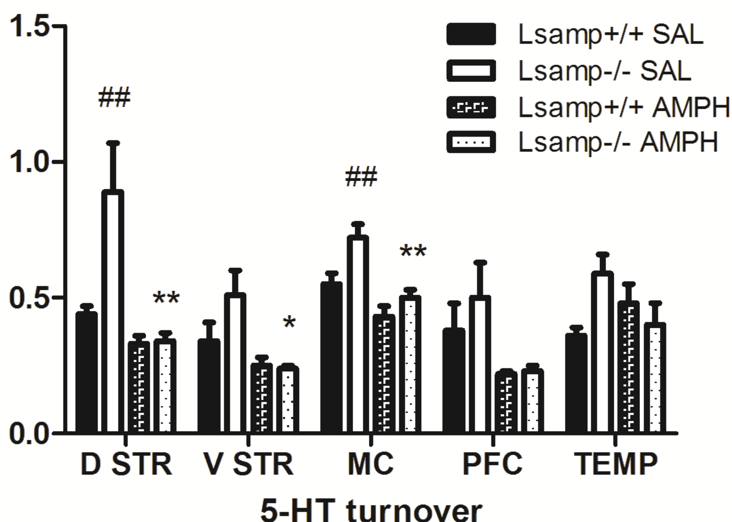

Figure 17. 5-HT turnover in the dorsal striatum (D STR), ventral striatum (V STR), mesencephalon (MC), prefrontal cortex (PFC) and temporal lobe (TEMP) in Lsamp$^{-/-}$ mice 30 min after saline (SAL) or 5 mg/kg of amphetamine (AMPH) administration. N = 6 per group. ## $P < 0.01$, Lsamp$^{-/-}$ vs. respective Lsamp$^{+/+}$ group; ** $P < 0.01$; * $P < 0.05$ Lsamp$^{-/-}$ amphetamine group vs. Lsamp$^{-/-}$ saline group.

Other significant differences between the genotypes included a stronger increase in the level of DA and 3-MT in response to amphetamine administration in Lsamp$^{-/-}$ mice in the dorsal striatum, a stronger decrease in the level of HVA and DA turnover in response to amphetamine administration in Lsamp$^{-/-}$ mice and a stronger increase in 3-MT level in response to amphetamine treatment in Lsamp$^{+/+}$ mice in the ventral striatum. As for the noradrenergic system, both genotypes reacted with an elevation in the level of NA and a decrease in NA turnover in response to amphetamine treatment and no clearcut differences between the genotypes could be observed.

3.4. Gene expression

In the ventral striatum, the expression level of dopamine D2 receptor in Lsamp$^{+/+}$ mice (0.26 ± 0.03) was not statistically different from that in Lsamp$^{+/-}$ (0.24 ± 0.03) and Lsamp$^{-/-}$ (0.21 ± 0.03) mice (Figure 18a). In the dorsal striatum, the small differences between the expression levels of dopamine D2 receptor between Lsamp$^{+/+}$ (0.26 ± 0.03), Lsamp$^{+/-}$ (0.24 ± 0.03) and Lsamp$^{-/-}$ (0.21 ± 0.03) mice were not significant (Figure 18b). In the mesencephalon, the expression level of dopamine transporter gene (Dat) was dependent on genotype ($F_{(2,19)} = 3.69$; $p < 0.05$) and post hoc analysis revealed that the expression level of Dat was in Lsamp$^{-/-}$ mice significantly ($p < 0.05$) lower than in Lsamp$^{+/+}$ mice (Figure 18c). In the mesencephalon, the differences in the expression level of Vmat2 between Lsamp$^{+/+}$ (0.055 ± 0.008), Lsamp$^{+/-}$ (0.044 ± 0.006) and Lsamp$^{-/-}$ (0.048 ± 0.009) mice were not significant (Figure 18d).

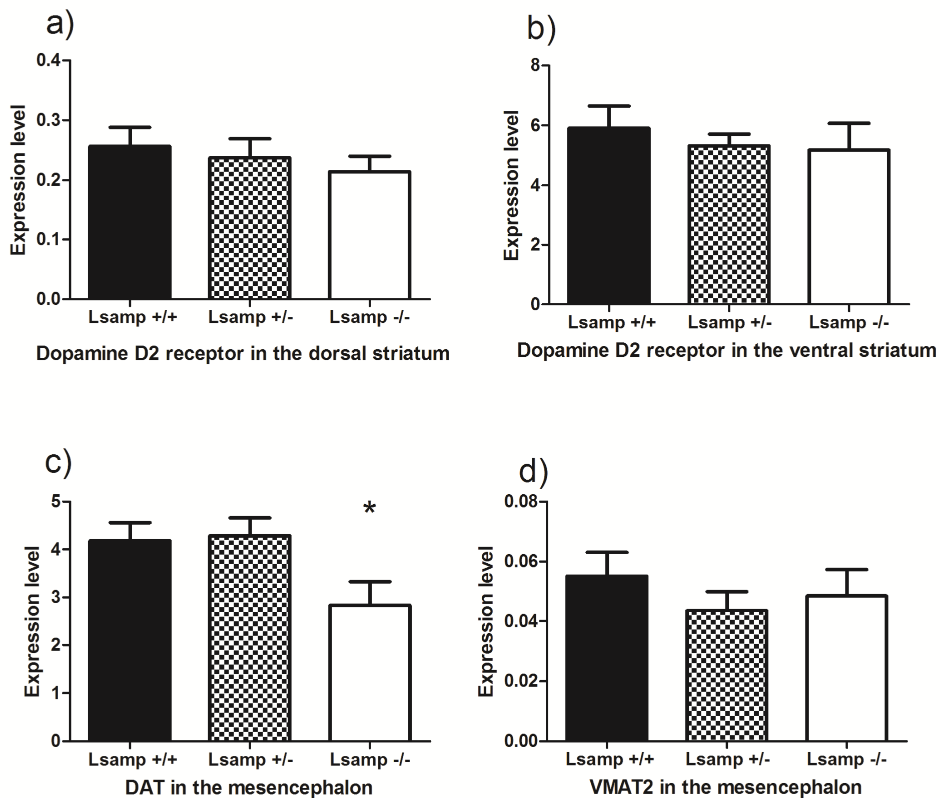

Figure 18. The expression level of dopamine D2 receptor gene in the ventral (a) and dorsal striatum (b), and dopamine transporter Dat gene (c) and brain vesicular monoamine transporter (Vmat2) gene (d) in the mesencephalon. N = 6–8 per group. * $P < 0.05$ Lsamp$^{-/-}$ vs. Lsamp$^{+/+}$ group.

DISCUSSION

1. General phenotype of Lsamp-deficient mice

Initial phenotyping (Paper I) revealed that, similarly to Lsamp$^{-/-}$ mice generated by Catania et al (2008), who produced their knockout mouse line by targeted deletion of exon 2 in the Lsamp gene and back-crossed their animals into the C57BL/6J strain for more than 10 generations, our Lsamp$^{-/-}$ mice were vital and fertile and displayed no gross abnormalities. Furthermore, our sensory-motor experiments showed that Lsamp$^{-/-}$ mice do not differ from their wild-type littermates in terms of motor abilities, muscle power, vision, hearing, olfaction and mechanical sensitivity. However, Lsamp$^{-/-}$ mice appeared to be slower swimmers in the water maze, but more active in the motility box than wild-type mice. When consistently slower swim velocity in Lsamp$^{-/-}$ mice was taken into account, their spatial memory and learning curve were similar to that of wild-type mice. In a study by Qiu et al (2010) Lsamp$^{-/-}$ mice exhibited a pronounced deficit in spatial memory acquisition, however, the authors did not report swim velocity, therefore it cannot be excluded that different swimming speed is responsible for the difference in the learning curve. We used also another learning paradigm, the active avoidance test, which also failed to reveal any differences between the two genotypes.

We found some other differences between our Lsamp-deficient mouse line and the mouse line described by Catania et al (2008). These slight phenotypic differences between the two mice lines are probably mainly due to different backgrounds. For example, in standard housing, we noted consistently lower body-weight in Lsamp$^{-/-}$ mice compared to wild-type mice. Also, our Lsamp$^{-/-}$ male mice displayed increased locomotor activity in the motility box only during the first 10 minutes of the test; by contrast, in the study of Catania et al (2008), it took over 30 minutes before Lsamp$^{-/-}$ male mice habituated with a novel arena. In general, however, the overlap between the phenotype of the two Lsamp knockout models is remarkable. Our results also show that targeted deletion of the Lsamp gene induces a robust behavioural phenotype that is detectable over and above the behavioural variability caused by a mixed genetic background, obviating the need for back-crosses (Crawley, 2008). Repeated testing of mice, used in this study, had probably no impact on the results as in experiments considered sensitive to repeated testing (Võikar et al, 2004) only naïve animals were used.

2. Anxiety-related phenotype of Lsamp-deficient mice

In Paper I, we explored the anxiety-related phenotype in more detail in our Lsamp$^{-/-}$ mice and tried to elucidate whether increased exploratory behaviour in Lsamp$^{-/-}$ mice could be interpreted as decreased anxiety or is only secondary to disinhibition in a novel environment as suggested by Catania et al (2008). Our Lsamp$^{-/-}$ mice showed a similar behavioural pattern in the plus maze to the results described by Catania et al (2008). In both studies, Lsamp$^{-/-}$ mice made significantly more open arm entries, closed arm entries and unprotected head-dippings. Catania et al (2008) interpreted unprotected head-dippings as risk assessment, however, according to our previous studies unprotected head-dippings show a very strong positive correlation with classical measures of anxiety in the plus maze, such as number of open arm entries, time spent in open arms, and ratio between open and total arm entries; by contrast, unprotected head-dippings were not correlated with stretch-attend postures, a measure of risk assessment (Nelovkov et al, 2006; Sütt et al, 2010). Catania et al (2008) hypothesized that Lsamp$^{-/-}$ mice may experience disinhibition in stressful environments. However, our Lsamp$^{-/-}$ mice behaved similarly in both stressful (illuminated) and unstressful (dim) experimental conditions. Pretreatment of wild-type mice in aversive conditions (illuminated room) with anxiolytic drug diazepam (1 mg/kg) raised their exploratory activity to the basal level of Lsamp$^{-/-}$ mice. Also, it has been shown previously that anxiogenic manipulations, such as exposure to cat odor, increase the expression level of the Lsamp gene in the amygdaloid area (Kõks et al, 2004), anxious rats have an elevated level of the Lsamp gene compared to non-anxious animals (Alttoa et al, 2010; Nelovkov et al, 2003, 2006), and fear conditioning raises Lsamp mRNA expression level 5 h after training in the lateral amygdala of rats (Lamprecht et al, 2009). Altogether, these data suggest that the behavioural profile of our Lsamp$^{-/-}$ mice is indicative of reduced anxiety.

We also studied the effects of diazepam on the behaviour of Lsamp$^{-/-}$ mice in the plus maze and investigated the expression level of transcripts encoding $GABA_A$ receptor subunits in the frontal cortex, mesolimbic area and temporal lobe of Lsamp$^{-/-}$ and wild-type mice. $GABA_A$ receptors are formed by the co-assembly of five subunits belonging to different families, which are heterogeneously distributed throughout the brain (Olsen and Sieghart, 2009). We selected two of these subunits: α1 and α2 subunit genes (Gabra1 and Gabra2, respectively), for the present study based on the findings that α1 subunit is responsible for the inhibitory effect of diazepam (McKernan et al, 2000) and α2 subunit plays a role in the stimulating (and therefore also anxiolytic) effect of the drug (Löw et al, 2000). In Lsamp$^{-/-}$ mice, the administration of diazepam had no effect on the frequency of open arm entries and head-dippings, mostly because the baseline values for Lsamp$^{-/-}$ mice were similar to values that were achieved in wild-type mice after administration of diazepam. However, administration of diazepam increased the time spent by

Lsamp$^{-/-}$ mice in the open arms by almost three times, indicating a strong stimulating effect of diazepam. Based on the gene expression data, we propose that the anxiolytic-like phenotype and lower sensitivity to diazepam in Lsamp$^{-/-}$ mice is related to altered proportional balance between Gabra1 and Gabra2 genes. We found a significant decrease in the Gabra1 gene in the temporal cortex and therefore there is proportionally significantly more transcript encoding the α2 subunit of $GABA_A$ receptors, related to the stimulating effect of diazepam, in the temporal cortex of Lsamp$^{-/-}$ mice.

3. Changes in social behaviour in Lsamp-deficient mice

Significant deviations in social behaviour could be observed in male Lsamp$^{-/-}$ mice, such as lack of whisker trimming. Whisker trimming or barbering is a social behaviour (Strozik and Festing, 1981) characteristic of both males and females from several mouse strains (Lijam et al, 1997). Our results suggest that prevalent barbering behaviour in wild-type mice is a manifestation of social hierarchy as barbering patterns are stable already from six weeks of age, which is the age period when male mice begin to develop a hierarchical structure under laboratory conditions (Hayashi, 1993). Strozik and Festing (1981) report that the barber is usually dominant in the "tube dominance" test and in several mutant mice lines the lack of barbering occurs together with subordinate behaviour in the tube test (Koh et al, 2008; Lijam et al, 1997). In our study, Lsamp$^{-/-}$ mice behaved in subordinate manner in the tube test, which indicates incompetence in inter-male social behaviour. The results of the social interaction test between male mice, where Lsamp$^{-/-}$ mice failed to display aggressive attacks that consistently occurred during interactions between wild-type mice, add credibility to the results of the tube test. Additionally, Lsamp$^{-/-}$ mice spent significantly less time sniffing partner's anogenital area in the social interaction test. According to our observations, anogenital sniffing tends to precede aggressive attacks in male mice. It is also known that naturally occuring pheromones in the urine of male mice significantly affect inter-male social interactions (Jones and Nowell, 1989). Therefore we propose that reduced aggressiveness in Lsamp$^{-/-}$ mice is related to reduced anogenital sniffing that is another indicator of impaired inter-male social communication in Lsamp$^{-/-}$ male mice. It is interesting to note that in interactions with wild-type ovariectomized oestrus-induced female mice, Lsamp$^{-/-}$ males displayed no changes in any behavioural parameters, such as anogenital sniffing of the female. However, differences in the levels of androgens is one possible mechanism behind the abnormal social behaviour observed in Lsamp-deficient male mice that should be studied in detail. It cannot be excluded that some aspects of deviant social behaviour in Lsamp-deficient mice are related to their mixed background, as 129/SV mice differ from most other strains in their behaviour; however, this is not very likely

as we have not observed any abnormal social behaviours in F2 hybrids of other knockout mouse lines, created by the same strategy as Lsamp-deficient mice.

4. Effect of environmental manipulations on Lsamp-deficient mice

In Paper II, we examined the impact of three different housing conditions – standard housing, environmental enrichment (EE) and isolation – on the phenotype of Lsamp-deficient mice and their wild-type littermates. In standard housing conditions, we failed to repeat our earlier findings in the motility box, where Lsamp-deficient mice had previously displayed slight hyperlocomotion (Paper I). Also, no statistically significant differences between the two genotypes were evident in the plus maze in the anxiety-related parameters. It should be noted, however, that these differences had been obtained with much larger experimental groups (Paper I) and in this study, due to three different housing conditions, it was neither possible nor sensible to use so large experimental groups. The results of all the other tests validated earlier findings.

The study demonstrated that while some phenotypic differences seen in Lsamp-deficient mice in standard housing conditions are also evident in other types of housing conditions, environmental manipulations differentially modified the behaviour of Lsamp-deficient and wild-type mice in several tests. Namely, EE abolished differences between the genotypes in body weight and inter-male anogenital sniffing, a behaviour often preceding aggressive attacks, and amplified the anxiolytic-like phenotype of Lsamp-deficient mice both in the plus maze and motility box. Isolation abolished differences between the genotypes in body weight and anxiety and increased inter-male anogenital sniffing, an aggression-related behaviour, in wild-type animals, but not in Lsamp gene-deficient mice. Isolation and EE lowered blood corticosterone concentrations somewhat, but not statistically significantly, in wild-type mice, while in Lsamp gene-deficient mice the concentrations remained stable in all three housing conditions. Environmental manipulations failed to modify the results as compared to standard housing conditions in whisker trimming, locomotor activity and marble burying. An overview of the impact of environmental manipulations is presented in Table 9 (only statistically significant differences are reported; tendency-level effects are ignored).

Table 9. An overview of the impact of environmental manipulations on the phenotype observed in standard housing conditions.

	Standard	EE	Isolation
Body weight	*Lsamp*$^{(-/-)}$ weigh less	No difference	No difference
Whisker trimming	*Lsamp*$^{(-/-)}$ fail to trim	*Lsamp*$^{(-/-)}$ fail to trim	Cannot be measured
Plus maze – anxiety	No difference	*Lsamp*$^{(-/-)}$ less anxious	No difference
Motility box – locomotor activity	No difference	No difference	No difference
Motility box – anxiety	No difference	*Lsamp*$^{(-/-)}$ less anxious	No difference
Swimming speed	*Lsamp*$^{(-/-)}$ swim slower	*Lsamp*$^{(-/-)}$ swim slower	*Lsamp*$^{(-/-)}$ swim much slower
Marble burying	No difference	No difference	No difference
Social interaction – agonistic behaviour	*Lsamp*$^{(-/-)}$ less agonistic	No difference	*Lsamp*$^{(-/-)}$ much less agonistic
Corticosterone	No difference	No difference	No difference

The following explanation for the differential impact of different housing conditions on the phenotype of Lsamp-deficient and wild-type mice remains speculative at this moment, however, given the fact that the data from many different experiments on the role of the Lsamp gene and the LSAMP protein point in the same direction, a logical theory starts to emerge. Namely, lack of LSAMP protein seems to lead to an inability to adapt or react to novel environments or stressful environmental manipulations in an evolutionarily sustainable way both externally (behaviourally) and internally (at the organismic level). Previously, Lsamp has been established as a tumour suppressor gene (Kresse et al, 2009; Yen et al, 2009; Pasic et al, 2010), hence the lack of LSAMP protein leads to an impaired ability to fight cancer cells (to adapt to an intra-organismic challenge or threat). Exposure to a novel environment like open field induces a bout of hyperactivity in Lsamp$^{-/-}$ mice that can be construed as maladaptive behaviour, since it would raise the chance to fall prey to a predator. The same can be said about the reduced anxiety-like behaviour observed in the plus maze, which seems to reflect maladaptive behaviour since entering the open arm without assessing the situation before is a behaviour that would decrease the chances to stay alive in nature. Lsamp-deficiency also leads to aberrant social behaviour, such as lack of whisker trimming, lack of agonistic behaviour and lack of aggressive contacts, which is indicative of lack of hierarchies, however, building a hierarchy is an evolutionarily innate behaviour in rodents. We have also observed that although Lsamp$^{-/-}$ mice display normal or even exaggerated locomotor activity, they are much easier to catch from the cage, i.e. their ability

and/or motivation to escape is much lower. In water, Lsamp-deficient mice swim much slower and, according to our unpublished results, also display more floating, however, increased floating explains only about 15% of the decrease in swimming speed during a 1-minute trial. A decrease in swimming speed most probably reflects lower anxiety level, but may also be related to decreased motivation. Furthermore, in this study, Lsamp$^{-/-}$ mice displayed decreased sensitivity to stressful environmental manipulations as confinement to solitary housing failed to disturb their weight gain pattern, but induced a typical sudden drop in body weight in their wild-type littermates. Isolation, which is a stressful manipulation, also expectedly increased aggressive behaviour in wild-type mice, but had no impact on the behaviour of the mutants. The relative stability of the levels of corticosterone, a biochemical stress marker, in Lsamp$^{-/-}$ in all three housing conditions compared to wild-type mice also supports this theory; however, as this difference remained at a tendency level, the result is preliminary and needs to be repeated with larger study groups.

In conclusion, both previous findings and the results of this study indicate that Lsamp-deficiency leads to an decreased sensitivity or inability to adapt to stressful or challenging environmental stimuli (such as isolation or exposure to novel lit environment). We therefore propose that the LSAMP protein, which guides axon targeting and growth in the brain, plays a crucial role in forming connections in the brain necessary for adapting to changes in the environment in an evolutionarily sustainable way. As impaired adaptation is a common denominator of almost all psychiatric disorders, it is unsurprising that so many connections between the LSAMP protein and psychiatric disorders have been found. In the light of these data it seems that altered social behaviour in these mice reflects a wider underlying phenotype – adaptation impairment.

5. Changes in major monoamine systems in Lsamp-deficient mice

Lsamp$^{-/-}$ mice had a blunted response to the locomotor effect of amphetamine at higher dose levels compared to wild-type littermates. In the conditioned place preference test, amphetamine at dose level 2.5 mg/kg induced place preference in wild-type mice, but not in Lsamp$^{-/-}$ mice. This indicates that the partial loss of sensitivity to amphetamine in Lsamp$^{-/-}$ mice is probably not confined to locomotor effects, but rather is systemic, comprising also the reward-related mechanisms.

Monoamine measurements showed that the level of 5-HT was lower and the turnover of 5-HT higher in Lsamp$^{-/-}$ mice in all five brain regions measured; amphetamine raised the level of 5-HT and lowered the turnover of 5-HT in Lsamp$^{-/-}$ mice to a greater extent than in wild-type mice. Thus, Lsamp$^{-/-}$ mice seem to have an increased endogenous 5-HT tone that might readily explain their lower anxiety and a decrease in agonistic behaviour and aggression (Paper I). Slightly exaggerated behavioural activation observed in Lsamp$^{-/-}$ mice

(Paper I; Catania et al, 2008) is harder to explain by this increase, because, for example, increasing the 5-HT levels by using 5-HT transporter blockers leads to hyperactive behaviour, however, the increase of 5-HT due to gene knockout strategy (5-HT transporter knockout) induce hypoactivity, thus the effect of serotonin on locomotor behaviour is non-linear and might be dependent on secondary effects on other neurotransmitter systems, such as dopamine (Viggiano, 2008). It has been shown that 5-HT neurons innervate both dopaminergic and non-dopaminergic neurons in the ventral tegmental area and may influence mesocortical and mesolimbic efferent systems through synaptic as well as non-synaptic mechanisms (Hervé et al, 1987) and that central 5-HT system exerts a tonic and phasic inhibitory control on mesolimbic DA neuron activity (Di Matteo et al, 1999), but the question, how and to what extent central 5-HT influences locomotor activity in rodents is open-ended. In this study, the acceleration of 5-HT turnover in several brain regions in Lsamp$^{-/-}$ mice is indicative of changes at the level of brainstem 5-HT-ergic neurons, i.e. the changes caused by the genetic invalidation of the Lsamp gene are rather presynaptic than receptor-related. However, in Lsamp$^{-/-}$ mice, amphetamine seems to release 5-HT more readily than in wild-type mice, suppressing its turnover rate. Blunted behavioural effect of amphetamine in Lsamp$^{-/-}$ mice could thus be explained by the antagonistic effect of 5-HT on the DA-ergic system, which leads to suppressed locomotor activity. For example, in the rat and monkey, elevated synaptic 5-HT level can dampen the behavioural effects, including locomotor activation, of DA-releasing agents (Rothman and Baumann, 2006). The increase of tissue level of 5-HT in Lsamp$^{-/-}$ mice in response to amphetamine compared to wild-type mice could develop as a result of inhibiting the reuptake of 5-HT and this in turn would suppress its fast turnover rate. Since 5-HT is not removed from the synaptic cleft, and its production rate is not changed, the effect of 5-HT on the DA-ergic system is elevated in Lsamp$^{-/-}$ mice. This could account for the blunted locomotor activity in Lsamp$^{-/-}$ mice in response to amphetamine. In wild-type mice the concentration of 5-HT also increased at the tissue level in response to amphetamine compared to saline administration, but less than in Lsamp$^{-/-}$ mice. It must be noted, however, that the changes in tissue levels seen in this study, and the synaptic levels are not necessarily equal and therefore, this topic should be studied further by means of in vivo microdialysis, for example.

For amphetamine, two alternative, but mutually not exclusive routes of action have been proposed: first, it exerts influence both at the vesicular level where it redistributes DA to the cytosol, promoting reverse transport, and DA release (Sulzer et al, 1995) and secondly, according to the “DAT hypothesis”, amphetamine exerts its effect by binding to DAT and being transported into the terminals, resulting in DA efflux. Therefore, DAT expression level is one of the factors likely influencing amphetamine-induced locomotor stimulation (Chen et al, 2006). This is in good accordance with the results of the present study where Lsamp$^{-/-}$ mice displayed both lower expression level of DAT in the mesencephalon and markedly blunted locomotor response to amphetamine.

6. Concluding remarks and future prospects

Overall, our results suggest that LSAMP protein is not crucial for the general development of anatomical brain structures, but is needed for specific neural circuits that regulate anxiety-related and social behaviour. Genetic invalidation of the Lsamp gene causes several major shifts in the activity of the monoamine systems. Reduced anxiety and reduced aggressiveness in Lsamp$^{-/-}$ mice are likely related to a shift in balance in the Gabra1 and Gabra2 genes and enhanced serotonergic tone. Besides regulating anxiety, the Lsamp gene seems to play a crucial role in the formation of pathways related to the development of social behaviours and inter-male hierarchy. Thus, Lsamp$^{-/-}$ mice might be a fruitful model to study the possible molecular mechanisms behind changes in social behaviour that accompany many psychiatric disorders.

Manipulations with environment revealed that Lsamp$^{-/-}$ mice are remarkably insensitive to changes in environment as in most of the tests their phenotype was much more stable, regardless of rearing conditions, than in wild-type littermates. In nature, such a lack of reaction to changes in environment would be disadvantageous, a sign of adaptation impairment.

The deletion of the Lsamp gene facilitates the release of 5-HT and suppresses the turnover rate of 5-HT in response to amphetamine administration, and Lsamp$^{-/-}$ mice display lower level of DAT mRNA in the mesencephalon. These effects may be responsible for the markedly blunted behavioural response to amphetamine in Lsamp$^{-/-}$ mice. Our preliminary experiments indicate that the sensitivity of Lsamp$^{-/-}$ mice to cocaine and morphine is also altered, but the extent and mechanisms of these effects remain to be elucidated in further studies.

However, it is clear that by studying Lsamp$^{-/-}$ mice, no definitive answers on the function of the Lsamp gene and LSAMP protein can be obtained. It is necessary to study the IgLON protein family as a whole, for example, to measure the expression levels of other members of the IgLON family in response to the genetic deletion of one member to reveal possible compensatory responses, to characterise the phenotype of neurotrimin, kilon and OBCAM knockout mice, to study double (or even triple, if possible) knockout effects (by crossing two or three different knockout mouse lines). Recently discovered zinc-finger technology enables to produce knockout rats that are a better and more relevant model than mice for studying the links between genes and psychiatric disorders. Also, it is necessary to add a developmental dimension, by studying the comparative expression patterns of the members of the IgLON family during embryonic and early life development.

All in all, the present thesis generated even more questions than it answered and opened many new paths to explore, but science has always been like a many-headed monster that grows back more heads than are cut off.

CONCLUSIONS

1. Our Lsamp$^{-/-}$ mice are vital and fertile, display no gross abnormalities and do not differ from their wild-type littermates in terms of motor abilities, muscle power, vision, hearing, olfaction and mechanical sensitivity. However, Lsamp$^{-/-}$ mice are less anxious and slightly hyperactive; swim slower, but perform normally in the Morris water maze and other learning and memory tests; and display serious deviations in social behaviour such as lack of whisker trimming and lack of aggressiveness. The anxiolytic-like phenotype of Lsamp$^{-/-}$ mice is probably related to altered proportional balance between Gabra1 and Gabra2 genes. There is proportionally significantly more Gabra2 transcript encoding the α2 subunit of $GABA_A$ receptors, related to the anxiolytic effect of diazepam, in the temporal cortex of Lsamp$^{-/-}$ mice.
2. Some phenotypic differences seen in Lsamp$^{-/-}$ mice in standard housing conditions are also evident in other types of housing conditions, environmental manipulations differentially modified the behaviour of Lsamp$^{-/-}$ and wild-type mice in several tests. Environmental enrichment (EE) abolished differences between the genotypes in body weight and anogenital sniffing, a behaviour often preceding aggressive attacks, and amplified the anxiolytic-like phenotype of Lsamp$^{-/-}$ mice. Isolation abolished differences between the genotypes in body weight and anxiety and increased inter-male anogenital sniffing in wild-type animals, but not in Lsamp$^{-/-}$ mice. Overall, Lsamp-deficiency leads to decreased sensitivity or inability to adapt to stressful or challenging environmental stimuli. It means that the effects seen in Paper I may reflect a wider underlying phenomenon – an adaptation impairment.
3. Lsamp$^{-/-}$ mice display lower expression level of DAT in the mesencephalon and have a blunted response to the locomotor effect of amphetamine at higher dose levels compared to wild-type littermates. Also, in the conditioned place preference test, amphetamine induced place preference in wild-type mice, but not in Lsamp$^{-/-}$ mice. This indicates that the partial loss of sensitivity to amphetamine in Lsamp$^{-/-}$ mice is probably not confined to locomotor effects, but rather is systemic, comprising also the reward-related mechanisms. Monoamine measurements showed that the level of 5-HT was lower and the turnover of 5-HT higher in Lsamp$^{-/-}$ mice; amphetamine raised the level of 5-HT and lowered the turnover of 5-HT in Lsamp$^{-/-}$ mice to a greater extent than in wild-type mice. Thus, Lsamp$^{-/-}$ mice seem to have an increased endogenous 5-HT tone that may explain their lower anxiety and a decrease in agonistic behaviour and aggression.

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SUMMARY IN ESTONIAN

Limbilise süsteemiga seotud membraanvalgu (LSAMP) geeni puudulikkusega hiire käitumuslik, farmakoloogiline ja neurokeemiline iseloomustus

Inimese genoomis on umbes 20 000 geeni, mis kodeerivad valku. Mitu tuhat nendest on praktiliselt läbi uurimata ning veel mitut tuhandet on võrdlemisi vähe uuritud. Lsamp geen on üks selline geen, mille kohta meie senised teadmised ei ole kaugeltki veel ammendavad. Lsamp geeni produkt – LSAMP valk – kuulub IgLON valguperekonda nagu ka neurotrimiin, kilon ja OBCAM. Need valgud moodustavad omavahel valgukomplekse (heterodimeere), mis suunavad ja stimuleerivad närvirakkude jätkete – aksonite – väljakasvu. Kui molekulaarsel ja strukturaalsel tasandil on Lsamp geen ja LSAMP valk võrdlemisi hästi iseloomustatud, siis funktsionaalsel ja organismi tasandil on teadmised selgelt ebapiisavad, eriti arvestades seda, et LSAMP näib olevat organismis väga olulist rolli etendav valk. Nimelt on leitud selgeid seoseid Lsamp geenis leiduvate ühenukleotiidsete polümorfismide ja psühhiaatriliste häirete esinemissageduse vahel ning loomuuringutes on leitud selgeid seoseid Lsamp geeni ekspressioonitaseme ja ärevuse taseme vahel. Lisaks sellele on Lsamp osutunud teatud vähitüüpide puhul tuumorsupressorgeeniks.

Käesolev doktoritöö koosneb kolmest artiklist, milles uuriti ja iseloomustati Lsamp geeni puudulikkusega hiiri. Lsamp geeni puudulikkusega hiir on transgeense *knockout* tehnoloogia abil loodud mudelorganism, milles Lsamp geen on väljalülitatud ning milles seetõttu funktsionaalset LSAMP valku ei leidu. Vaadeldes muutusi organismis, kus LSAMP valku ei leidu, saab teha järeldusi selle valgu funktsiooni kohta ning kuna inimese ja hiire Lsamp geeni järjestus on 99% ulatuses kattuv, saab hiirtega tehtud uuringute põhjal teha ettevaatlikke järeldusi ka Lsamp geeni funktsiooni kohta inimorganismis. Tuleb aga meeles pidada, et geenipuudulikkusega hiire organismis leiavad arengu käigus aset paljud kompensatoorsed muutused ning seetõttu ei väljenda Lsamp geeni puudulikkusega hiirte fenotüüp ilmtingimata üks-üheselt Lsamp geeni puudumise mõju. Sellest hoolimata on *knockout* hiire uurimine oluline ja kasulik meetod, mida kasutatakse tänapäeval iga geeni funktsiooni uurimisel rutiinselt.

Esimene artikkel kirjeldab põhjalikumalt Lsamp geeni puudulikkusega hiireliini loomist, sensoorset fenotüüpi ja käitumuslikke eripärasid nagu vähenenud ärevus, suurenenud spontaanne liikumisaktiivsus, vähenenud ujumiskiirus, vähenenud vastastikune vurrude pügamine ja vähenenud agressiivsus. Lisaks leidsime selles artiklis, et Lsamp geeni puudulikkusega hiirtel on suurenenud tundlikkus bensodiasepiin diasepaami ärevust vähendava toime suhtes ning see võib tuleneda GABA A retseptori kahe põhilise alaühiku – alfa1 ja alfa2 – suhte muutumisest. Teine artikkel kirjeldab fenotüübilisi muutusi erinevates keskkondades kasvanud Lsamp geeni puudulikkusega hiirtel võrreldes nende metsiktüüpi (s.o normaalsete) pesakonnakaaslastega. Leidsime, et rikastatud

keskkonnas (suurem puur, kus on jooksurattad, puidust majakesed jmt) kasvades Lsamp geeni puudulikkusega hiirte mõned fenotüübilised iseärasused (nt vähenenud ärevus) võimenduvad ning mõned (nt väiksem kehakaal) kaovad. Isolatsioon (üksikpuuris kasvamine) tekitab tavaliselt isasloomadel tugevat stressi, kuid Lsamp geeni puudulikkusega hiired olid selle stressi suhtes tähelepanuväärselt tundetud. Kolmandas töös mõõtsime Lsamp geeni puudulikkusega hiirte tundlikkust amfetamiini stimuleerivale ja sarrustavale toimele ning leidsime, et see oli mõlemal juhul langenud. Samuti mõõtsime olulisemate virgatsainete ja nende metaboliitide taset *knockout* hiirte ja metsiktüüpi hiirte viiest ajupiirkonnast nii füsioloogilise lahuse kui ka amfetamiini manustamise järgselt. Leidsime, et Lsamp geeni puudulikkusega hiirtel on madalam serotoniinitase, kuid suurem serotoniinisüsteemi „käive" kui nende metsiktüüpi pesakonnakaaslastel ning et amfetamiini manustamine suurendab serotoniinitaset ja langetab serotoniinisüsteemi käivet Lsamp geeni puudulikkusega hiirtel rohkem kui metsiktüüpi hiirtel. Samuti tuvastasime geeniekspressiooni määramise meetodi (qRT-PCR) abil, et Lsamp geeni puudulikkusega hiirtel on keskajus vähem dopamiini transporteri (DAT) mRNA-d. Muutused serotoniinisüsteemis ja DAT-i mRNA madalam tase aitavad seletada nii Lsamp geeni puudulikkusega hiirte vähenenud ärevust, vähenenud agressiivsust kui ka nende vähenenud tundlikkust amfetamiinile.

Kokkuvõtteks, Lsamp geeni väljalülitamine hiire organismis põhjustab ulatuslikke muutusi nii käitumises kui kesksetes virgatsainesüsteemides. Nende muutuste põhjalik uurimine võib aidata leida uusi molekulaarseid sihtmärke uute paremate psühhiaatriliste ravimite väljatöötamiseks. Kindlasti tuleb Lsamp geeni edasistes uuringutes võtta paralleelselt vaatluse alla ka IgLONi valguperekonna teised liikmed.

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ORIGINAL PUBLICATIONS

CURRICULUM VITAE

Name: Jürgen Innos
Date of birth: 22.11.1976, Tartu
Citizenship: Estonian
Address: University of Tartu, Institute of Physiology, Ravila 19, Tartu 50411, Estonia
Phone: +372 737 4335
E-mail: innos@ut.ee

Education:

1984–1995	Tartu Secondary School No 2 (Miina Härma Gymnasium)
1995–1997	University of Tartu, Faculty of Law
1997–2004	University of Tartu, Faculty of Social Sciences, psychology, B.Sc.
1998–1999	Military service in Kuperjanov Infantry Batallion, Võru, 120 mm mortar group (one year), ensign
2000–2001	University of Toronto, Department of Psychology (one year)
2004–2006	University of Tartu, Faculty of Medicine, master studies in biomedicine, M.Sc.
2006–2013	University of Tartu, Faculty of Medicine, doctoral studies in neuroscience

Career:

1999–	Freelance translator and interpreter
2008–2011	University of Tartu, Institute of Physiology, research fellow
2011–	University of Tartu, Institute of Physiology, research fellow (physiology)

Practical courses:

2009	Competence course on Laboratory Animal Science (Tartu)
2012	Course „Assessment of rodents' behaviour: methods and rationale" (Helsinki)

Research:
Characterisation of the phenotype of transgenic mouse lines (Lsamp, Wfs1, Myg1, CCK2R, α-synuclein point mutation). Sixteen publications in international peer-review journals.

List of publications:

1. **Innos J**, Koido K, Philips MA, Vasar E (2013) Limbic system associated membrane protein as a potential target for neuropsychiatric disorders. *Frontiers in Pharmacology*, 4: 32. doi: 10.3389/fphar.2013.00032.

2. **Innos J**, Leidmaa E, Philips MA, Sütt S, Alttoa A, Harro J, Kõks S, Vasar E (2013) Lsamp (–/–) mice display lower sensitivity to amphetamine and have elevated 5-HT turnover. *Biochemical and Biophysical Research Communications*, 430(1): 413–418.
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4. **Innos J**, Philips MA, Leidmaa E, Heinla I, Raud S, Reemann P, Plaas M, Nurk K, Kurrikoff K, Matto V, Visnapuu T, Mardi P, Kõks S, Vasar E (2011) Lower anxiety and a decrease in agonistic behaviour in Lsamp-deficient mice. *Behavioral Brain Research*, 217(1): 21–31.
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9. Abramov U, Raud S, **Innos J**, Lasner H, Kurrikoff K, Türna T, Puussaar T, Okva K, Matsui T, Vasar E (2008) Different housing conditions alter the behavioural phenotype of CCK(2) receptor-deficient mice. *Behavioural Brain Research*, 193(1), 108–116.
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12. Abramov U, Raud S, **Innos J**, Kõks S, Matsui T, Vasar E (2006) Gender specific effects of ethanol in mice, lacking CCK(2) receptors. *Behavioural Brain Research* 175(1): 149–156.

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ELULOOKIRJELDUS

Nimi: Jürgen Innos
Sünniaeg: 22.11.1976, Tartu
Kodakondsus: Eesti
Aadress: TÜ füsioloogia instituut, Ravila 19, Tartu 50411, Eesti
Telefon: +372 737 4335
E-post: innos@ut.ee

Haridus:

1984–1995	Tartu 2. keskkool (Miina Härma Gümnaasium)
1995–1997	Tartu Ülikooli õigusteaduskond
1997–2004	Tartu Ülikooli sotsiaalteaduskond, psühholoogia eriala, B.Sc.
1998–1999	Ajateenistus Kuperjanovi Üksik-Jalaväepataljonis, 120 mm miinipildujapatarei (üks aasta), lipnik
2000–2001	Toronto Ülikool, psühholoogia eriala (üks õppeaasta)
2004–2006	Tartu Ülikooli arstiteaduskond, biomeditsiini magistriõpe, M.Sc.
2006–2013	Tartu Ülikooli arstiteaduskond, neuroteaduste doktorantuur

Töökogemus:

1999–	Vabakutseline tõlk ja tõlkija
2008–2011	Tartu Ülikooli füsioloogia instituut, teadur
2011–	Tartu Ülikooli füsioloogia instituut, füsioloogia teadur

Erialane enesetäiendus:

2009	Katseloomateaduse kursus (Tartu)
2012	Loengukursus „Assessment of rodents' behaviour: methods and rationale" (Helsingi)

Teadustöö:

Transgeensete hiireliinide (Lsamp, Wfs1, Myg1, CCK2R, α-sünukleiini punkt-mutatsioon) fenotüübi iseloomustamine. Rahvusvahelise levikuga eelretsen-seeritavates ajakirjades on ilmunud 16 publikatsiooni.

Artiklid eelretsenseeritavates ajakirjades:

1. **Innos J**, Koido K, Philips MA, Vasar E (2013) Limbic system associated membrane protein as a potential target for neuropsychiatric disorders. *Frontiers in Pharmacology*, 4: 32. doi: 10.3389/fphar.2013.00032.
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DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
3. **Dzhamilja Safiulina.** The studies of mitochondria in cultured cerebellar granule neurons: characterization of mitochondrial function, volume homeostasis and interaction with neurosteroids. Tartu, 2006.
4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
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