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Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

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ABBREVIATIONS

129Sv	–	129S6/SvEv mouse strain
Abbr	–	abbreviations
Amph	–	amphetamine
B6	–	C57BL/6 mouse strain
Bdnf	–	Brain derived neurotrophic factor gene
Bdnf	–	Brain derived neurotrophic factor protein
cDNA	–	complementary DNA
DNA	–	deoxyribonucleic acid
EE	–	environmental enrichment
IH	–	individual housing
LacZ	–	beta-galactosidase gene
LacZNeo	–	beta-galactosidase/neomycin fusion gene
Lsamp	–	limbic system associated membrane protein gene
Lsamp	–	limbic system associated membrane protein
LSAMP	–	human limbic system associated membrane protein
mRNA	–	messenger ribonucleic acid
NLS	–	nuclear localization signal
PBS	–	phosphate buffered saline
PCR	–	polymerase chain reaction
PFA	–	paraformaldehyde
qRT-PCR	–	quantitative real-time polymerase chain reaction
RNA	–	ribonucleic acid
SAP	–	stretch attend posture
SH	–	standard housing
X-Gal	–	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

LIST OF ORIGINAL PUBLICATIONS

I **Heinla, I.,** Leidmaa, E., Visnapuu, T., Philips, M. A., & Vasar, E. (2014). Enrichment and individual housing reinforce the differences in aggressiveness and amphetamine response in 129S6/SvEv and C57BL/6 strains. *Behav Brain Res, 267*, 66–73. doi: 10.1016/j.bbr.2014.03.024

II **Heinla, I.,** Leidmaa, E., Kongi, K., Pennert, A., Innos, J., Nurk, K., Tekko, T., Singh, K., Vanaveski, T., Reimets, R., Mandel, M., Lang, A., Lilleväli, K., Kaasik, A., Vasar, E., Philips, M. A. (2015). Gene expression patterns and environmental enrichment-induced effects in the hippocampi of mice suggest importance of Lsamp in plasticity. *Front Neurosci, 9*, 205. doi: 10.3389/fnins.2015.00205

III Philips, M. A., Lillevali, K., **Heinla, I**., Luuk, H., Hundahl, C. A., Kongi, K., Vanaveski, T., Tekko, T., Innos, J., Vasar, E. (2015). Lsamp is implicated in the regulation of emotional and social behavior by use of alternative promoters in the brain. *Brain Struct Funct, 220*(3), 1381–1393. doi: 10.1007/s00429-014-0732-x

Contribution of the author:

I The author designed the study, performed the behavioural and gene expression experiments, carried out the statistical analysis, wrote the manuscript and handled the correspondence.

II The author participated in designing the study, performed the behavioural and part of gene expression experiments, carried out the statistical analysis, wrote the manuscript and handled the correspondence.

III The author participated in designing the study, performed the behavioural and part of gene expression experiments, carried out the statistical analysis and participated in writing the manuscript.

INTRODUCTION

Rodent models are an unavoidable component of contemporary drug discovery and basic neuroscience. Before a candidate compound or treatment can be approved for phase I clinical studies in humans, it goes through thorough efficacy and safety testing in preclinical laboratories. Rodents are also used to model diseases and disorders in basic science.

After quick rise in popularity of transgenic technology in 1990-s and 2000-s (the first knockout mouse was created in 1989 (Thompson et al 1989)) many animals have been used in this field. Transgenetics allows studying functions of a single gene in a way that was unimaginable before. Detailed functional characterisation of most of the mammalian genes was enthusiastically believed to be available within a decade or two. A thorough understanding of genetic diathesis of diseases seemed to be leading to rapid improvement in treatment. Despite accumulating number of models and experimental animals, it has become clear that knock-out and knock-in models do not always produce clear and easy answers about the functions of molecular targets, mainly due to complex compensatory mechanisms. Nevertheless, transgenic experiments are an important addition to the immensely popular descriptive genome wide association studies. Transgenic models, nowadays often double-, triple-, or conditional and tissue-specific knock-out/in models, allow specific and in-depth research of biological phenomena.

Modern regulation on laboratory animal husbandry is guiding us to gradually enrich the standard environment in animal facilities for welfare reasons. Rodents raised in enriched environment are better suited for modelling human conditions; animals grow, develop and behave differently from their counterparts raised in a drab environment. Enriched animals are accustomed to changes in their surroundings and day-to-day challenges and their anxiety profile in the new environment is more versatile. Another commonly used environmental manipulation is social isolation, which acts as a chronic stressor allowing us to assess the reaction of animals with different biological diathesis.

A methodological shift is taking place in rodent phenotyping. The attitude towards animal experimentation and ethical standards posed for researchers and animal maintenance conditions are changing. The expectations towards the results are rising with the necessity to test more complex hypotheses and distinguish small differences while using as few animals as possible. On the other hand, we have the new generation of observation equipment available, which requires less interference and the amount of data that can be collected has increased in magnitudes compared to the possibilities of manual scoring. Changing paradigm in the living and rearing conditions of laboratory animals along with technological changes rise the question whether the current results obtained in modern conditions are comparable to those that were obtained ten or twenty years ago.

In the current dissertation I will evaluate mice from two inbred genetic backgrounds; B6 and 129Sv strains, that are widely used in pharmacological studies and in creating transgenic animals. The behavioural profile and molecular changes of the two strains are followed in three different environmental conditions: environmental enrichment, isolation and standard home cage. From the previous studies we know that B6 strain has an active coping style whereas 129Sv has a passive one. Higher anxiety and deviations in adaptive behaviour in 129Sv mice are some of the most obvious behavioural differences between the strains.

In our study we have chosen to investigate Limbic system associated membrane protein (Lsamp) as a sample molecular target, considering accumulating evidence that has linked Lsamp to neural pathways involved in anxiety reaction and coping strategies. Two independently created *Lsamp*-knockout lines (Catania et al 2008, Innos et al 2011) are both characterised by reduced anxiety-like behaviour and deviations in adaptive behaviour. Increased trait anxiety and increased acute fear reaction in rodents have been shown to be related to the elevated level of the *Lsamp* transcript in numerous brain structures (Alttoa et al 2010, Koks et al 2004, Lamprecht et al 2009, Nelovkov et al 2006). The positive correlations between anxiety measures and the levels of *Lsamp* transcripts were reproduced in the current study.

We also demonstrated the differences in the levels of *Lsamp* transcripts between B6 and 129Sv strains. Significant strain differences in *Lsamp* expression were detected in the hippocampus, frontal cortex and thalamus that could be related to the different behavioural phenotype of B6 and 129Sv mice. Furthermore, environmental enrichment elevated the expression levels of *Lsamp* 1b transcript specifically in the hippocampus in B6 mice. Finally, we present a thorough analysis of the anatomical distribution of *Lsamp* transcripts in the mouse brain allowing better insight of the neurobiological connections of our molecular target.

The research presented in the current thesis will broaden our knowledge about the molecular mechanisms of anxiety, emphasising the impact of biological predisposition which demands careful control over the background strains. It is also important to interpret and prepare for the inevitable changes in our subjects´ behaviour and neurochemistry following the changes in animal welfare in regard to the recent directives (Directive 2010/63/EU). Even though the initial enthusiasm about transgenic technology has calmed down, I encourage you to follow my train of thought through the following dissertation and I hope to convince you that this approach can provide useful information for contemporary translational neuroscience.

REVIEW OF THE LITERATURE

1. Transgenic research and inbred mouse lines 129Sv and B6

Transgenic mice are a standard, widely spread and cost-effective method for investigating functions of different genes with unknown function. Standard approach of creating transgenic mouse lines combines 129Sv and B6 strains. 129Sv-derived mouse embryonic stem (ES) cell line is most commonly used for introducing targeted mutation into mouse genome (Hedrich 2004) and C57BL/6 is the most widely used background line in biomedical research (Yoshiki & Moriwaki 2006). This combination of two lines results in heterogeneous and somewhat unpredictable genetic background. One could suggest that the variable genetic background may result in low reproducibility of studies using transgenic mice, e.g. the effects established by one group cannot always be repeated by the others (Crabbe et al 1999, Mandillo et al 2008, Wahlsten et al 2003). These effects may be caused by the confounding factors (either genetic or environmental), instead of being the outcome of performed manipulation; genetic modification or drug administration.

In order to challenge the issue of unreliable results arising due to the mixed background, several groups (Abramov et al 2008, Contet et al 2001, Rodgers et al 2002, Voikar et al 2001) have compared B6 and 129Sv mouse strains to map their behavioural and biochemical differences. Indeed, thorough understanding of two background strains would make it easier to detect all possible variations induced by the genetic manipulations, but it is also important to be able to disclaim false positive artefacts. Moreover, better characterization of B6 and 129Sv behavioural profiles is not only necessary in the context of transgenic studies, as 129Sv and especially B6 are also common strains used in non-transgenic neuroscientific research.

These background strains are demonstrating the substantial differences in the behavioural performance (the data analysed and referenced in the original publication I) which provides the baseline of behavioural parameters against which genetically modified lines, derived from these particular strains, have to be assessed.

We do already know that EE produces substantial changes in the brain. The upregulation of Bdnf due to EE was shown two decades ago by Falkenberg et al. (Falkenberg et al 1992) and since then Bdnf has been one of the most common markers in enrichment studies. Chourbaji et al. (Chourbaji et al 2012b) showed that EE induced Bdnf increase in C57Bl6/N mice hippocampus but had no significant effect in frontal cortex in neither mRNA or protein expression profiles. However, earlier studies have shown that the biochemistry of the frontal cortex is affected by EE (Brenes et al 2008). Despite apparent difference in behavioural phenotype between B6 and 129Sv strains, the two lines have been reported to be genetically relatively similar (Morris et al 2010). This

means that the alternative regulation of relatively few genes counts for the phenotype difference. We decided to find out if the reported effect of enrichment on *Bdnf* gene expression is differently regulated in the hippocampus or frontal cortex of either of the two strains.

2. Environmental enrichment and isolation

A growing field of social genomics research has begun to identify the specific types of genes that are subject to social-environmental regulation, the neural and molecular mechanisms that mediate the effects social processes have on gene expression (Slavich & Cole 2013). Enrichment and social isolation are the most common environmental manipulations used in laboratory rodent housing. In certain study designs rodents must stay in isolation; e.g. studies which require surgical intervention (Bailey & Crawley 2009) or investigation of feeding behaviour (where food intake per animal has to be measured with great precision) (Ellacott et al 2010). At the same time different kinds of enrichment equipment is applied to standard rodent laboratory housing conditions worldwide. Therefore it is vital to understand the molecular and functional impact of these environmental manipulations on the brain and behaviour. Environmental enrichment is known to profoundly affect the central nervous system at the transcriptome level (Rampon et al 2000) and influence the fine structural anatomy of neural networks (Freund et al 2013, Kempermann et al 1997) during the critical developmental period and during adulthood (Baroncelli et al 2010). Social isolation and rejection can influence the activity of a broad set of genes (Bibancos et al 2007, Sestito et al 2011) and cause permanent changes in the brain and behaviour throughout lifespan (Fone & Porkess 2008). The discovery that social-environmental factors can substantially alter the expression of meaningfully identified gene profiles represents a paradigm shift in thinking.

3. Functions of *Lsamp* gene

Limbic system-associated membrane protein (LSAMP) is a neural cell adhesion molecule expressed on the neuronal dendrites and somata (Zacco et al 1990) on structures known to be especially important for emotional and motivational functions (Heimer & Van Hoesen 2006, Levitt 1984). Recently, LSAMP has been linked with a spectrum of psychiatric disorders in humans. The levels of the LSAMP protein have been found to be increased approximately 20% in the *postmortem* frontal cortex both in patients with schizophrenia and bipolar disorder (Behan et al 2009). Polymorphisms in the *LSAMP* gene have been associated with depression (Koido et al 2012) and *LSAMP* has been suggested to have a role in the neurobiology of male completed suicide (Must et al 2008).

Functional studies have shown that LSAMP can promote or inhibit neurite outgrowth depending on interactions with other members of the IgLON family

(Gil et al 2002, Mann et al 1998) indicating its prominent role in neurite formation and synaptogenesis (Hashimoto et al 2009). Before second post-natal week of development, LSAMP is transiently expressed in developing axons and growth cones (Horton & Levitt 1988) indicating importance in developing of the brain structures. However, the lack of obvious deviations in brain organization in both of the two independently created *Lsamp*-deficient mouse strains (Catania et al 2008, Innos et al 2011) suggests that LSAMP is mediating finely specialized aspects of circuit formation and maturation of the limbic system. Genetic deletion of the *Lsamp* gene in mice induced no detectable changes in sensory and motor development, but caused increased activity in novel environments and reduced anxiety-like behaviour in both knockout models (Catania et al 2008, Innos et al 2011). Increased trait anxiety in rats has been shown to be related with increased level of the *Lsamp* transcript in the amygdaloid area, periaqueductal gray (Nelovkov et al 2006), raphe, hippocampus and frontal cortex (Alttoa et al 2010). Elevated levels of the *Lsamp* transcript in the amygdaloid area of rats have been associated with acute fear reaction (Koks et al 2004) and fear conditioning (Lamprecht et al 2009). The amino acid sequence of LSAMP is highly conserved among species. There is 99% sequence identity between human and rodent LSAMP (Pimenta & Levitt 2004) and 91% identity with chicken (Brummendorf et al 1997), indicating remarkable phylogenetic conservation of protein structure and associated functional properties. Growing evidence indicates that LSAMP is involved in the formation of anatomical substrate for emotional behaviour both in rodents and humans.

4. Two alternative promoters of *Lsamp* gene

Pimenta and Levitt (Pimenta & Levitt 2004) reported revised genomic structure of the mouse *Lsamp* gene, demonstrating that, besides the well-known exon 1 (now referred as exon 1b), the *Lsamp* gene has an alternative exon 1 (currently exon 1a) located 1.6 Mbp upstream, and both of them have separate promoter sequences. The two-promoter structure is conserved and has been described in mouse, rat, human (Pimenta & Levitt 2004) and also in chicken (Brummendorf et al 1997). LSAMP distribution in the whole adult mammalian brain was originally described in rat using immunohistochemistry (Levitt 1984) and *in situ* hybridization (Reinoso et al 1996). The anatomical distribution of the *LSAMP* transcript and protein has been extensively described in various species (Chesselet et al 1991, Cote et al 1995, Prensa et al 2003, Yamamoto & Reiner 2005), but the anatomical distribution of the alternative transcripts has not been reported. One purpose of the present study was to characterize the distribution of the two alternatively transcribed *Lsamp* isoforms in the mouse. Additionally, we investigated the relation between *Lsamp* expression and the regulation of emotional and social behaviour.

Accumulating evidence suggests that *Limbic system associated membrane protein* (*Lsamp*) gene expression is sensitive to changes in external social and environmental conditions and it could mediate neural plasticity. The anatomical distribution of LSAMP is controlled by complex regulation of alternative 1a and 1b promoters. The impact of LSAMP protein on neurite outgrowth (Gil et al 2002, Mann et al 1998) and neuronal connectivity has been established in a wide spectrum of psychiatric disorders in humans (Behan et al 2009, Koido et al 2012). In mice, lack of LSAMP protein leads to inability to adapt or react to novel environments or stressful environmental manipulations in an evolutionarily sustainable way (Catania et al 2008, Innos et al 2013, Innos et al 2011). *Lsamp*-deficient mice are less sensitive to social isolation which is usually stressful for wild-type mice; furthermore, inadequately reduced anxiety reaction in potentially threatening situations is amplified if *Lsamp*-deficient mice have been reared in an enriched environment (Innos et al 2012).

LSAMP protein has been shown to increase synaptogenesis in the hippocampal neurons *in vitro* (Hashimoto et al 2009) indicating its role in plasticity. Furthermore, loss of LSAMP *in vivo* results in altered synaptic transmission and impaired plasticity in adult hippocampus (Qiu et al 2010). Synaptic plasticity has been considered to be one of the main mechanisms responsible for the neuronal changes that occur in response to complex stimulation by enriched environment (van Praag et al 2000). *Lsamp* gene expression, however, had not been studied in different environments. We sought to investigate how the environmental manipulations influence the complex regulation of alternative 1a and 1b promoters of *Lsamp* gene in the mouse brain. We evaluated the role of genetic background which is known to influence the phenotypes caused by single genes (Navarro et al 2012) thus we used two inbred mouse lines described above.

AIMS OF THE STUDY

The purpose of the dissertation is to provide a better frame of reference for studies and models using *Lsamp*-deficient mice or common inbred strains 129Sv and B6, thereby increasing their translational value. The aims of the dissertation are:

1) To compare the response of two inbred mouse lines B6 and 129Sv to three rearing environments: environmental enrichment, individual housing and standard laboratory environment. To evaluate the possible mechanisms of established differences.
2) To demonstrate comparatively the expression of *Lsamp* in two genetic backgrounds (B6 and 129Sv) and in three environments.
3) To display the anatomical distribution of *Lsamp* gene as a sample molecular target over the brain and describe the links between behavioural adaptability phenotype and *Lsamp* 1a and 1b promoter expression.

MATERIAL AND METHODS

1. Animals

Breeding was conducted in the Institute of Biomedicine and Translational Medicine, University of Tartu.

For behavioural experiments male mice (C57BL/6 Bkl; Scanbur AB, Sollentuna, Sweden; 129S6/SvEv/Tac; Taconic Europe, Bomholt, Denmark and *Lsamp*-deficient mice – produced in the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia and backcrossed into B6 for at least 10 generations) were housed under a 12-hour light/dark cycle with lights on at 7:00 a.m. Animals from different genotypes were housed in their respective home cages. The animals had free access to food and water except during testing. The bedding (aspen chips) and nesting material (aspen wool) were changed once a week. All of the mice in same housing condition in same batch were housed in a single cage (5–8 animals per cage; see table 1), except for IH mice.

2. *Lsamp*-deficient Mice

Exon 1b of mouse *Lsamp* gene was replaced by an in-frame NLSLacZNeo cassette resulting in an insertion of gene encoding beta-galactosidase immediately after *Lsamp* 1b promoter. As a result, these mice could not express functional Lsamp protein from either of the promoters. Detailed description of the creation of *Lsamp*-deficient mice with LacZ transgene can be found in (Innos et al 2011).

3. Environmental Enrichment (EE) and Individual Housing (IH)

After weaning at 3 weeks, mice were randomly allocated to either standard, enriched or individual housing conditions for 7 weeks (129Sv and B6) or 8 weeks (Lsamp-deficient mice and their wild-type littermates) before the start of the experiments.

Standard housing consisted of standard laboratory cages (425 mm × 266 mm × 155 mm) with bedding and nesting material.

Mice in the environmental enrichment group were housed in larger cages (595 mm × 380 mm × 200 mm) containing double amount of nesting material, stainless steel running wheels, aspen houses, igloos, tubes or labyrinths, which were changed and repositioned once a week. Each enriched cage always had five items, always including at least one running wheel and either a house or an igloo for shelter.

Individually housed mice lived in smaller (220 mm × 160 mm × 140 mm) cages with standard bedding and a small amount of nesting material.

4. Drugs

Amphetamine (amphetamine sulphate, Sigma-Aldrich, St Louis, MO, USA) was injected intraperitoneally (i.p.) 15 min prior to testing. Compound was diluted in 0.9% NaCl (B. Braun Melsungen AG, Germany). Dose was 5 mg/kg of amphetamine per animal (injection volume 10 ml/kg). Saline group animals received similar volume of 0.9% NaCl i.p.

5. Behavioural Tests

Male mice aged between 11 and 15 weeks were used unless stated otherwise. Behavioural testing was carried out between 10:00 and 17:00 of the light phase (except Phenotyper which started at 16:00 and ran 20 hours). Before each experiment, mice were allowed to habituate to the experimental room and the lighting conditions therein for at least 1 h.

The order in which the tests were carried out was chosen from more sensitive (to handling, stress or interference) to less sensitive tests as described before in (Contet et al 2001).

The phenotyping was carried out in different testing batteries (Tables 1 to 3) that measured locomotion, sensory functions and response to amphetamine.

Table 1. Order of tests in study I

Test battery	Behavioural test	Batch of mice (letter) and testing order (nr)	Number of mice					
			Enrichment		Individual housing		Standard	
			129Sv	B6	129Sv	B6	129Sv	B6
	Open field	A1 B1 C1	8 8 8	8 7 8	8 8 8	8 8 8	8 8 8	8 5 8
	Phenotyper	C2	8	8	8	8	8	8
	Hot Plate	C4	8	8	8	8	8	8
	Social interaction	C3	8	8	8	8	8	8
	Open field with and without amphetamine	A2 B2	8 8	8 7	8 8	8 8	8 8	8 5

Table 2. Order of tests in study III

Test battery	Behavioural test	Batch of mice (letter) and testing order (nr)	Number of animals		
			Standard conditions B6 males		
			Naïve	Pre-Conditioning	Conditioned fear
	Elevated plus-maze	D1	15		
	Locomotor activity/ open field	D2	15		
	Social interaction	D3	14		
	Acute Fear	E1	5	5	6

5.1. Open field

Locomotor activity was automatically registered for 30 min in photoelectric plexiglass motility boxes (448 mm × 448 mm × 450 mm, TSE, Technical and Scientific Equipment GmbH, Germany). The distance travelled, number of rearings and time spent in the centre were registered for study I and time spent moving, distance travelled, time spent in the centre and time spent in the corners for study III. Illumination level in the motility boxes during the experiments was approximately 400 lux. The floors of the motility boxes were cleaned thoroughly with 5% ethanol solution and dried after each animal.

5.2. Phenotyper

Animals were tested for 20 hours on the PhenoTyper® (EthoVision 3.0, Noldus Information Technology, Wageningen, The Netherlands). It is a 30 × 30 cm plexiglass box with similar sawdust bedding as in home cage. Each animal had an individual cage. Food and water were available ad libitum throughout the test.

Data for covered distance, time spent moving, hyperactivity episodes and velocity was recorded automatically by EthoVision software and exported for analysis.

5.3 Hot plate

The hot plate test was conducted by placing the mouse on a metal surface maintained at 52 °C. The hot plate was surrounded by transparent plastic barrier (diameter 15 cm, height 20 cm). Latency to show hind paw response (licking or

shaking) and latency to jump (end-point) from the plate were measured. The cut-off time was set at 120s (if animal did not show jump response earlier).

5.4. Direct social interaction (DSI)

Two unfamiliar male mice (in study I one mouse from the SH, EE or IH group and one age- and weight-matched unfamiliar partner from standard housing) were simultaneously placed into a cage (22 cm × 16 cm × 14 cm) with standard sawdust bedding and a cover made of transparent Plexiglas. 129Sv interaction partner was used with batch C animals and B6 interaction partner with batch D animals. In study III 14 mice were matched into 7 pairs of two unfamiliar mice. Illumination level of the testing arena was 50 lx. Mice were videotaped for 10 min. The videotapes were later scored by a trained observer blind to the experimental design. The following measures were registered for each mouse: (1) sniffing the body of the other mouse, (2) anogenital sniffing of the other mouse, (3) time and (4) number of aggressive attacks. The following were considered aggressive behaviours: attacking, biting, chasing and rattling the tail

5.5. The effect of amphetamine on locomotor activity (open field)

Half of the animals were injected with amphetamine solution (5 mg/kg; ip), the other half with saline and then placed in the motility box (448 mm × 448 mm × 450 mm, TSE, Technical and Scientific Equipment GmbH, Germany). Presented data was recorded from 15 to 45 minutes after injection. Same animals were tested similarly after 72hr with saline and amphetamine groups switched.

5.6. Elevated Plus-Maze

The apparatus consists of two opposite open (17.5 cm × 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 brightly lit room (450 lx in open arms). Pre-experimental social separation for 15 min was employed in order to increase exploratory activity. Testing began by placing a mouse on the central platform facing an open arm. Standard 5 min test duration was used, and the maze was thoroughly cleaned with damp and dry towels between the subjects. Test sessions were video-recorded and the videotapes were analysed by a trained observer unaware of testing conditions (eariler described in (Philips et al 2010)). Eight behavioural parameters were recorded: (1) the number of closed arm entries, (2) the number of open arm entries, (3) the ratio between open and closed arm entries, (4) the latency to enter open arm (latency, s), (5) time spent on open arms, (6) the number of protected head-dips, (7) the number of unprotected head-dips and (8) the number of stretch-attend postures (SAPs).

5.7. Fear conditioning

Fear conditioning was carried out by means of a computer-controlled Multi Conditioning System (TSE). Sixteen mice were divided into 3 groups: “Naïve” (n = 5); “Pre-Conditioning” (n = 5) and “Conditioned fear” (n = 6). Training for the “Conditioned fear” and “Pre-conditioning” groups was performed in a dimly illuminated (15 lx) acrylic cage (30 × 30 × 30 cm) with stainless steel rod floor. Between subjects the cages were cleaned with isopropanol. On the first day after 150 s acclimation period animals received 6 trials with the following stimuli: 15 s tone (12 kHz; 70 dB) and bright light (pulsing at 200 ms) were terminated by a 2 s electric shock (0.6 mA) during which the light was constant. Inter-trial interval was 120 s (± 50%). After the last trial the animals were returned to their home cages. On the second day, animals in the “Conditioned fear” group were placed into the conditioning cages and exposed to similar stimuli without an electric shock for about 45 minutes (20 trials). The animals were sacrificed immediately afterwards. We chose a 45 min duration as it has been shown that neuronal stimulation induces gene expression between time-points 30–60 min (Lamperech, 2009). “Naïve” and “Pre-conditioning” groups received no treatment on the second day. The hippocampus and temporal lobe (including the temporal cortex and the lateral, basolateral, central and medial nuclei of the amygdala) were dissected from the brains.

6. qRT-PCR analysis in mouse brain areas

6.1. The expression of *Lsamp* transcripts

The C57BL/6 (n=3×8) and 129S6/SvEv (n=3×8) mice were sacrificed by decapitation 10 days after the last experiment at the age of 15 weeks and brain regions of interest were collected into Eppendorf tubes and kept at 80 °C. Six different brain areas were dissected for the current study: the hippocampus, temporal lobe (including temporal cortex and the complex of amygdaloid nuclei), the frontal cortex, ventral striatum (including olfactory tubercles), thalamus and hypothalamus. The dissections of the brain areas were performed according to the mouse brain atlas (Franklin & Paxinos 1997). *Lsamp* mRNA level was determined by quantitative real-time PCR (qRT-PCR) in 6 brain regions. Total RNA was extracted individually from each brain structure by using Trizol® Reagent (Invitrogen, USA) according to the manufacturer’s protocol. First strand cDNA was synthesized by using Random hexamerprimer-mix (Applied Biosystems) and SuperScript™ III Reverse Transcriptase (Invitrogen, USA). TaqMan Assay was designed for the detection of 1a and 1b specific transcripts. FAM-MGB-probe AACCGAGGCACGGACAAC was used with universal reverse primer combined with alternative forward oligos specific for either 1a allele or 1b allele. TaqMan® Universal PCR Master Mix was used in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems,

USA). Reactions were carried out in 10 µl reaction volumes in four replicates. qRT-PCR data in figures is presented in a linear scale, calculated as 2 -ΔCT, where ΔCT is the difference in cycle threshold (CT) between the target gene and housekeeper gene HPRT-1 (VIC-TAMRA-labelled probes were used), which we found to be most reliable in a comparison of several housekeeper genes in the brain (Raud et al 2009). *Bdnf* and *synaptophysin* mRNA levels were determined by quantitative real-time PCR (qRT-PCR) by using the pre-designed Taqman Gene Expression Assays (Applied Biosystems): Mm01334042 m1(*Bdnf)* and Mm00436850_m1 (*synaptophysin*; the assay previously used in (Abel & Rissman 2013).

6.2. The expression of *Bdnf* transcript

The third batch of animals (batch C in Table 1) was sacrificed by decapitation 10 days after last experiment and brain regions of interest were collected into Eppendorf tubes and kept at 80 °C.

Bdnf mRNA level was determined by quantitative real-time PCR (qRT-PCR) in 24 mice from B6 strain and 24 mice from 129S6/SvEv strain (8 from each condition). Total RNA was extracted individually from each brain structure by using Trizol® Reagent (Invitrogen, USA) according to the manufacturer´s protocol. First strand cDNA was synthesized by using random hexamer oligo-nucleotides and SuperScriptTM III Reverse Transcriptase (Invitrogen, USA). *Bdnf* Taqman Gene Expression Assay (Applied Biosystems, Assay ID: Mm01334042 m1) (similarly to (Lockrow et al 2011)) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were used in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in 10 µl reaction volumes in four replicates. qRT-PCR data in figures is presented in a linear scale, calculated as 2 -ΔCT, where ΔCT is the difference in cycle threshold (CT) between the target gene (BDNF) and housekeeper gene HPRT-1 (VIC-TAMRA-labelled probes were used).

7. Histological stainings of the mouse brain slices

7.1. In situ RNA hybridization analysis with digoxigenin-UTP

Male C57BL/6 strain mice were used for all histological stainings. Mouse Lsamp cDNA fragments were cloned from a cDNA pool from C57BL/6 mouse hippocampus and inserted into pGEM7-Zf(+) vector (Promega) to create an in situ probe. cDNA fragment specific for 1a promoter (400 bp) consisted of 1a-specific 50UTR, exon 1a and exon 1a´. Universal Lsamp probe (567 bp) consisted of a cDNA fragment consisting of exons 2–6. RNA in situ hybridization on free-floating PFA-fixed 40 µm thick mouse brain cryosections using

digoxigenin-UTP (Roche) labeled *Lsamp* sense and antisense RNA probes was performed as described previously (Braissant & Wahli 1998). As a major modification, active DEPC treatment was avoided and 0.25 % Triton X-100 was added to the PBS to improve probe penetration.

7.2. Radioactive in situ hybridization with oligonucleotides

Antisense 400mer DNA oligonucleotide probes complementary to mouse *Lsamp* gene (Accession No. uc007zfr.1, UCSC Genome Browser, genome.ucsc.edu) Lsamp1a (5´-accccagcacccagacgctgtgcagccagtaggtcctcat-3´), Lsamp1b (5´-gaa-gaaggcagagcagtctcagtaggaccagcggcaactg-3´) and LsampUNI (5´-agagcatggcg-cttctccagctcaacccgagggtccagag-3´) were labeled with 33P-dUTP by use of terminal transferase (Sigma-Aldrich, Europe). Free-floating in situ hybridization was carried out essentially as described previously (Hundahl et al 2010).

7.3. X-Gal stainings

Lsamp-deficient mice with a LacZ transgene were used for visualizing the anatomical distribution of *Lsamp* 1b promoter activity. In BrdU and X-Gal co-stainings, male adult *Lsamp*-deficient mouse received two injections of 5-bromodeoxyuridine (BrdU; 100µg/g) with 2-hour interval and was sacrificed 24 hours after the last injection. For X-Gal staining the brains were cut into 100 µm coronal sections and transferred to glass slides. The contours and abbreviations in all the figures representing anatomical data have been adopted from the mouse brain atlas (Franklin & Paxinos 1997). For embryonic brains, the embryos were dissected from timed matings. E13.5 (embryonic day 13.5) and E15.5 brains and/or embryos were fixed in 4 % PFA/PBS at 4 _C overnight for in situ hybridization, or for 30 min for X-gal staining. For cutting 50-µm vibratome sections, the stained (E13.5 and E15.5) specimens were inserted into 1 ml of 0.5% gelatine/30% BSA/20% sucrose/PBS, wherein 140 µl of 25% glutaraldehyde was added immediately before insertion and incubated for 10 min. The sections were mounted into 70% glycerol and microphotographed.

BrdU incorporation was detected immunohistochemically using monoclonal rat anti-BrdU (AbDSerotec), biotinylated donkey anti-rat (Dako) antibodies, and Vectastain Elite ABC Kit (Vector Laboratories). Peroxydase reaction was detected by DAB detection kit (Vector Laboratories).

Dissociated primary hippocampal neuronal cultures were prepared according to (Chatterjee & Sikdar 2014) from the whole hippocampus of 0–2 days old mouse pups (*Lsamp* knockout). Hippocampus was digested in papain/DNase solution and neuronal cells were suspended in culture media consisting Dulbecco's modified Eagle's medium F12 HAM supplemented with N1, 10% fetal bovine serum and 1% antibiotic antimycotic. Cells were plated on 0.1 mg/mL poly-D lysine coated white microwell plates (96 F Nuclon Delta, Nunc)

at a density of 20,000–50,000 cells in 2 ml media in 12 mm diameter of culture area of 35 mm culture dish. For X-Gal staining primary hippocampal neurons were fixed with 2% PFA in PB Buffer for 15 minutes and washed three times in PBS for 15 minutes. Neurons were stained overnight with X-Gal solution. After staining again neurons were treated with 4% PFA for complete fixation.

8. Data analysis and statistics

Mean values and S.E.M. are presented in the figures. All data were analysed using Statistica version 10 (StatSoft, Inc., USA). Factorial ANOVA (strain × environment as grouping variables) was performed to compare the mRNA expression of experimental groups. For comparing 1a and 1b promoter expressions, repeated measures ANOVA (strain and environment as grouping variables and promoters as within subject factor) was used. Tukey HSD *post hoc* analysis was used when applicable after statistically significant ANOVA. For easier comparability coefficient of variation (CV) was used to analyse variance (table 3). Sample variations were analysed with Friedman ANOVA and Kendall´s concordance test.

The qRT-PCR data in figures is presented on a linear scale, calculated as $2^{-\Delta CT}$, where ΔCT is the difference in cycle threshold (CT) between the target gene (*Lsamp*) and housekeeper gene *Hprt-1* (VIC-MGB). Reported correlations were calculated using Pearson's Product-Moment correlation method.

9. Ethics

All the experiments were performed in accordance with the EU guidelines directive 86/609/EEC) and permit (No. 59, September 5, 2006) from the Estonian National Board of Animal Experiments.

RESULTS

1. B6 and 129Sv mice react differently to changes in rearing conditions (Paper I)

1.1. Phenotyper

Overall greater activity of B6 mice compared to 129Sv appears from the very first hour in Phenotyper ($F_{(1, 42)}$= 165.9; p<0.001) and is sustained throughout the test.

Next we looked at the housing effects and animals raised individually covered the longest distance in both strains (housing effect in first hour $F_{(2, 42)}$= 42.6; p<0.001 Figure 1a). We could also see that compared to standard housing, enrichment decreased the locomotion (for the first hour effect is significant for both 129Sv and B6 strain; see Fig 1a).

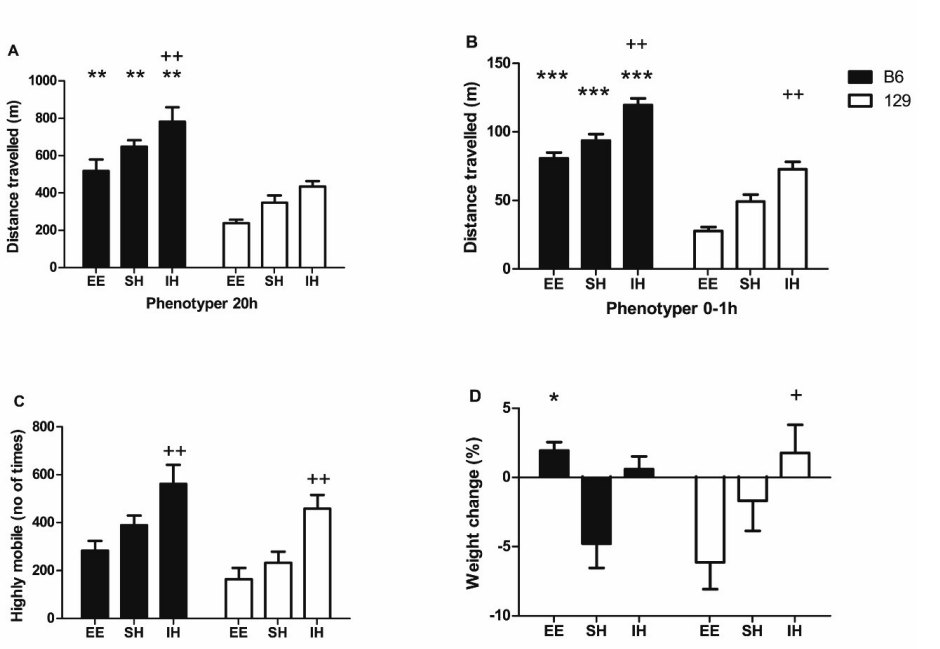

Figure 1. Phenotyper data illustrates basic strain differences and housing effects. (A) Distance travelled in 20 h Phenotyper test. ** p < 0.01 (Tukey HSD test after significant two-way ANOVA; compared to the respective 129 Sv group); ++ p < 0.01 (compared to the EE group of the same strain). (B) Distance travelled during first 60 min of Phenotyper test. *** p < 0.001 (compared to the respective 129 Sv group); ++ p < 0.01 (comparison of individual housing mice with EE and SH groups of same strain). (C) Number of highly mobile episodes. ++ p < 0.01 (compared to the EE group of same strain). (D) Weight change during Phenotyper test. * p < 0.05 (compared to the respective 129Sv group); + p < 0.05 (compared with EE group of the same strain).

After the Phenotyper test we observed an interesting interaction between strain and environment in the animals' weight (strain x housing x time $F_{(2, 42)}$=7.1; p<0.01; Figure 1d). As expected, animals from both strains that had been in social isolation for a while before the test, did not experience stress-induced weight loss but instead gained a little weight (1.7% for 129Sv and 0.6% for B6; Figure 1d). Animals raised in standard conditions from both strains lost weight (–1.7% and –4.8% respectively). We could see an interesting picture with the animals raised in EE. 129Sv animals lost more body weight than any other group (–6.1%) and their B6 counterparts gained more than any other group (+1.9%).

1.2. Social interaction

Our findings confirm that B6 animals are more aggressive than 129Sv mice ($F_{(2, 42)}$=13.7, p<0.0001; Figure 2B). No other group showed significant tendency to fight with an unfamiliar mouse but socially isolated B6 males (on average 89s of aggressive behaviour per interaction vs 0–8s for other groups). Vast difference suggests that both environmental factors and genetic predisposition need to be present to evoke violent behaviour in these conditions.

In time spent sniffing other animal we could see the strain effect (Figure 2A; $F_{(1, 42)}$=9.6, p<0.01), 129Sv spent more time sniffing partner than B6, and the housing effect ($F_{(2, 42)}$=5.7, p<0.01), isolated animals sniff more than mice from enriched or standard housing, but there was no interaction effect between strain and environment.

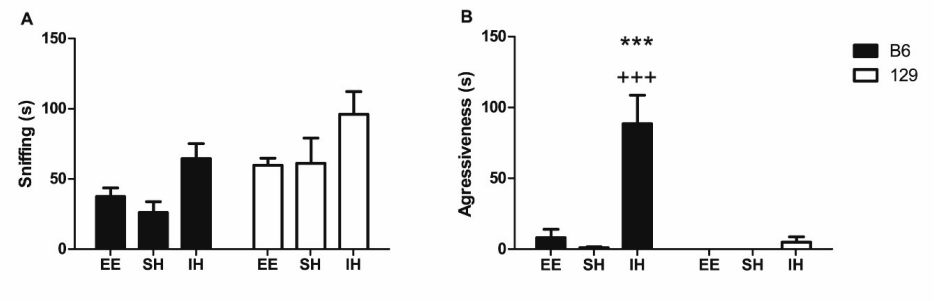

Figure 2. Social Interaction
(A) 129 mice sniff the partner significantly more than other groups. Individual housing increases sniffing in both strains. (B) Aggression is only displayed by individually housed B6 mice. *** p < 0.001 (Tukey HSD test after significant two-way ANOVA, compared to the respective 129 group); +++ p < 0.001 (compared to B6 mice kept in other conditions)

1.3. Locomotor activity in motility box (open field)

B6 mice proved to be significantly more mobile both horizontally and vertically (Figure 3). Strain effects for distance covered ($F_{(1, 86)}$=239.5, p<0.001), time spent rearing and observing ($F_{(1, 86)}$=480.4, p<0.0001), time in centre ($F_{(1, 86)}$=12.8, p<0.001) and visits to the corners ($F_{(1, 86)}$=125.6, p<0.001) were all highly significant showing higher averages for B6.

Environmental conditions affected the distance covered ($F_{(2, 86)}$=10.4, p<0.001), rearings ($F_{(2, 86)}$=8.8, p<0.001) and visits to the corners ($F_{(2, 86)}$=8.9, p<0.001). Throughout these parameters enrichment decreased the activity and isolation elevated it. In all three parameters difference between B6 IH and EE groups was confirmed by significant Tukey HSD post hoc test (p<0.05) but in 129Sv animals the effects of similar nature in distance travelled and visits to corners remain weaker and therefore not statistically significant.

Significant strain × housing interaction appeared in rearing behaviour ($F_{(2, 86)}$=6.4, p<0.01): environmental conditions affect B6 animals in a manner described above (IH increases, EE decreases) but do not change already very low rearing activity of 129Sv.

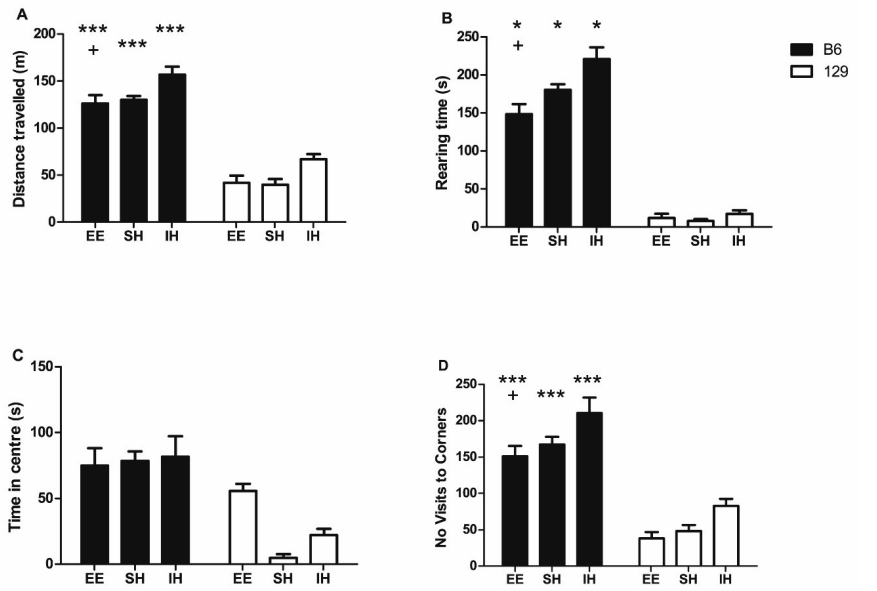

Figure 3. Motility box

(A) Distance covered in motility box. *** p < 0.001 (Tukey HSD test after significant two-way ANOVA; compared to the respective 129 group); + p < 0.05 (compared to B6 mice kept in individual housing). (B) Rearing time in seconds. * p < 0.05 (compared to the respective 129 group); + p < 0.05 (compared to B6 mice kept in individual housing). (C) Time spent in the centre of the motility box(s). (D) No of visits to corners. *** p < 0.001 (compared to the respective 129Sv group). + p < 0.05 (compared to B6 mice kept in individual housing).

1.4. The effect of amphetamine on locomotor activity in motility box (open field)

Amphetamine causes significant elevation in horizontal activity and decreases vertical activity(Figure 4). Amphetamine expectedly has strong stimulating effect on all groups (treatment effect on distance $F_{(1, 172)}$=145.7; p<0.01 and on time in the centre $F_{(1, 172)}$=8.5; p<0.01).

Individual housing stimulates mice similarly to Phenotyper (housing effect on distance on open field $F_{(2, 172)}$=5.7; p<0.01).

There were significant three-way interactions (strain × housing × treatment) in time spent rearing ($F_{(2, 172)}$=7.4; p<0.001) and on covered distance ($F_{(2, 172)}$=3.17; p<0.05). We discovered strain × treatment interaction in both parameters displayed in Figure 3 (distance travelled $F_{(1, 172)}$=10.9; p<0.01 and time in the centre $F_{(1, 172)}$=5.; p<0.05). The stimulating effect of amphetamine is significantly weaker in 129Sv EE mice compared to 129Sv animals from SH. There is no such effect in B6 strain. This likely demonstrates different reactivity of dopamine system of the studied strains.

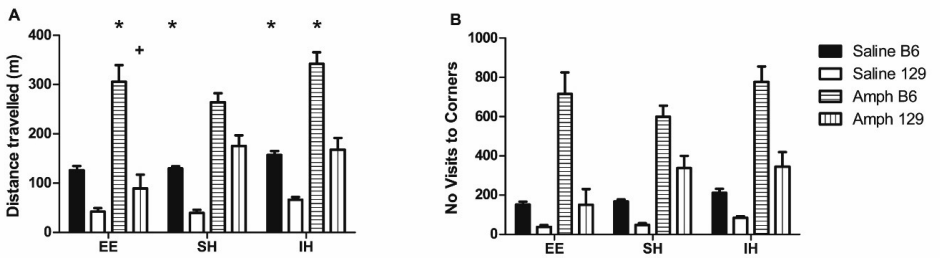

Figure 4. The effect of amphetamine on motility
(A) EE increases the stimulating effect of amphetamine in B6 but decreases it in the 129 strain. *p < 0.05 (Tukey HSD test after significant three-way ANOVA; compared to the respective 129 similarly treated group); + p < 0.05 (compared to amphetamine effect in the standard housing in 129 Sv mice). Note: Amphetamine effect in standard housing 129 vs. B6, p = 0.053. (B) Time spent in the centre of open field shows abnormal tendencies of enriched 129 mice.

1.5. Hot plate

We can see clear strain differences in both lick/shake ($F_{(1, 42)}$=13.7; p<0.001) and jump ($F_{(1, 42)}$=130.6; p<0.001) latencies between 129Sv and B6, latter scoring lower latencies for all groups and both parameters (Figure 5). Social isolation raised thresholds for lick/shake which was an expected result (housing effect $F_{(2,42)}$=5.7; p<0.01). Influence was stronger for 129Sv (average latency 43s compared to 22s in SH and 23s in EE) but there were no significant interactions. There were no housing effects regarding jump latency.

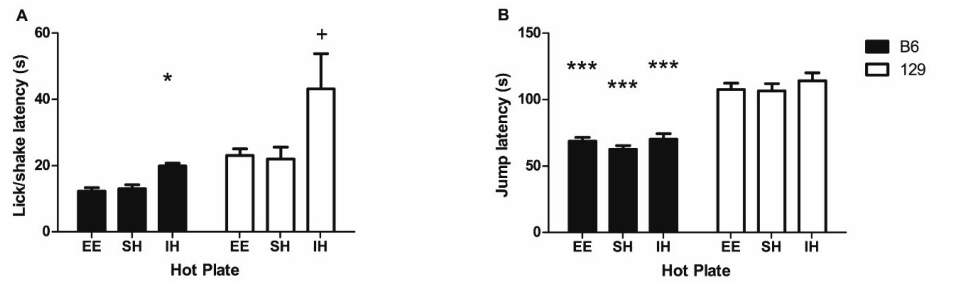

Figure 5. Hot Plate
A) Anxiety dependent licking/shaking response is delayed in animals from IH. * $p < 0.05$ (Tukey HSD test after significant two-way ANOVA; compared to the respective 129 group); + $p < 0.05$ (compared to the 129 mice kept in other conditions). (B) Escape response is not influenced by housing conditions in either strain. *** $p < 0.001$ (compared to the respective 129 group).

1.6. Bdnf expression

We can see significantly stronger *Bdnf* expression in frontal cortex of the B6 compared to 129Sv mice ($F_{(1,42)}$=9.1; $p<0.01$)(Figure 6).

In hippocampus there is an opposite strain effect ($F_{(1,42)}$=6; $p<0.05$) for 129Sv showing stronger expression and also a significant housing effect ($F_{(2,42)}$=9.5; $p<0.001$), EE showing higher expression than SH or IH.

There were no strain × housing interactions effects present in BDNF mRNA expression in either of the studied brain regions.

No significant correlations appeared between measured behavioural parameters and *Bdnf* expression in hippocampus or frontal cortex.

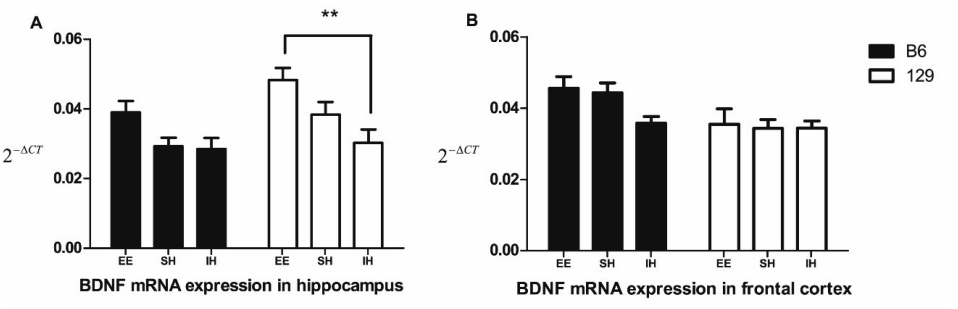

Figure 6. BDNF mRNA expression
(A) BDNF mRNA expression levels in hippocampus are elevated by EE. ** $p < 0.01$ (Tukey HSD test after significant two-way ANOVA, 129 EE compared to the 129 in IH). (B) BDNF mRNA expression levels in frontal cortex are higher in B6 strain.

1.7. Sample variation in different housing conditions

Since Coefficients of variation (CV; standard deviation [SD]/mean value) are independent from mean values, these were used to compare the variation between the housing conditions (same parameter was used by Tsai et al (Tsai et al 2002)). There was no significant difference between the 3 groups (Chi.Sqr.(N_{12}, df_2)=3.2; p=0.2; Coeff. of Concordance = 0.13; Av. rank r= 0.053)

Table 3. Sample variation in different housing conditions

Measured parameter	Variation in respective housing condition		
	SH	EE	IH
Highly mobile in Phenotyper	Medium 0.459	Higher 0.611	Lower 0.386
Distance in Phenotyper	Lower 0.371	Higher 0.502	Medium 0.393
Sniffing in Social Interaction	Higher 0.966	Lower 0.396	Medium 0.511
Lick latency in Hot Plate	Medium 0.492	Lower 0.406	Higher 0.762
Jump latency in Hot Plate	Higher 0.307	Lower 0.259	Medium 0.289
Distance in Motility w Amph	Medium 0.481	Higher 0.798	Lower 0.473
Distance in Motility w Saline	Medium 0.605	Higher 0.636	Lower 0.457
Rearings in Motility w Amph	Higher 1.632	Lower 1.382	Medium 1.574
Rearings in Motility w Saline	Higher 1.163	Medium 1.123	Lower 1.070
Time in centre w Amph	Lower 1.591	Higher 3.717	Medium 2.092
Time in centre w Saline	Medium 1.331	Higher 1.695	Lower 1.044

2. The effect of environment and genetic background on *Lsamp* gene expression (paper II)

2.1. The effect of environment and genetic background on *Lsamp* gene expression

In general, the expression levels of *Lsamp* transcripts in different brain areas were stable regardless of different rearing conditions. A remarkable environmental effect was the increase of *Lsamp* transcripts in mice raised in enriched environment. *Lsamp* 1b transcript level was significantly elevated in the hippocampal area of B6 mice (Figure 7A, B6 standard housing vs enriched environment $F_{(2, 20)}=4.47$; $p<0.05$). The trend of enrichment-induced elevation of both 1a and 1b transcripts exists in 129Sv and B6 background but is only statistically significant in the case of 1b promoter in B6 mice (Figure 7A, $F_{(2, 42)}=3.98$; $p<0.05$); in pooled backgrounds the effect is again significant in the case of 1b (*Lsamp* 1a $F_{(2, 45)}=0.83$, $p=0.44$; *Lsamp* 1b $F_{(2, 45)}=3.82$ $p<0.05$). There were no other enrichment- or isolation-induced effects on *Lsamp* expression.

We detected strain differences between B6 and 129Sv mice in *Lsamp* expression levels. *Lsamp* 1a transcript was higher in the hippocampal area of 129Sv mice (Figure 7A; $F(1, 46)=6.92$, $p<0.05$) while *Lsamp* 1b had higher expression levels in the frontal cortex (Figure 7B; $F_{(1, 46)}=4.92$ $p<0.05$) and thalamus (Figure 7E; $F_{(1, 46)}=10.05$ $p<0.01$) of B6 mice.

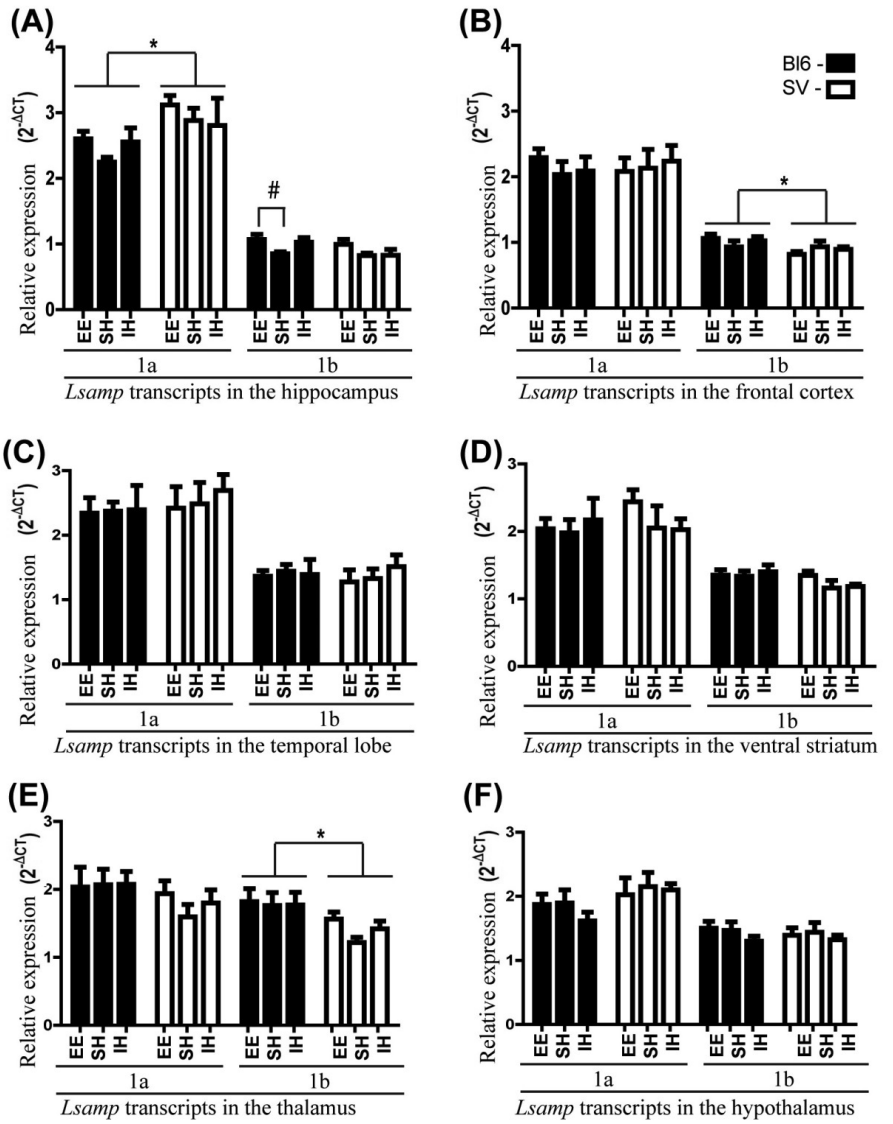

Figure 7. *Lsamp* 1a and 1b promoter mRNA expression in 6 different brain structures of mice raised in three different environmental conditions. The data has been presented separately for the mice with B6 and 129Sv background.
(A) In the hippocampus environmental enrichment elevates *Lsamp* expression; # $p < 0.05$ (Tukey HSD test after significant ANOVA). 1a expression is higher in 129Sv mice compared to B6; *$p < 0.05$ (Main effect of genotype in ANOVA). **(B)** In the frontal cortex 1b promoter is more prominent in B6 mice compared to 129Sv; *$p < 0.05$ (Main effect of genotype in ANOVA). **(C)** In the temporal lobe there were no significant differences in *Lsamp* promoter expression. **(D)** In the ventral striatum there were no significant differences in *Lsamp* promoter expression. **(E)** In the thalamus1b promoter is more prominent in B6 mice compared to 129Sv; *$p < 0.01$ (Main effect of genotype in ANOVA). **(F)** In the hypothalamus there were no significant differences in *Lsamp* promoter expression.

2.2. The quantitative analysis of *Lsamp* 1a and 1b transcript levels in six brain areas

1a promoter had significantly higher expression levels in all measured brain areas compared to 1b transcript [Figure 7 A-F; difference is significant in all tissues: p-values <0.001]. The relative expression levels of *Lsamp* 1a and 1b transcripts in six different brain areas were well in line with the data from our analysis about the anatomical distribution of alternative promoters of *Lsamp* gene (Figures 11–14). The expression level of *Lsamp* 1a promoter was highest in the hippocampus compared to other brain areas (Figure 7). In the mouse hippocampal formation 1a promoter was almost exclusively expressed in the pyramidal cell layer in CA1, CA2 and CA3 regions (Figure 8A) and in the granule cell layer (GL) of the dentate gyrus (DG, Figure 8D). There were a few 1a positive cells spread all over hilus. In the mouse hippocampal formation 1b promoter was sparsely expressed all over the structure (Figure 8B). In the DG, there were notably more concentrated 1b signals in the subgranular zone (SGZ) and 1b staining was nearly missing in the granule cell layer (Figure 8E). Summarized staining reveals strong expression of *Lsamp* in the pyramidal cell layer in CA1, CA2 and CA3 regions and evidently less intensive staining in the GL of the DG (Figure 8C), confirming that both promoters of the *Lsamp* gene are active in the pyramidal cells of the hippocampus but only 1a promoter is active in the granule cell layer in the gyrus dentatus in both B6 and 129Sv mice. The analysis of neurogenesis in the adult mouse DG showed a remarkable spatial overlap between the expressional activity of *Lsamp* 1b (X-Gal staining) transcript in BrdU positive proliferating cells. Namely, both stainings were prominent in the subgranular zone of DG, moreover many X-Gal positive cells also showed clearly BrdU staining (arrow in Figure 8F). Still, not all proliferating cells showed 1b promoter activity. In the primary culture analysis, all the cells that displayed X-Gal staining were morphologically clearly identified as neurons in both 10-day (Figure 8G) and 21-day (Figure 8H) hippocampal cultures and approximately 8–10% of all neurons were X-Gal positive.

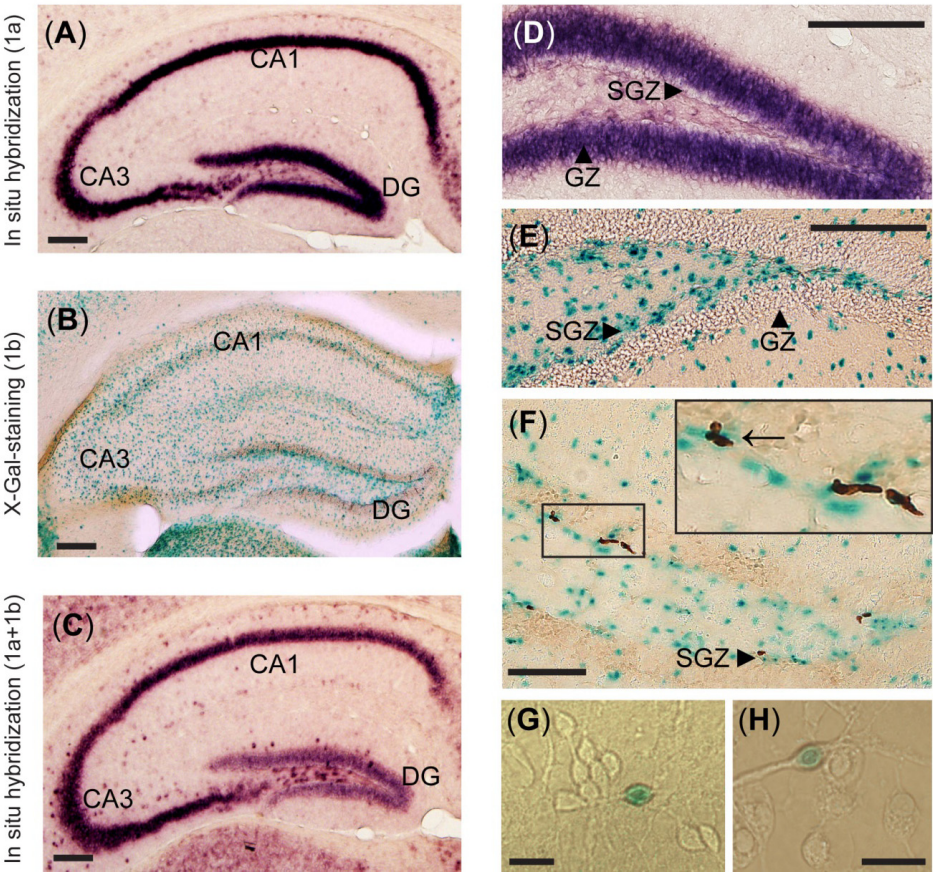

Figure 8. The anatomical distribution of alternative promoter activities of *Lsamp* gene. mRNA in situ hybridization indicates *Lsamp* 1a (A,D) and summarized (1a plus 1b; C) promoter activity, X-Gal staining indicates 1b activity (B,E,F) and BrdU incorporation indicates proliferation (F). Arrow on (F) points to X-Gal positive cells that show also BrdU staining. Arrowheads on (D), (E) and (F) point to the specific compartments of hippocampal formation. (G-H): X-Gal positive cells in 10-day old (G) and in 21-day old (H) hippocampal culture. Abbreviations: dentate gyrus (DG), granular zone (GZ), subgranular zone (SGZ). Scale bars represent 0.2 mm (A-F) and 30μm (G-H).

2.3. The effect of environment on *Bdnf* and synaptophysin gene expression

The expressional analysis of the well-studied biomarker *Bdnf* (*Brain-derived neurotrophic factor*) was used as a control for the efficacy of environmental manipulation. Levels of *Bdnf* gene were upregulated in the hippocampi of mice raised in enriched environment compared to mice raised in isolation or standard housing (Figure 9A) which is well in line with data from previous studies (Novkovic et al 2014). If the data from different housing conditions were pooled, the expression levels of both *Lsamp* transcripts correlated significantly with *Bdnf* expression levels in the hippocampus and frontal cortex. In the case of hippocampus, the correlations between *Lsamp* 1a and 1b transcripts and *Bdnf* transcript were significant in both genetic backgrounds (Figure 10): in 129Sv mice the correlation between *Bdnf* and *Lsamp* 1a levels was 0.61 ($p<0.05$) and between *Bdnf and* 1b levels 0.58 ($p<0.05$). In B6 strain, the correlation between *Bdnf* and *Lsamp* 1a levels was 0.62 ($p<0.05$) and between *Bdnf* and *Lsamp*1b levels 0.44 ($p<0.05$).

The previously reported enrichment-induced increase of *synaptophysin* (Nithianantharajah et al 2004) in the hippocampi of wild-type animals did not reach significance in our study (Figure 9B). It is possible that enrichment failed to induce *Syp* upregulation because of a late time-point of measurement. It has been shown previously that the exercise-induced upregulation of *Syp* starts to decline as soon as 15 days after the beginning of exercise (Ferreira et al 2013). It is likely that the same (acute upregulation only during the first weeks) is also true in environmental enrichment which lasted 8 weeks in the current study.

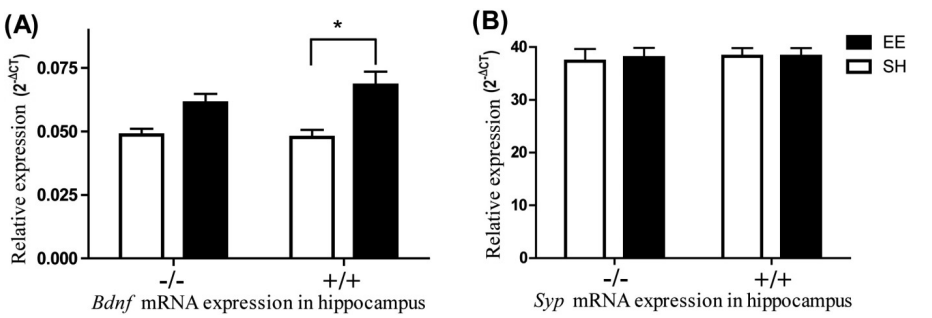

Figure 9. The influence of environmental enrichment on the *Bdnf* and Syp mRNA expression in the hippocampus of *Lsamp*-deficient mice and their wild-type littermates.

(A) Environmental enrichment increases *Bdnf* mRNA expression in wild-type mice but the same effect is diminished in the hippocampi of *Lsamp*-deficient mice. **(B)** *Syp* mRNA expression does not hange in response to environmental enrichment. *$p < 0.05$ (Main effect of environment in ANOVA).

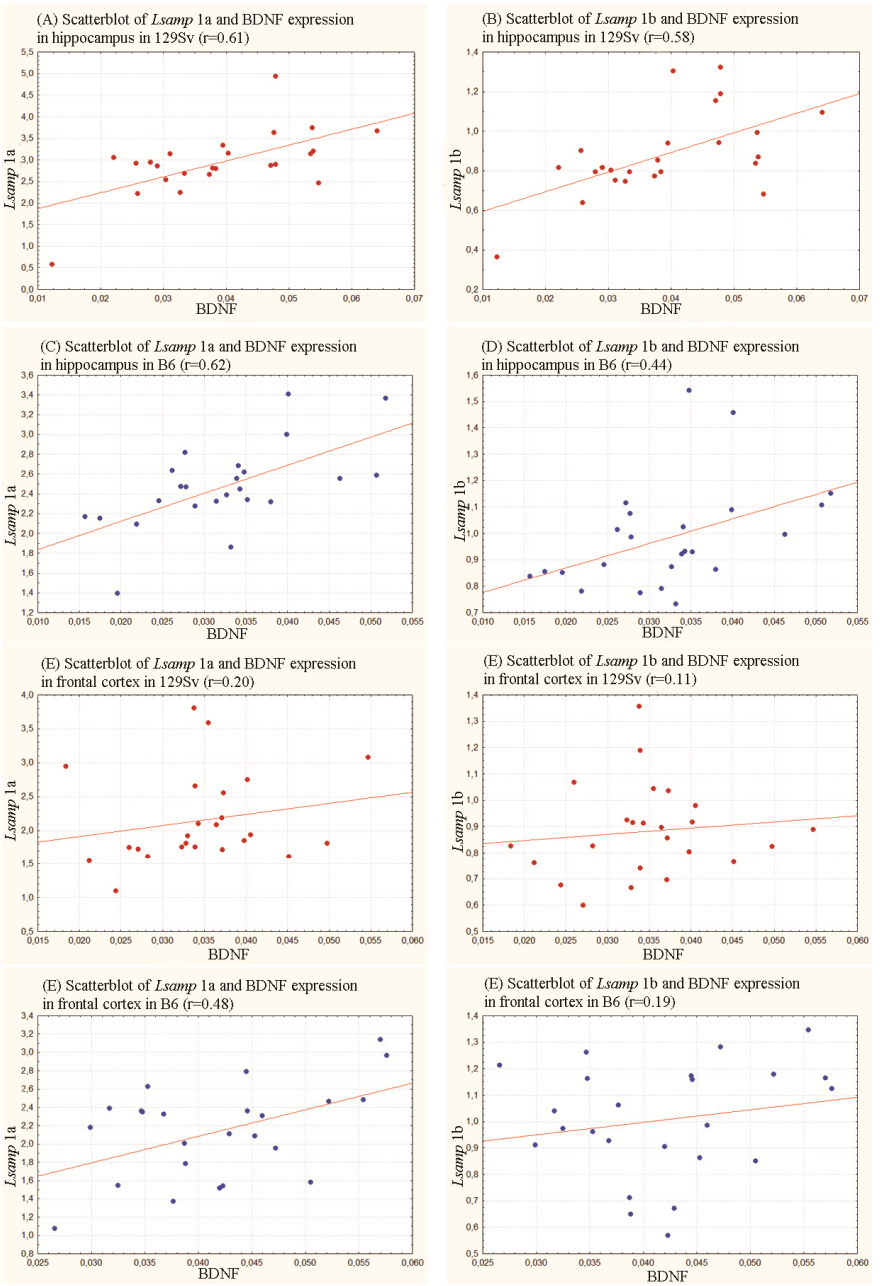

Figure 10. Scatterblots of *Bdnf* and *Lsamp* promoter expression in hippocampus and in frontal cortex.

In hippocampus in 129Sv mice the correlation between *Bdnf* and *Lsamp* 1a levels (A) was 0.61 ($p<0.05$) and between *Bdnf* and 1b levels (B) 0.58 ($p<0.05$). In B6 strain, the correlation between *Bdnf* and *Lsamp* 1a levels (C) was 0.62 ($p<0.05$) and between *Bdnf* and *Lsamp*1b levels (D) 0.44 ($p<0.05$). In frontal cortex in 129Sv mice the correlation between *Bdnf* and *Lsamp* 1a levels (E) was 0.20 and between *Bdnf* and 1b levels (F) 0.11. In B6 strain, the correlation between *Bdnf* and *Lsamp* 1a levels (G) was 0.48 ($p<0.05$) and between *Bdnf* and *Lsamp* 1b (H) levels 0.19.

3. The anatomical distribution of *Lsamp* 1a and 1b promoter activity (paper III)

3.1. *Lsamp* 1a promoter activity predominates in "classic" limbic structures

Limbic system-associated membrane protein 1a transcript is intensively and specifically expressed in the brain areas that are commonly considered to be limbic structures (Heimer & Van Hoesen 2006, Morgane et al 2005). Transcript 1a-specific staining is pronounced in the cingulate cortex (Cg, Figure 12B, F), insular cortex (Ins, Figure 12B, f; Figure 13E, F), prelimbic cortex (PrL, Figure 13E) and infralimbic cortex (IL, Figure 13E). Extensive 1a-specific staining can be seen in the hippocampal formation (CA1, CA3 and DG; Figures 10E–H, 11J), amygdalohippocampal area (AHi, Figure 11G, H), lateral amygdaloid nucleus (La, Figures 10E–G, 11J, 13C), basolateral (BL, Figure 11E–H and BLA, Figure 15C) and basomedial (BM, Figure 11E, F) amygdaloid nuclei, medial amygdaloid nucleus (Me, Figure 11F) and posterolateral (PLCo, Figure 11E–G) and posteromedial (PMCo, Figure 11G, H) cortical amygdaloid nuclei. Transcript 1b-specific X-Gal staining and in situ signal are much weaker in these areas. However, there is moderate 1b-specific staining in the central amygdaloid nucleus (Ce, Figures 10I, 11L, 13F) and cortical amygdaloid nuclei (PLCo/PMCo, Figure 11J–L). Expression of 1b isoform in the hippocampal formation is moderate and homogeneous (Figures 10I–L, 11K, L; Figure 13M). qRT-PCR results confirm the prevalent expression of 1a transcript in the hippocampal area and temporal lobe (Figure 15E).

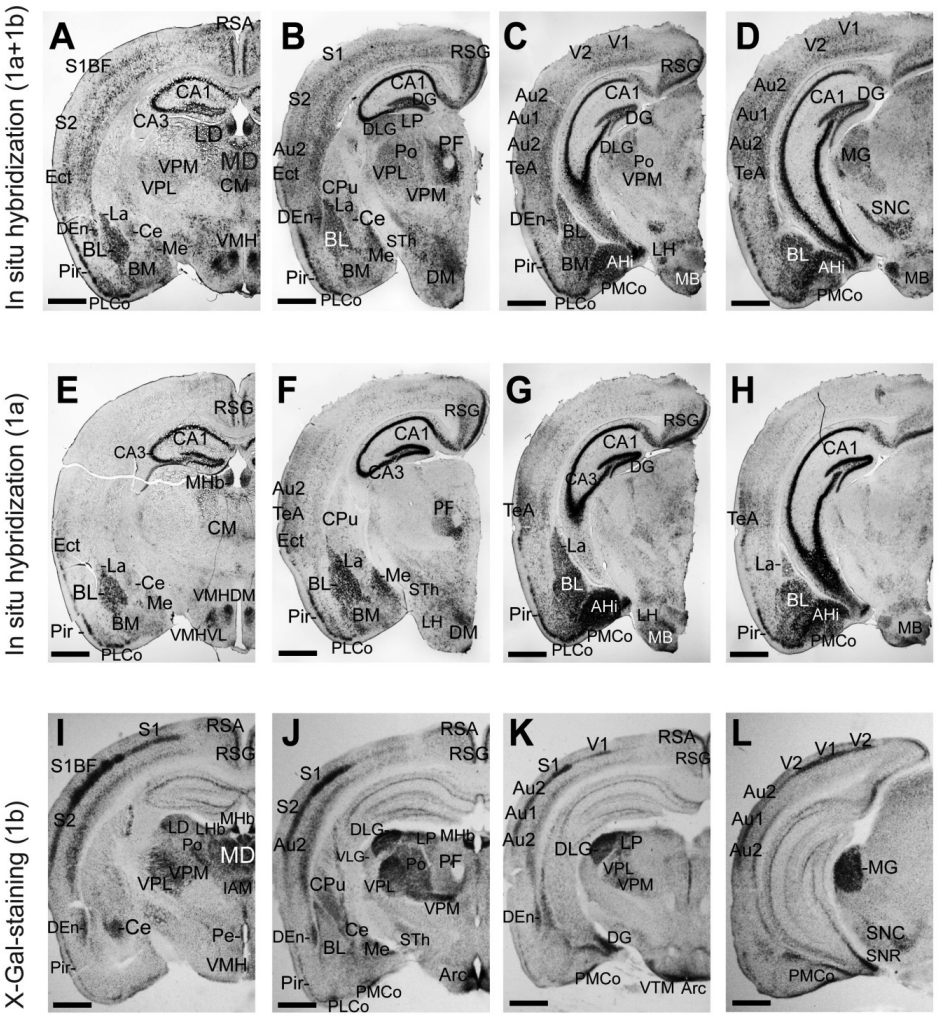

Figure 11. Non-radioactive *in situ* RNA hybridization analysis with digoxigenin-UTP representing universal *Lsamp* transcript (A–D) or *Lsamp* 1a transcript (E–H) and X-Gal staining expressing 1b promoter activity (I–L). Abbreviations: AHi (amygdalohippocampal area), Arc (arcuate hypothalamic nucleus), Au1/Au2 (primary /secondary auditory cortex), BL/BM (basolateral/ basomedial amygdaloid nucleus), CA1/CA3 (CA1/CA3 field of hippocampus), Ce (central nucleus of amygdala), CM (central medial thalamic nucleus), CPu (caudate putamen), DEn (dorsal endopiriform nucleus), DG (dentate gyrus), DLG/VLG (dorsal/ventral lateral geniculate nucleus), DM (dorsomedial hypothalamic nucleus), Ect (ectorhinal cortex), Ins (insular cortex), La (lateral amygdaloid nucleus), LHb/MHb (lateral/medial habenular nucleus), LD/MD (laterodorsal/ mediodorsal thalamic nucleus), LP (lateral posterior thalamic nucleus), LH (lateral hypothalamic area), MB (mammillary bodies), Me (medial amygdaloid nucleus), MG (medial geniculate nucleus), Pe (periventricular hypothalamic nucleus), Pir (piriform cortex), PF (parafascicular thalamic nucleus), PLCo/ PMCo (posterolateral/posteromedial cortical amygdaloid nucleus), Po (posterior thalamic nuclear group), RSA/RSG (retrosplenial agranular/granular cortex), S1/S2 (primary/ secondary somatosensory cortex), S1BF (S1, barrel field), SNC/SNR (substantia nigra, compact/ reticular part), STh (subthalamic nucleus), TeA (temporal association cortex), V1/V2 (primary/ secondary visual cortex), VMH, VMHDM/VMHVL (ventromedial thalamic nucleus, dorsomedial part/ ventrolateral part), VPM/VPL (ventral posteromedial/posterolateral thalamic nucleus), VTM (ventral tuberomammillary nucleus). Scale bar = 1 mm.

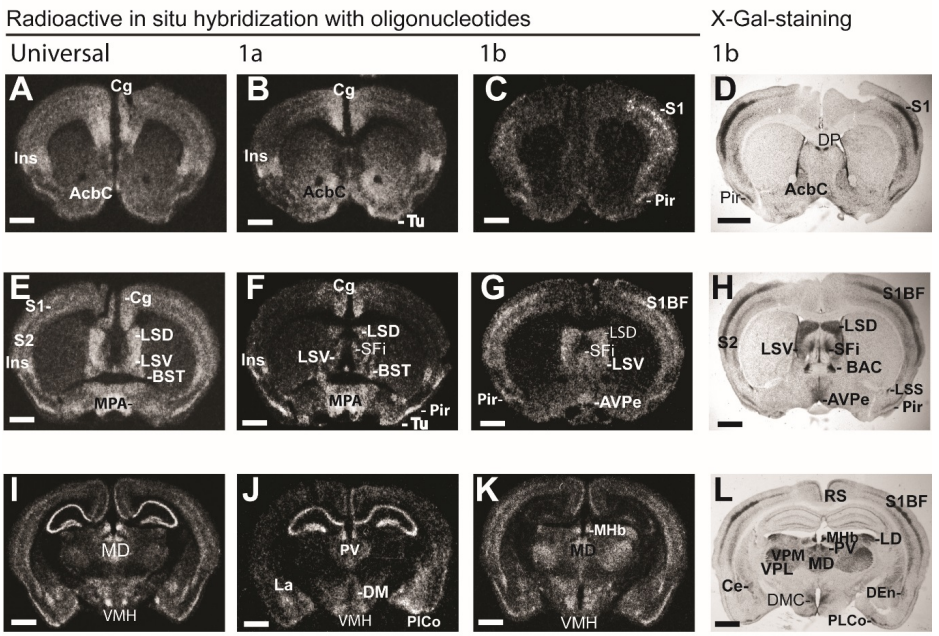

Figure 12. Radioactive in situ hybridization with oligonucleotides. Universal probe (A,E,I); 1a transcript specific probe (B,F,J) or 1b specific probe (C,G,K) has been used and complementary X-Gal staining in respective brain areas representing 1b-specific staining (D,H,L) has been shown. Abbreviations: AcbC (accumbens nucleus, core), AVPe (anteroventral periventricular hypothalamic nucleus), BAC (bed nucleus of the anterior commissure), BST (bed nucleus of stria terminalis), Ce (central nucleus of amygdala), Cg (cingulate cortex), DP (dorsal peduncular cortex), DM (dorsomedial hypothalamic nucleus), Ins (insular cortex), LSD/LSV (lateral septal nucleus, dorsal part/ventral part), LSS (lateral stripe of striatum), LD/MD (laterodorsal/mediodorsal thalamic nucleus), MPA (medial preoptic area), Pir (piriform cortex), PV (paraventricular thalamic nucleus), S1/S2 (primary/ secondary somatosensory cortex), S1BF (S1, barrel field), SFi (septofimbrial nucleus), Tu (olfactory tubercle), VPM/VPL (ventral postero-medial/posterolateral thalamic nucleus). Scale bar = 1 mm.

3.2. *Lsamp* 1b promoter activity is prevalent in the sensory nuclei and primary cortex areas

Many of the sensory systems are distinguished by 1b promoter-specific staining. In the major afferent pathways for somatosensory information, intense *Lsamp* 1b-specific staining is seen in the ventral posterior lateral thalamic nucleus (VPL; Figures 11I–K, 12K, L) and primary somatosensory cortex (S1, Figures 11J, K, 12C, D, G, K). The expression signal is the highest in the barrel field (S1b, f, Figures 11I, 12H, L). There is moderate 1b transcript-specific staining in the gracile, cuneate (Cu, Figure 13S) and spinal trigeminal (Sp5, Figure 13S)

nuclei and strong staining in the laterodorsal ((Bezdudnaya & Keller 2008)) thalamic nucleus (LD, Figure 11I). In the ascending auditory pathway there is strong 1b-specific staining in the dorsal and ventral cochlear nuclei (DC and VC, respectively, Figure 13O, P) and moderate 1bspecific staining in the superior olivary complex (SOC, Figure 13P) and trapezoid body (Tz, Figure 13P). Isoform 1b staining is strong in the nuclei of lateral lemniscus (LL, Figure 13N), in the inferior colliculus (IC, Figure 13O), in the medial geniculate nucleus (MG, Figure 11L) and also in the primary and secondary (Au1/Au2, Figure 11K, L) auditory cortex. In the visual pathway there is intensive 1b transcript-specific staining in the dorsal lateral geniculate thalamic nucleus (DLG, Figure 11J, K; Figure 13M) and primary visual cortex (V1, Figure 11L; Figure 13N). Strong 1b-specific X-Gal staining is also found in other brain areas receiving major projections from the retina: the superior colliculus (SC, Figure 13N) and suprachiasmatic nucleus (SCh, Figure 13I, J, L), and weak in the ventral lateral geniculate thalamic nucleus (VLG, Figure 11J; Figure 13M). In the sensory areas of the cortex, Lsamp 1b staining forms two distinct lines corresponding to layers 4 and 6 of the cortex (Lein et al 2007) as estimated by comparing X-Gal staining (Figure 13D) with NeuN immunoreactivity in the cortex (Figure 13C). Staining reflecting promoter 1a activity is weak in the sensory areas of the cortex (Figure 13A), and summarized expression of both isoforms reveals two moderate but distinct lines (Figure 13B). Both 1a and 1b promoters are expressed in brain areas involved in the processing of gustatory and olfactory information. In the gustatory system, 1b-specific staining is strong in the ventral posteromedial nucleus (VPM, Figure 11I– K) and weak in the solitary nucleus (Sol, Figure 13S). In the insular cortex only 1a isoform is expressed (Ins, Figure 14B, F; Figure 13E, F). In the olfactory system, the activity of 1b promoter is remarkable in the mediodorsal (Tham et al 2009) thalamic nucleus (MD, Figures 10I, 11K, L), and prevalent in the olfactory bulb (data not shown) and entorhinal cortex (Ent, Figure 13N). The expression of 1a promoter is distinct in the nucleus of the lateral olfactory tract (LOT, Figure 13K), and it dominates over 1b signal in the olfactory tubercle (Tu, Figure 12B) and piriform cortex (Pir, Figure 11E–H).

3.3. Differences in the activity of *Lsamp* 1a and 1b promoters in adult brain

Thalamic and hypothalamic nuclei are distinguished by the isoform-specific expression of *Lsamp*. Only two hypothalamic nuclei display activity for both 1a and 1b promoters: the paraventricular nucleus (Pa, Figure 13K, L) and mammillary bodies (MB, Figure 11C, G, K). High expression of 1a isoform is seen in the ventromedial hypothalamic nucleus, namely in the ventrolateral (VMHVL, Figure 11E) and dorsomedial parts (VMHDM, Figure 11E); weak 1b-specific staining is present in the anterior part of the VMH (Figure 12K). Promoter 1a is active in the dorsomedial hypothalamic nucleus (DM, Figure

11F), while the activity of 1b promoter is limited to the compact part of the DM (DMC, Figure 12L). Strong 1a promoter-specific expression can be seen in the medial preoptic area (MPA, Figure 12B), including medial preoptic nucleus, and also in the ventromedial preoptic nucleus (VMPO, Figure 13F). Promoter 1a-specific staining is moderate in the anterolateral (LA, Figure 13K) and lateral hypothalamus (LH, Figure 11F, G; Figure 13K, L). Strong 1b promoter-specific expression can be detected in the periventricular hypothalamic nucleus (Pe, Figure 11I; Figure 13L), anteroventral periventricular nucleus (AVPe, Figure 12H), suprachiasmatic nucleus (SCh, Figure 13I, J, L), supraoptic nucleus (SO, Figure 13L) and arcuate nucleus (Arc, Figure 11J, K).

Promoter 1a is specifically active in the anterior thalamus: the anteroventral thalamic nucleus (AV, Figure 13G), reticular thalamic nucleus (Rt, Figure 13K) and central medial thalamic nucleus (CM, Figure 11E). Weak 1a specific expression can be detected in the anteromedial thalamic nucleus (AM, Figure 13G). Isoform 1b-specific staining can be seen in the sensory thalamic nuclei as described above, but also in the posterior thalamic nuclei (Po, Figure 11I, J), lateral habenular nucleus (LHb, Figure 11I), lateral posterior thalamic nucleus (LP, Figure 11J, K), paratenial thalamic nucleus (PT, Figure 13H) and reuniens thalamic nucleus (Re, Figure 13L). There are numerous thalamic nuclei where both 1a and 1b promoters are active: the anterodorsal thalamic nucleus (AD, Figure 13G, H), paraventricular thalamic nucleus (PV, Figure 13K, L), paracentral thalamic nucleus (PC, Figure 13G, H), parafascicular thalamic nucleus (PF, Figure 11B, F, J), medial habenular nucleus (Mhb, Figure 11A, E, I) and subthalamic nucleus (STh, Figure 11B, F, J). Alternative expression of Lsamp 1a and 1b promoters can be seen throughout the brain. Both 1a and 1b promoters are active in the dorsal (LSD, Figure 12E–H) and ventral (LSV, Figure 12E–H) part of the lateral septal nucleus, septofimbrial nucleus (SFi, Figure 12F–H), subfornical organ (SFO, Figure 13G, H) and retrosplenial granular cortex (RSG, Figure 11E–G, I–K), whereas only 1b promoter is active in the retrosplenial agranular cortex (RSA, Figure 11I–K). On the level of anterior commissure, 1b promoter is active in the bed nucleus of anterior commissure (BAC, Figure 12H); 1a transcript is prevalent in the bed nuclei of stria terminalis (BST, Figure 12F, Figure 13F) and in the core of the nucleus accumbens (AcbC, Figure 12B). Isoform 1b-specific staining is present in the dorsal peduncular cortex (DP, Figure 12D), caudate putamen (CPu, Figure 11J), dorsal endopiriform nucleus (Den, Figure 11I– K), claustrum (Cl, Figure 12H) and lateral stripe of striatum (LSS, Figure 12H). The expression of the Lsamp transcript in the cerebellum is mostly initiated from promoter 1b, which is abundant in the Purkinje cell layer (Pc, Figure 13R). There is moderate 1b expression in the molecular layer (Mc, Figure 13R) and weak 1a expression in the granule cells (Gc, Figure 13Q). Limbic system-associated membrane protein expression is moderate in the spinal cord: both 1a (Figure 13T) and 1b (Figure 13U) isoforms are expressed in the ventral and dorsal horns. Overview of the anatomical findings is presented in the table 4.

Table 4. Distribution of *Lsamp* 1a, 1b and combined transcripts in the mouse brain. (1a+1b) summarized distribution refers to the staining with universal *Lsamp* probes that do not distinct different isoforms. The table represents distribution data from all the alternative staining methods that were used in the current study. The intensity of the signal has been estimated on a scale: 0 – baseline; 1 – weak, 2 – modest, 3 – strong, 4 – very strong. The brain areas are categorized as sensory (auditory, visual, somatosensory, gustatory and olfactory) or "limbic". The brain areas were categorized as "limbic" according to (Heimer & Van Hoesen 2006) and (Morgane et al 2005).

	Area	**Abbr**	**1a**	**1b**	**1a+ 1b**	
FOREBRAIN	Olfactory bulb	OB	1	1	1	Olfactory
	Cerebral cortex					
	Orbital cortex	LO, VO	2	0	1	Limbic
	Prelimbic/ Infralimbic cortex	PrL/IL	3	0	3	Limbic
	Prelimbic/ Infralimbic cortex	PrL/IL	3	0	3	Limbic
	Dorsal peduncular cortex	DP	0	3	2	
	Cingulate cortex	Cg	4	0	2	Limbic
	Retrosplenial agranular cortex	RSA	0	2	2	
	Retrosplenial granular cortex	RSG	3	1	2	
	Granular insular cortex	G Ins	3	1	2	Gustatory/ Limbic
	Agranular insular cortex	A Ins	3	0	2	Gustatory/ Limbic
	Ectorhinal cortex	Ect	1	0	1	
	Entorhinal cortex	Ent	0	1	1	Limbic
	Claustrum	Cl	0	1	1	
	Dorsal endopiriform nucleus	DEn	0	2	2	
	Piriform cortex	Pir	3	2	3	Olfactory
	Temporal association cortex	TeA	2	1	2	
	Parietal association cortex	PtA	1	1	1	
	Primary auditory cortex	Au1	1	2	2	Auditory
	Secondary auditory cortex	Au2	1	2	2	Auditory
	Primary visual cortex	V1	0	3	2	Visual
	Secondary visual cortex	V2	0	1	1	Visual

	Area	Abbr	1a	1b	1a+ 1b	
	Primary somatosensory cortex	S1	0	4	2	Somatosensory
	Secondary somatosensory cortex	S2	1	3	2	Somatosensory
	Primary motor cortex	M1	1	1	1	Motor
	Secondary motor cortex	M2	1	0	1	Motor
	Septal and basal forebrain regions					
	Medial septal nucleus	MS	1	0	1	
	Lateral septal nucleus, dorsal part	LSD	2	3	3	
	Lateral septal nucleus, ventral part	LSV	2	2	2	
	Lateral septal nucleus, intermediate part	LSI	0	1	1	
	Septofimbrial nucleus	SFi	2	3	2	
	Subformical organ	SFO	1	2	2	
	Bed nucleus of the anterior commissure	BAC	0	4	2	
	Lateral bed nucleus of stria terminalis	BSTL	1	1	2	Limbic
	Medial bed nucleus of stria terminalis	BSTM	3	1	3	Limbic
	Basal ganglia and striatum					
	Olfactory tubercle	Tu	2	0	1	Olfactory
	Nucleus of the lateral olfactory tract	LOT	3	0	2	Olfactory
	Nucleus accumbens core	AcbC	3	1	1	Limbic
	Nucleus accumbens shell	AcbSh	3	1	1	Limbic
	Caudate Putamen	CPu	0	1	1	Motor
	Globus pallidus	GP	0	1	1	Motor
	Lateral stripe of striatum	LSS	0	3	1	
	Hippocampal formation					
	CA1	CA1	4	1	4	Limbic
	CA2	CA2	4	1	4	Limbic
	CA3	CA3	4	1	4	Limbic
	Dentate gyrus	DG	4	1	3	Limbic
	Subiculum	S	2	0	2	Limbic
	Amygdala					

	Area	Abbr	1a	1b	1a+ 1b	
	Amygdalohippocampal area	AHi	4	0	3	Limbic
	Intercalated nuclei of the amygdala	I	0	1	0	
	Central amygdaloid nucleus	Ce	1	3	2	Limbic
	Lateral amygdaloid nucleus	La	3	1	3	Limbic
	Basolateral amygdaloid nucleus	BL	3	0	3	Limbic
	Basomedial amygdaloid nucleus	BM	3	0	3	Limbic
	Medial amygdaloid nucleus	MeA	3	1	3	Limbic
	Medial amygdaloid nucleus, posterodorsal	MePD	2	1	3	Limbic
	Medial amygdaloid nucleus, posteroventral	MePV	4	0	3	Limbic
	Posterolateral cortical amygdaloid nucleus	PLCo	3	1	3	Limbic
	Posteromedial cortical amygdaloid nucleus	PMCo	4	1	4	Limbic
DIENCEPHALON	*Hypothalamus*					
	Medial preoptic nucleus	MPN	4	0	3	Limbic
	Medial preoptic area	MPA	2	0	2	Limbic
	Lateral preoptic area	LPO	2	0	2	Limbic
	Periventricular hypothalamic nucleus	Pe	0	4	3	
	Anteroventral periventricular nucleus	AVPe	0	4	3	
	Lateral hypothalamus	LH	2	0	2	
	Lateroanterior hypothalamus	LA	2	0	2	Limbic
	Supraoptic nucleus	SO	0	2	2	
	Suprachiamatic nucleus	SCh	0	4	2	
	Paraventricular nucleus	Pa	3	4	4	Limbic
	Medial Tuberal nucleus	Mtu	3	0	3	
	Dorsomedial hypothalamus, compact	DMC	3	2	3	
	Dorsomedial hypothalamus	DM	3	0	3	
	Ventromedial hypothalamic nucleus, anterior part	VMH	0	2	2	

	Area	Abbr	1a	1b	1a+ 1b	
	Ventromedial hypothalamic nucleus, ventrolateral part	VMHDM	3	0	3	
	Ventromedial hypothalamic nucleus, dorsomedial part	VMHVL	3	0	3	
	Arcuate nucleus	Arc	0	4	3	
	Mammilary bodies	MB	4	4	4	Limbic
	Ventral tuberomammillary nucleus	VTM	2	4	4	Limbic
	Ventromedial preoptic nucleus	VMPO	3	0	3	
	Thalamus					
	Anterodorsal thal. n.	AD	3	4	4	Limbic
	Anteroventral thal. n.	AV	2	0	2	Limbic
	Anteromedial thalamic nucleus	AM	1	0	1	Limbic
	Paraventricular thal. n.	PV	2	3	3	
	Ventrolateral thal. n.	VL	0	1	0	Motor
	Ventromedial thalamic nucleus	VM	0	0	0	Motor
	Ventral posterolateral nucleus	VPL	0	3	1	Somatosensory
	Ventral posteromedial nucleus	VPM	0	3	2	Gustatory
	Paratenial thal. n.	PT	1	4	3	
	Mediodorsal thal. n.	MD	0	3	2	Olfactory
	Laterodorsal medial nucleus	LD	0	2	1	Somatosensory
	Central medial thal. n.	CM	2	0	1	
	Paracentral nucleus	PC	1	2	1	
	Parafascicular thalamic nucleus	PF	3	2	3	
	Reuniens thalamic nucleus	Re	0	1	1	
	Reticular thal. n.	Rt	2	0	2	
	Xiphoid thalamic nucleus	Xi	1	2	2	
	Interanteromedial thalamic nucleus	IAM	0	2	1	
	Medial habenular n.	Mhb	4	4	4	
	Lateral habenular n.	LHb	0	2	2	

	Area	Abbr	1a	1b	1a+ 1b	
	Posterior thalamic nuclear group	Po	0	2	1	
	Lateral posterior thalamic nucleus	LP	0	2	1	
	Dorsal lat. Geniculate n.	DLG	0	4	1	Visual
	Ventral lat. Geniculate n.	VLG	0	1	0	Visual
	Medial geniculate n.	MG	0	3	2	Auditory
	Subthalamic nucleus	STh	1	2	3	
	Zona Incerta	ZI	0	0	0	
MIDBRAIN	Superior colliculus	SC	0	2	1	Visual
	Inferior colliculus, central nucleus	IC	0	3	1	Auditory
	Substantia nigra, pars reticulata	SNR	1	2	2	
	Substantia nigra, pars compacta	SNC	1	2	2	
	Periaqueductal gray	PAG	1	1	2	
BRAINSTEM	Nuclei of lateral lemniscus	LL	0	3	2	Auditory
	Solitary nucleus	Sol	0	1	1	Gustatory
	Superior olivary complex	SOC	0	1	1	Auditory
	Nucleus of the trapezoid body	Tz	0	2	1	Auditory
	Dorsal cochlear nucleus	DC	0	3	2	Auditory
	Ventral cochlear nucleus	VC	0	3	2	Auditory
	Cuneate nucleus	Cu	0	2	1	Somatosensory
	Gracile nucleus	Gr	0	2	1	Somatosensory
	Spinal trigeminal nucleus	Sp5	0	2	1	Somatosensory
CEREBELLUM	Molecular layer	Mc	0	1	1	
	Granule cell layer	Gc	1	0	1	
	Purkinje cell layer	Pc	0	3	2	
SPINAL CORD	Dorsal horn	DH	2	2	2	
	Ventral horn	VH	2	2	2	

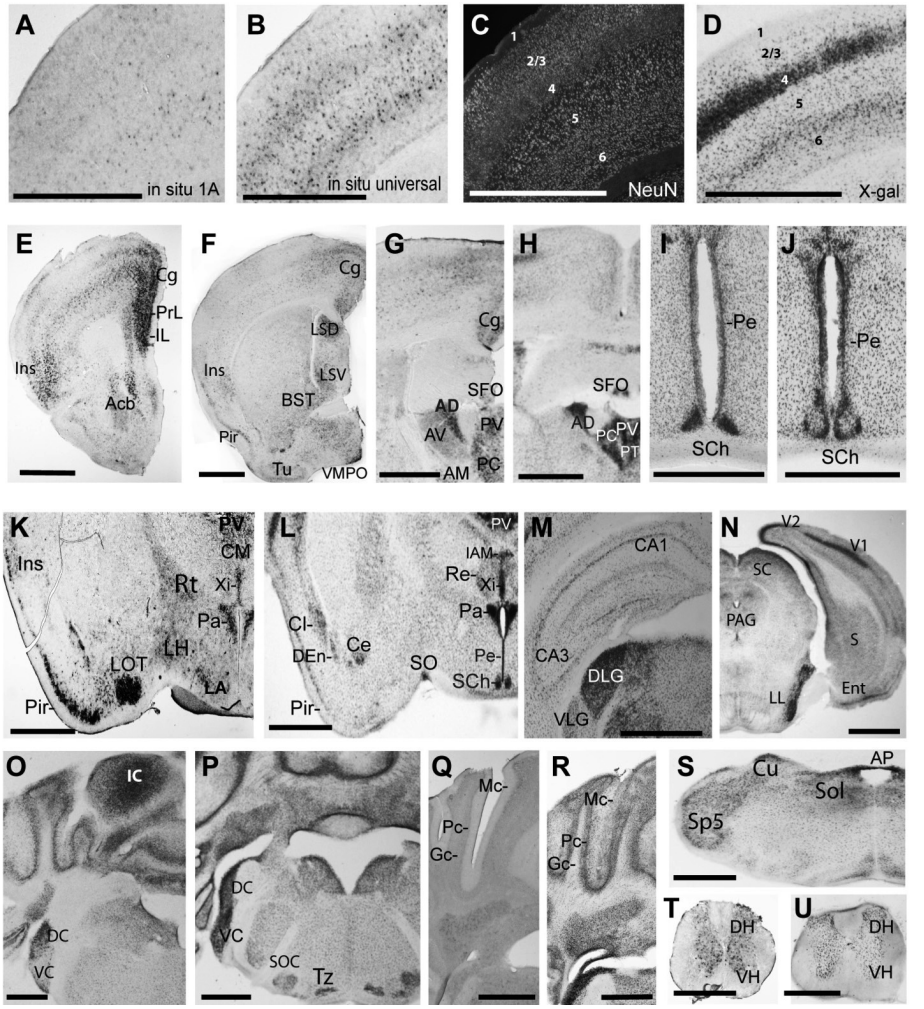

Figure 13. Non-radioactive *in situ* RNA hybridization analysis representing the distribution of *Lsamp* 1a transcript (**A**,E-G,K,Q,T) and universal *Lsamp* transcript (**B**) and X-Gal staining representing 1b promoter activity (D,H-J,L-P,R,A,U) and NeuN immunostaining (**C**). Abbreviations: Acb (accumbens nucleus), AD/AM/AV (anterodorsal/ anteromedial/ anteroventral thalamic nucleus), BST (bed nucleus of stria terminalis), CA1/CA3 (CA1/CA3 field of hippocampus), Ce (central nucleus of amygdala), CM (central medial thalamic nucleus), CPu (caudate putamen), DEn (dorsal endopiriform nucleus), DLG/VLG (dorsal/ventral lateral geniculate nucleus), Cg (cingulate cortex), Cl (claustrum), Cu (cuneate nucleus), DC/VC (dorsal/ventral cochlear nucleus), DH/VH (dorsal/ventral horn of the spinal cord), Gc/Mc/Pc (granule/molecular/ Purkinje cell layer of the cerebellum), IAM (interanteromedial thalamic nucleus) IC/SC (inferior/superior colliculus), IL (infralimbic cortex), Ins (insular cortex), LA/LH (lateroanterior/lateral hypothalamus), LSD/LSV (lateral septal nucleus, dorsal part/ventral part), LL (nuclei of lateral lemniscus), LOT (nucleus of the lateral olfactory dract), Pa/Pe (paraventricular/ periventricular hypothalamic nucleus), PV (paraventricular thalamic nucleus), PAG (periaqueductal gray), Pir (piriform cortex), PrL (prelimbic cortex), PC (paracentral thalamic nucleus), PT (paratenial thalamic nucleus), Rt (reticular thalamic nucleus), Re (reuniens thalamic nucleus), SFO (subfornical organ), SCh (suprachiasmatic nucleus), Sol (solitary nucleus), Sp5 (spinal trigeminal nucleus), S (subiculum), SO (supraoptic nucleus), SOC (superior olivary complex), Tz (trapezoid body), Tu (olfactory tubercle), VMPO (ventromedial preoptic nucleus), Xiphoid thalamic nucleus (Xi). Scale bar = 1 mm.

3.4. Differences in the activity of *Lsamp* 1a and 1b promoters in embryonic brain

We detected the first signals for both promoters of the *Lsamp* gene at around E12.5. Limbic system-associated membrane protein 1a transcript is first activated in the midbrain, being prominent in the outer layers of the neural tube (Figure 14A, B), in the forebrain the first signs were detected at around E13.5 also in the outer surface of the neuroepithelium. The first signs of 1b transcript expression were detected in the lateral side of the lateral ventricle (Figure 14D, E). During later embryonic development (E15.5), strong signal is detectable also in the lining of the aqueduct and in the deepest layers of the sensory region of the neocortex (S1, Figure 14F). At E15.5, *Lsamp* 1a promoter is especially active in the caudate putamen (CPu, Figure 14C), whereas this activity shades off during the first postnatal week (data not shown) and is not detectable in adult brain (CPu, Figure 11F). The expression of *Lsamp* 1b promoter in the CPu is weak during development [as shown in E15,5; CPu (Figure 14F) and moderate in adulthood (CPu, Figure 11J)].

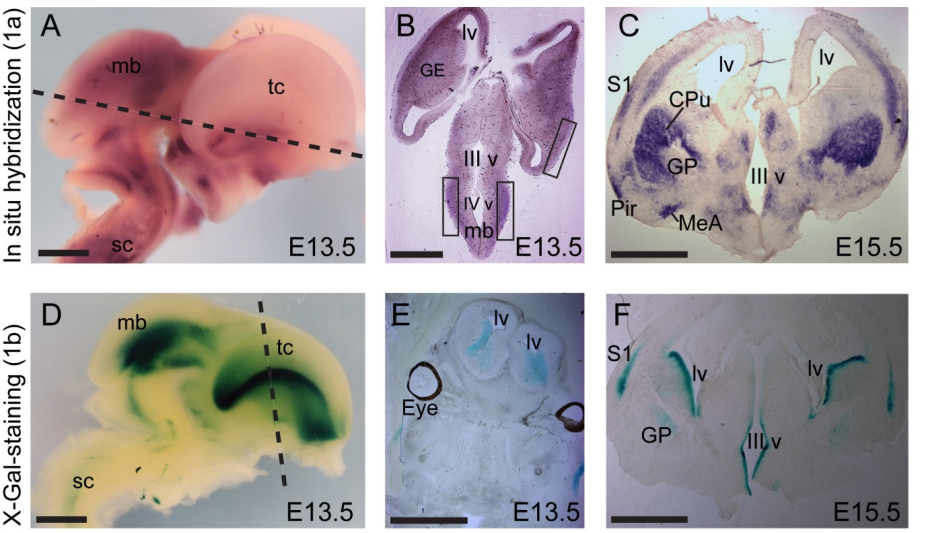

Figure 14. Distribution of *Lsamp* 1a and 1b transcripts during development. Non-radioactive in situ RNA hybridization analysis representing *Lsamp* 1a transcript (A–C) and X-Gal staining expressing 1b promoter activity (D–F) in the embryonic mouse brain. Abbreviations: CPu (caudate putamen), GE (ganglionic eminence) GP (globus pallidus), lv (lateral ventricle), mb (midbrain), Pir (piriform cortex), S1 (primary somatosensory cortex), sc (spinal cord), tc (telencephalon), III v (third ventricle), IV v (fourth ventricle). Dashed lines in a and d represent approximate cross-sections for b and e, respectively. Scale bar = 1 mm.

3.5. Expression of *Lsamp* transcripts correlates with behavioural measures of trait anxiety and social interaction

We looked for the correlations between various behavioural parameters and the activity of *Lsamp* transcript expression in three brain areas (ventral striatum, hippocampus and temporal lobe). Most of the significant correlations emerged between *Lsamp* expression in the temporal cortex and behavioural parameters in the elevated plus maze (Table 5). Both *Lsamp* 1a and 1b transcript levels were negatively correlated with time on open arms in the elevated plus maze (Figure 15a). Furthermore, *Lsamp* 1a transcript levels were negatively correlated with unprotected head-dips in the elevated plus maze (Figure 15B), with the number of open arm entries and with the ratio between open and closed arm entries. There was also a significant positive correlation between *Lsamp* 1a transcript levels and the latency to enter open arm. Additionally, *Lsamp* transcript levels in the hippocampus and ventral striatum correlated with behavioural parameters in the social interaction test. Both *Lsamp* 1a and 1b transcript levels were negatively correlated with the time that the mice spent sniffing the other animal. The time of anogenital sniffing positively correlated with *Lsamp* 1a activity in the ventral striatum. In the experiment for acute fear response, all the mice in the ‘‘Conditioned fear’’ group displayed an obvious fear reaction as evidenced by startling response and freezing (data not shown). The ‘‘Conditioned fear’’ group had significantly higher c-Fos expression in the amygdala [$F(2,13) = 11.6$, $p<0.01$] and hippocampus [$F(2,13) = 8.8$, $p<0.01$] than the ‘‘Naive’’ or ‘‘Pre-conditioning’’ groups (Figure 15d). Furthermore, in the ‘‘Conditioned fear’’ group, the c-Fos activation in the temporal lobe was significantly higher than in the hippocampus ($p<0.05$), indicating specific activation of the amygdaloid area in response to conditioned fear. There were no statistically significant changes in the expression levels of *Lsamp* 1a or 1b transcripts at the 45 min timepoint (Figure 15E).

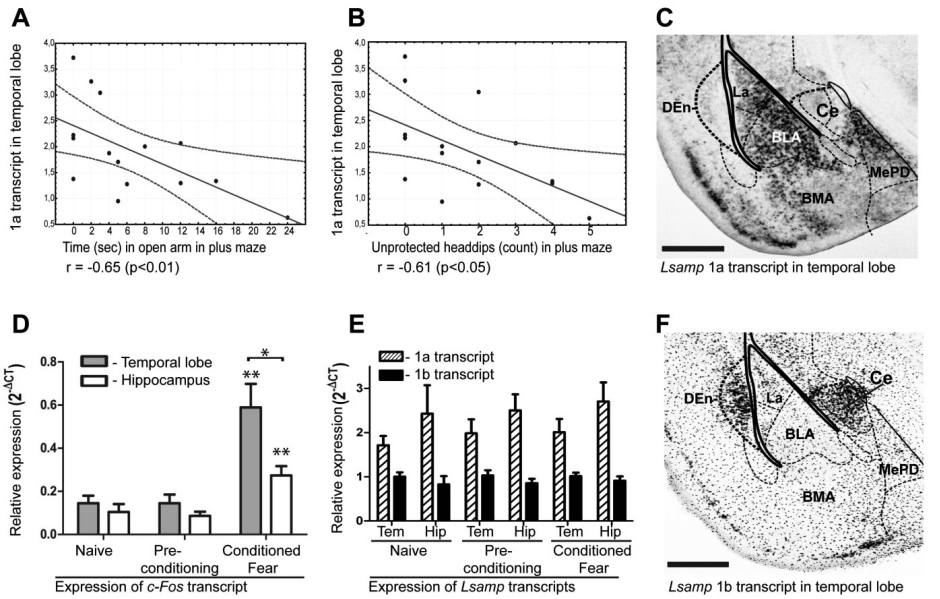

Figure 15. *Lsamp* expression in temporal lobe correlates with measures of trait anxiety (A–B), but was not altered 45 min after acute conditioned fear experience (E) that significantly raised c-Fos transcript in the temporal lobe and hippocampal area (D). Distribution of *Lsamp* 1a (C) and 1b (F) in the temporal lobe. Dashed lines indicate 95% confidence limits (A–B). The whiskers represent SEM; **p<0.01; *p<0.05 (d-e). Abbreviations: BLA (basolateral amygdaloid nucleus, anterior part), BMA (basomedial amygdaloid nucleus, anterior part), Ce (central amygdaloid nucleus), Den (dorsal endopiriform nucleus), Hip (hippocampus), La (lateral amygdaloid nucleus), MePD (medial amygdaloid nucleus, posterodorsal part), Tem (temporal lobe). Scale bar = 500μm (c and f).

Table 5. Correlation analysis between relative expression levels ($2^{-\Delta CT}$) of *Lsamp* 1a and 1b transcript in 3 brain areas and behavioural parameters in the motility box, elevated plus maze and social interaction test. The behavioural measures have been presented in either counts, seconds (s) or meters (m). **p<0.01; *p<0.05 (Spearman's rank-order correlation).

	Hippocampus		Temporal lobe		Ventral striatum	
	1A	1B	1A	1B	1A	1B
Motility box						
Move, s	–0.01	–0.06	0.11	0.06	0.12	–0.34
Distance, m	–0.06	–0.16	0.19	0.08	0.05	–0.30
Time center, s	–0.40	–0.36	0.43	0.36	0.05	0.03
Time corner, s	0.21	0.33	–0.38	–0.10	–0.13	0.17
Elevated plus maze						
Closed arm entries	–0.20	–0.43	0.17	–0.02	0.08	–0.14
Open arm entries	0.16	0.16	–0.57*	–0.40	0.01	–0.34
Ratio open/closed arm entries	0.08	0.13	–0.63*	–0.38	–0.11	–0.34
Latency, s	0.03	–0.01	0.53*	0.22	–0.15	0.45
Time on open arms, s	0.24	0.15	–0.65**	–0.66**	0.14	–0.21
Protected headdips	0.17	0.08	–0.06	–0.06	–0.12	–0.26
Unprotected headdips	0.10	0.12	–0.61*	–0.50	0.20	–0.40
SAPs	–0.05	0.10	0.24	0.23	0.11	0.17
Social interaction test						
Anogenital sniffing, s	–0.10	–0.22	–0.09	–0.26	0.54*	0.11
Sniffing other body parts, s	–0.67**	–0.65*	0.06	0.15	–0.19	–0.07
Time of social sniffing, s	–0.51	–0.60*	–0.01	–0.09	0.14	–0.06

DISCUSSION

Both genetic background and environment are involved in the formation of characteristics that are coded by specific genes. *Limbic system associated membrane protein* (*Lsamp*) gene modulates behavioural adaptation in social or anxiogenic environments.

B6 and 129Sv are common inbred strains used in behavioural neuroscience and are of special interest to transgenic researchers. Despite their comparatively similar genetic background (Morris et al 2010) these strains display rather different behavioural phenotypes. With results presented in this dissertation I have demonstrated that EE is likely more beneficial for B6 male mice compared to more anxious and inhibited 129Sv.

1. Differences in 129Sv and B6 behavioural phenotype

Both strains analysed in this paper consistently show vast differences in their phenotypes both in previous studies and the ones performed by me and my colleagues. Our design allowed us to investigate which behaviours are subject to environmental influences and which are more stable (likely genetically determined). B6 is commonly known as the more agile, mobile and venturous strain. Various tests in different laboratories have shown higher spontaneous locomotor activity in the B6 strain, which is displayed in longer distances covered and significantly higher number of rearings compared to 129Sv mice (Figures: 1–3 and (Contet et al 2001, Voikar et al 2001, Voikar et al 2004)). Seeing the same phenomenon in different laboratories implies that this characteristic is innate to the strain and thus genetically determined.

Lower sensitivity and higher tolerance for pain repeatedly displayed by 129Sv mice (Figure 5; (Abramov et al 2008, Voikar et al 2004)) is also a clear indication of differences in pathways and mechanisms shaped by genes rather than environment.

2. Individual housing stimulates rodents

Individual housing is often believed to be stressful for men and mice, however, adult male mice do not necessarily prefer company of other males to solitude (Hunt & Hambly 2006). Individual housing often promotes curiosity and exploratory behaviour. To make rodents kept in standard conditions more venturous in anxiety and locomotion tests, short-time isolation is often used in many models. Indeed, our results confirmed that individually housed animals do have slightly increased locomotion and slightly heightened threshold for pain.

The most salient result regarding IH appeared in social interaction test. B6 is generally described as more aggressive of the two strains. Social isolation is

known to increase activity and induce aggression (Toth et al 2012). Our results, which showed that only group significantly aggressive in these settings was that of individually housed B6 mice, are well in line with that knowledge. The difference in social behaviour after having been reared in an isolation has also been displayed by (Heitzer et al 2012) in Fmr1 knockout mice.

In the hot-plate test we could see that animals from both strains that had been reared in IH displayed an increase in the lick/shake latency which is an anxiety-dependent primary noxious-evoked behaviour, whereas environmental conditions did not change the jump latency, which is an escaping behaviour (or a straightforward pain response) (Casarrubea et al 2012, Veraksits et al 2003).

3. Environmental enrichment reinforces the existing differences between strains

Environmental enrichment is increasingly viewed as a significant component of refinement in animal studies. We found some evidence supporting the popular argument of increased behavioural variation in EE animals, which is often used as an argument against EE in rodent facilities (Table 3), but the differences were not significant. Therefore, in addition to enrichment already compulsory by the new legislation, we encourage the usage of different enrichment tools available for researchers to discover new phenotypes and explain known effects in more detail. (Van de Weerd et al 2002) advocate EE, saying that because animals can perform more of their species-specific behaviour in EE, they may be more able to cope with novel and unexpected changes, thus showing a uniform response in test paradigm. Our data support that claim because we found that B6 animals kept in EE seem to acquire necessary toolset for withstanding stress and adjusting to new environments better than animals from SH. However, 129Sv mice are often just overwhelmed by redundant stimuli: inability to cope (decreased movement and bigger weight loss) in a novel environment in Phenotyper is a good example of that. In the open field, big variance in time spent in the centre (0s to 720s; Figure 3) is also an indication of abnormal behaviour in enriched 129Sv mice and this effect is strengthened by amphetamine (Figure 4). Altogether we have shown that EE reinforces predispositions of B6 strain having an active coping strategy, and of 129Sv having a more passive one.

In the current research it was found that environmental enrichment enhances the expression level of *Lsamp* 1b transcript specifically in the hippocampus in B6 mice; the same tendency existed across both mouse lines and both transcripts. The enrichment-induced elevation in *Lsamp* 1b expression in the hippocampal area was also significant when the data from 129Sv and B6 genotypes was pooled, indicating that the environmental effect persists regardless of genetic background. We did not detect any statistically relevant enrichment-induced differences of *Lsamp* expression in the frontal cortex, hypothalamus, thalamus, ventral striatum or temporal lobe.

It is common knowledge that exploratory drive and motivational systems for 129Sv and B6 strains are vastly different. We found that 129Sv animals whose monoaminergic systems have been constantly excited (in this case, by EE) and who are further stimulated by amphetamine or new conditions (exposure to Phenotyper apparatus), have decreased exploratory and locomotor activity compared to 129Sv animals from SH. On the contrary, in the B6 mice the stimulating effect of amphetamine is stronger in animals raised in EE compared to those raised in SH. As the expression of dopamine related genes has been shown to be fairly similar in the brains of 129Sv and B6 mice (Morris et al 2010), this effect is likely caused by desensitisation or inhibition of the dopamine system.

4. *Lsamp* 1a and 1b expression in the mouse brain

Several *Lsamp* expression differences were found between the mouse strains. In the hippocampus of 129Sv mice *Lsamp* 1a promoter had significantly higher expressional activity compared to B6 mice. In another dataset 1a transcript was strongly correlated with behavioural parameters associated with higher anxiety (Figure 15). Hippocampus has also been found to modulate anxiety-related behaviours by other authors (Fournier & Duman 2013). Dorsal hippocampus was shown to be necessary for contextual fear encoding (Kheirbek et al 2013); inactivation of ventral hippocampus has been indicated to reduce anxiety (Bannerman et al 2004). Therefore it can be hypothesized that the significant elevation of *Lsamp* 1a in the hippocampi of anxious 129Sv can be related to the highly anxious phenotype they show when compared to B6 mice. *Lsamp* 1b (that is more specific to sensory systems) was more highly expressed in the thalamus and frontal cortex of B6 mice. This may help explain well known differences in physical activity, spatial memory and coordination between these mouse lines. It is important to note that the activation of ventral hippocampus has also been found to be anxiolytic in novel environments (Kheirbek et al 2013). So far the expression differences of *Lsamp* have not been analysed longitudinally; this discrimination could be made in the future studies. Another consideration is that new-born neurons that migrate to the DG initially exhibit increased excitability and may have distinct and yet unknown functions regarding emotional behaviour (Fournier & Duman 2013).

The studies in this dissertation provide the first evidence that the activity profile of the two alternative promoters of the *Lsamp* gene have a heterogenous anatomical distribution in the developing and adult brain and the activity of these two promoters correlates with trait anxiety and social behaviour in mice.

Lsamp 1a promoter is transcriptionally active in the “classic” limbic structures known to be especially important for emotional and motivational functions (Heimer & Van Hoesen 2006). Namely, 1a promoter is specifically active in the hippocampal formation, temporal cortex and amygdaloid area and also in the

ventral striatum that includes nucleus accumbens and olfactory tuberculi; furthermore, 1a transcript is expressed specifically in the limbic, cingulate and insular cortex. Promoter 1a is active in the anterior thalamic nuclei that have been specified as "limbic thalamus" (Vogt & Gabriel 2013, Marchand et al 2014) and in the anterior hypothalamus, including preoptic area that is a major interacting structure of the limbic system (Morgane et al 2005). Promoter 1b of the *Lsamp* gene is notably active in the sensory pathways ranging from the brainstem and sensory nuclei in the thalamus and up to the primary sensory areas in the cortex. In the cerebral cortex, the signal from 1b promoter can be seen in layers 4 and 6 of the cortex (Figure 13) emphasizing systematic expression in the areas involved in the processing of sensory input. Layer 4 is the primary recipient of sensory input from the thalamus (Liao & Lee 2012) and most thalamic relay neurons receive feedback from layer 6 of the same cortical column they innervate (Lam & Sherman 2010). The specific activity of 1b promoter is obvious in the sensory pathways in visual, auditory and somato-sensory areas. However, both 1a and 1b promoters are active in the neural pathways transmitting olfactory and gustatory information. This finding can be anticipated as brain regions associated with olfactory and gustatory perception (e.g., the piriform cortex and insular cortex) are often overlapping with brain regions that are involved in emotional processing (Gutman et al 2013).

While *Lsamp* 1b promoter is predominantly active in sensory areas, it is also highly expressed in areas that are either traditional components of the limbic system and/or actively involved in regulating stress and arousal, such as the mammillary bodies and the paraventricular nucleus of the hypothalamus. Additionally, 1b promoter is prevalently active in the central nucleus of amygdala that is commonly referred to as the central part of the limbic structures (Heimer & Van Hoesen 2006). However, according to a recent study, the central nucleus of amygdala gets projections from several sensory-related regions (Bienkowski & Rinaman 2013). 1b promoter activity is highly enriched but not strictly limited to sensory areas; however, connections with the sensory systems can be found in most of the areas expressing 1b transcript. The expression of *Lsamp* in the brain areas processing sensory information has been reported in earlier studies (Reinoso et al 1996, Yamamoto & Reiner 2005). Yet, the discussion of whether the distribution of LSAMP is really specific for limbic structures has not been raised. Our results indicate that it is questionable to use the summarized LSAMP staining as a marker of the limbic regions, however, we propose that *Lsamp* 1a transcript is intensively and specifically expressed in the brain areas that are commonly considered to be limbic structures (Heimer & Van Hoesen 2006, Kotter & Stephan 1997, Morgane et al 2005).

5. The impact of *Lsamp* expression on anxiety phenotype

Increased levels of the *Lsamp* transcript have been associated with lower activity and higher levels of trait anxiety or acute fear reaction; and the genetic deletion of the *Lsamp* gene in mice resulted in increased activity in novel environments and reduced anxiety (Catania et al 2008, Innos et al 2011). To get further insight of how LSAMP is involved in the regulation of adaptive and emotional behaviour by the usage of alternative promoters, we studied behavioural correlates for *Lsamp* 1a and 1b transcripts in three brain areas. Most of the significant correlations appeared between *Lsamp* expression in the temporal lobe and behavioural parameters in the elevated plus maze. Higher levels of *Lsamp* 1a transcript had significant correlations with all of the measures indicating higher anxiety (Cruz et al 1994) in the elevated plus maze test. Higher levels of *Lsamp* 1b in the temporal cortex correlated significantly with the time that mice spent on open arm that is again, a common measure of anxiety. Current results are correlative in nature, but well in line with previous loss-of-function studies with *Lsamp*-deficient mice displaying decreased anxiety (Innos et al 2011).

In the current research we did not detect any expressional changes in *Lsamp* transcripts after acute fear reaction although there is evidence that *Lsamp* is also activated in reaction to acute fear in the amygdaloid area of rats (Koks et al 2004) and in the lateral amygdaloid nuclei of rats after fear conditioning (Lamprecht et al 2009). It is most likely that acute changes are limited to specific subnuclei in the amygdala that can be more precisely separated from the rat brain. In our study, the temporal lobe of mice was used, including all the amygdaloid nuclei and also the temporal cortex. However, the expression of *Lsamp* transcripts of the same area was significantly correlated with trait anxiety of the mice. We provide further evidence that the *Lsamp* gene is implicated in the formation of fear and anxiety processing circuits in the temporal cortex/ amygdaloid area (Nieh et al 2013), but this influence seems to be mediated differentially in acute fear reaction and trait anxiety. Although related, fear and trait anxiety are distinctly different – fear is an emotional reaction triggered by an immediate threat, while anxiety is a state of heightened apprehension in the absence of an immediate threat (Davis et al 2010). Taken together, current results fit with previous evidence relating increased levels of *Lsamp* with heightened trait anxiety (Alttoa et al 2010, Nelovkov et al 2006); the implication of *Lsamp* in acute fear reaction seems to be more complicated and might be related to certain subnuclei in the amygdala and/or specific time points.

Both *Lsamp* 1a and 1b transcript levels in the hippocampus correlated negatively with social sniffing and *Lsamp* 1a transcript in the ventral striatum was positively correlated with the time of anogenital sniffing in the social interaction test. The implication of LSAMP in the regulation of social activity is again in line with the behavioural phenotype of *Lsamp*-deficient mice displaying lack of inter-male dominance hierarchy and whisker trimming (Innos et al 2011). The

positive correlation of *Lsamp* 1a transcript with anogenital sniffing fits with reduced anogenital sniffing accompanying reduced inter-male aggressiveness reported in *Lsamp*-deficient mice (Innos et al 2011). General social sniffing is not altered in *Lsamp*-deficient mice; therefore the correlation between higher level of *Lsamp* in the hippocampal area and shorter time of social sniffing may reflect higher *Lsamp* levels correlating with higher trait anxiety as the social interaction test was initially designed to measure anxiogenic and anxiolytic drug effects (File & Hyde 1978). Current data is in line with our previous reports showing that *Lsamp*-deficient mice have decreased anxiety and alterations in social behaviour. Our results provide further evidence that *Lsamp* is functional in brain areas processing emotional reactions, particularly those related to anxiety/hyperactivity and social behaviour.

6. *Lsamp* expression pattern is conserved across species

Comparison of anatomical data from different species reveals high levels of conservation in the anatomical distribution of LSAMP transcript/protein. The summarized anatomical distribution from the current study is in line with the data from humans and primates: in humans LSAMP expression is intensive in the paraventricular thalamic nucleus (Uroz et al 2004) and moderate in the nucleus accumbens and claustrum (Prensa et al 2003). In primates, the hippocampus displays the strongest immunoreactivity, amygdala has a highly heterogeneous staining pattern (Cote et al 1996) and ventral striatum displays more intense LSAMP immunostaining than the dorsal striatum (Cote et al 1995). Furthermore, the twin promoter structure of the *LSAMP* gene seems to be essential also in humans. In the human genome, the exon 1a′ has been mutated by insertion of 2 nucleotides introducing a frame shift and resulting in a termination codon. Surprisingly, consequent loss of the acceptor site prevents the inclusion of the mutated exon 1a′ (Pimenta & Levitt 2004). The consequence of these two evolutionary events suggests that two promoters and alternatively regulated expression is needed for functional emotional responses in humans. Altered expression of *LSAMP* has been demonstrated in brain areas of human psychiatric patients in the frontal cortex (Behan et al 2009) and hippocampus, but in the previous studies 1a and 1b transcripts of the *LSAMP* gene in the nervous system have not been distinguished. Certain SNPs that reside in the first intron flanking exon 1b of *LSAMP* are associated with major depressive disorder (Koido et al 2012). Furthermore, lower expression level of *LSAMP* 1a transcript has been linked with the susceptibility allele for coronary artery disease (Vance et al 2007). These data emphasize the importance of studying 1a and 1b isoforms separately to find out relevant information that can be used in diagnostic panels in the future.

The distinct system-specific use of alternative promoters reveals highly organized transcriptional regulation of LSAMP gene/protein associated with a

broad spectrum of emotional behaviours. We propose that LSAMP is involved in emotional and social operating systems by complex regulation of two alternative promoters that guide the development of neural circuits in the limbic and sensory brain areas.

The highest expression level of the *Lsamp* gene (namely 1a promoter) in the hippocampal formation compared to other regions in the brain was confirmed by using quantitative RT-PCR analysis. Also a detailed description of the anatomical distribution of *Lsamp* promoter activity in the hippocampus was provided. 1a promoter is highly expressed in the pyramidal and granule cell layers. The overall expression level of *Lsamp* 1b promoter is evidently less intensive compared to 1a promoter, but the 1b-transcript positive cells are scattered all over the hippocampal formation. Although the anatomical distribution of X-Gal positive cells in the hippocampus reveals no clear neuronal staining, in the primary cell culture 1b promoter activity was detected only in the cells that had the morphology of neurons. Additionally, there is a discrepancy between alternative stainings as 1a staining is highly intensive in the granule cells of the dentate gyrus (DG) whereas universal staining reveals only moderate signal in the DG (Figures 8 A and C). The reliability of this picture is confirmed by the 1a and universal stainings made by 40 bp radioactive oligo in situ probes and also by the universal staining provided by Allen brain atlas (http://www.brain-map.org/). The presence of four short transcripts initiated from 1a promoter (Lsamp-006, Lsamp-007, Lsamp-008 and Lsamp-009 according to ensemble.org database) expressed specifically in the DG could be a potential explanation for the somewhat discordant stainings of alternative transcripts in the DG of the mouse hippocampus.

7. *Lsamp* has a role in the neurogenesis and synaptogenesis

In the DG, there are occasional 1b-positive cells in the granular zone, but the density of 1b promoter-positive cells is remarkably higher in the subgranular zone (SGZ) of the DG in the hippocampal formation which is known to be a specific area of enrichment-induced neurogenesis in adult rodents (Brown et al 2003, Lois & Alvarez-Buylla 1993, Peretto & Paredes 2014). According to our current results there is a remarkable spatial overlap of the expressional activity of *Lsamp* 1b transcript and BrdU positive proliferating cells in the SGZ. However, *Lsamp* 1b is not expressed in all the newborn neurons and 1b transcript is occasionally active in the neurons that are surrounding and supporting new neurons. Currently it can be hypothesized that the complex regulation of the alternative promoters in *Lsamp* could be related to the maturation of neurons as newborn neurons from the SGZ eventually migrate to the GZ (Gage et al 1998) – the area with intensive and ubiquitous *Lsamp* 1a transcript expression where only few cells are expressing 1b transcript. The vast majority of *Lsamp*

1b-positive cells in the primary hippocampal cultures showed a neuronal morphology, nevertheless, the precise phenotype of the cells in hippocampal sections remains to be defined in future studies. The study of alternative promoter activity is limited to transcript analysis at the moment as the 1a or 1b specific regions in the transcript encode for a signal peptide which is cleaved from a mature protein (Pimenta & Levitt 2004), therefore it is impossible to separate these isoforms by using an antibody.

The eminent expressional density of *Lsamp* 1b transcript in the SGZ is in compliance with specific elevation of *Lsamp* 1b transcript in the hippocampal area of B6 mice reared in enriched environments suggesting that Lsamp is involved in the enrichment-induced neurogenesis and synaptogenesis. Furthermore, the involvement of Lsamp in synaptogenesis and synaptic transmission (Hashimoto et al 2009, Qiu et al 2010) in the hippocampal neurons has been shown in previous studies. The basal synaptic transmission in *Lsamp*-deficient mice is not affected but CA1 long term potentiation (LTP) in slices from *Lsamp*-deficient animals has been shown to be significantly reduced suggesting that loss of Lsamp results in altered synaptic transmission and impaired plasticity in adult hippocampus (Qiu et al 2010). As the previous evidence points that LSAMP serves as an adhesion molecule that is implicated in target recognition during synaptogenesis and in integrity and stability of the synapses, we suggest that the enrichment-induced elevation of *Lsamp* in the hippocampal area is related to promoting synaptic connections in newborn neurons.

The elevation of BDNF specifically in the hippocampus is one of the most extensively described molecular changes (Chourbaji et al 2012a, Kazlauckas et al 2011, Kuzumaki et al 2011) induced by environmental enrichment. Our results confirmed that effect. BDNF is one likely mediator of the long-term effects of enrichment on the phenotype doing so by promoting neuronal survival, differentiation and synaptic plasticity (Huang & Reichardt 2001). The reduction of enrichment-induced *Bdnf* increase in the hippocampus of *Lsamp*-deficient mice further indicates that LSAMP could serve as a positive modulator of the BDNF regulated neuronal pathways. Enrichment-induced molecular changes and synaptogenesis in the brain are not specific to the hippocampus (Rampon et al 2000), however as for BDNF, the enrichment-induced expressional increase of *Lsamp* transcript was evident only in the hippocampus. The synaptogenesis-inducing effect of *Lsamp* could also be specific for the hippocampus as, according to our preliminary results, we have detected no effect of *Lsamp* on the rate of synaptogenesis in the primary culture of cortical neurons (data not shown) by using identical study design with Hashimoto *et al* (Hashimoto et al 2009) who demonstrated synaptogenesis-inducing effect of *Lsamp* in hippocampal cell culture. The expression levels on *Lsamp* transcripts correlated significantly with *Bdnf* expression levels in the hippocampus and frontal cortex, further suggesting a functional relationship between *Lsamp* and *Bdnf*.

8. Future perspectives

The increased attention to the animal welfare is positive development in the field. The new facilities ensure more humane conditions; the new models and methods are designed to be more ethological. In western society the rearing environment for people has changed dramatically over the last few generations. Different but equally effective changes have happened in laboratory rodent environments. This does not have to be annoyance for the researcher, but can also be seen as an opportunity. The animals from the enriched environment can reveal phenotypes that could not have been discovered before. It is crucial to continue to investigate gene-environment interactions in trans-genetic and other animal models.

We have described the expression of the *Lsamp* promoters in the brain. The expression differences of *Lsamp* have not been analysed longitudinally yet. This discrimination should also be a major target for the future studies.

CONCLUSIONS

1) The behavioural and physiological evidence presented in this dissertation demonstrate that EE is likely more beneficial for B6 male mice compared to more anxious and inhibited 129Sv. Our data suggests that the underlying mechanism behind this phenomenon could be explained with different reactivity of the monoaminergic system.
2) Genetic background and rearing environment influence the expression of *Lsamp* gene. We found that EE enhances the expression level of *Lsamp* 1b transcript specifically in the hippocampus in B6 mice; the same tendency existed across both mouse lines and both transcripts. The significant elevation of *Lsamp* 1a transcript in the hippocampi of anxious 129Sv can be related to the highly anxious phenotype they showed when compared to B6 mice. *Lsamp* 1b transcript was more highly expressed in the thalamus and frontal cortex of B6 mice. This may help to explain well known differences in physical activity, spatial memory and coordination between these mouse lines.
3) We demonstrated differential expression of 1a and 1b promoters of the *Lsamp* gene in the mouse brain. 1b promoter is transcriptionally active in the sensory pathways ranging from the brainstem and sensory nuclei in the thalamus and up to the primary sensory areas in the cortex, whereas 1a promoter is expressed in the "classic" limbic areas of the brain. There appears to be a connection between *Lsamp* expression in the hippocampus (which in both humans and rodents is linked to plasticity and adaptability) and adaptability phenotype in rodents.

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SUMMARY IN ESTONIAN

B6 ja 129Sv hiireliinide käitumuslik ja geneetiline võrdlus, mis keskendub ärevuskäitumisele ja Lsamp geeni ekspressioonile

Kaasaegne neuroteadus vajab hiiri nii baasteaduses haiguste ja häirete mudeldamiseks kui ravimiarenduse valdkonnas ohutuse testimisel. Alles pärast loomkatseid tohib ravimikandidaati hakata inimeste peal testima.

Tänapäevased näriliste mudelid püüdlevad etoloogilisema lähenemise poole. Loomade elutingimused ja ka katsed peavad toimuma võimalikult loomuomases keskkonnas. Surve selliseks lähenemiseks kasvab nii teadlaste kogukonnast, kes tahavad, et nende tulemused oleksid võimalikult valiidsed ja kõrge translatsioonilise väärtusega, kui ka seadusloome tasandilt (direktiiv 2010/63/EU), mis tuleb vastu heaoluühiskonna eeldustele loomade heaolu kohta.

See, kuidas närilisi laborites kasvatatakse ja milliseid käitumiskatseid nendega tehakse, mõjutab katsetest saadud tulemusi. Käesolev doktoritöö hindab erinevates keskkonnatingimustes, see tähendab individuaalselt, tavapuuris või rikastatud keskkonnas üles kasvamise mõju klassikalistele hiireliinidele B6 ja 129Sv. Lisaks keskendub töö eelmainitud taustaliinidesse loodud *Lsamp*-puudulikkusega hiire iseloomustamisele.

B6 hiired kasutavad varasemalt teadaolevalt keskkonnas toime tulemiseks ja kohanemiseks aktiivset toimetulekustrateegiat; samades tingimustes käituvad 129Sv liini hiired alalhoidlikult ja passiivselt. Tuvastasime uuringutega, et rikastatud keskkonnas elamine mõjub aktiivsetele B6 loomadele paremini kui ärevatele 129Sv liini hiirtele. Farmakoloogilised ja käitumiskatsete andmed viitavad, et 129Sv hiirte monoamiinsüsteem on rikastatud keskkonnas üle stimuleeritud, mis tingib ebaadekvaatse kohanemisreaktsiooni.

Lsamp geen juhib närvisüsteemi kujunemisel neuronite ühenduste kujunemist. Mutatsioone selles geenis seostatakse ärevus- ja meeleoluhäiretega. *Lsamp*-puudulikkusega hiirtel, kellel see geen on välja lülitatud, esineb kõrvalekaldeid sotsiaalses käitumises ning uudses ja ärevas olukorras kohanemisel. *Lsamp* geenil on kaks promootorit: 1a ja 1b. Käesolevas töös näitasime esmakordselt nende erinevaid ekspressioonimustreid hiire ajus. 1b on transkriptsiooniliselt aktiivne sensoorsetes juhteteedes alates tuumadest ajutüves ja taalamuses kuni primaarsete sensoorsete aladeni ajukoores. 1a promootor ekspresseerub klassikalistes limbilistes struktuurides nagu hippokampus ja hüpotaalamus. *Lsamp* promootorite ekspressiooni tasemed hippokampuses käitumiskatsete järgselt viitavad, et koos *Bdnf*iga mängib *lsamp* olulist rolli aju plastilisuses ja kohanemiskäitumise reguleerimisel.

Nii geneetiline taust kui kasvukeskkond mõjutavad *Lsamp* geeni ekspressiooni. Käesolevas töös näitame, et rikastatud keskkond suurendab *Lsamp* 1b transkripti ekspressiooni taset B6 hiirte hippokampuses; sama tendents ilmneb ka teise uuritud 129Sv liini juures ja mõlemal liinil 1a promootori puhul. Ka liinide vahel esineb märkimisväärseid erinevusi. Ärevate 129Sv loomade ajus

on *Lsamp* 1a transkripti ekspressioon hippokampuses oluliselt kõrgem (hoolimata kasvukeskkonnast) kui B6 hiirtel. Samas *Lsamp* 1b transkript ekspresseerub kõrgemal tasemel B6 liini taalamuses ja ajukoores, mis võiks aidata seletada liinidevahelisi erinevusi füüsilises aktiivsuses, ruumilises mälus ja peenmotoorikas. Edasistes uuringutes tuleb vaadelda *Lsampi* ekspressiooni muutumist erinevates ajapunktides.

Viimaste põlvkondade jooksul on läänemaailmas inimeste kasvukeskkond dramaatiliselt muutunud. Teisel viisil, kuid samaväärselt muutub ka laborinäriliste kasvukeskkond. Lisaks ebamugavustele, mis selliste muutustega kaasnevad, saab seda vaadelda kui võimalust avastamaks uusi fenotüüpe, mis varem ei saanud avalduda. Geenide ja keskkonna vahelised interaktsioonid väärivad süvitsi edasi uurimist nii transgeensetes loommudelites kui ühiskonnas laiemalt.

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Kask, K.; Bull, R.; **Heinla, I**.; Davies, G. (2007). The effect of a standard to improve person descriptions by children. Journal of Police and Criminal Psychology, 22(2), 77–83.

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