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# Alterations of emotional behaviour induced by the genetic invalidation of the limbic system associated membrane protein (Lsamp) – potential implications for neuropsychiatric disorders

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# LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, referred to in the text by Roman numerals I–IV.

I. Mazitov, Timur, **Aleksandr Bregin**, Mari-Anne Philips, Jürgen Innos, and Eero Vasar. 2017. "Deficit in Emotional Learning in Neurotrimin Knockout Mice." *Behavioural Brain Research* 317 (January): 311–18. https://doi.org/10.1016/j.bbr.2016.09.064.

II. **Bregin, Aleksandr**, Timur Mazitov, Ingrid Aug, Mari-Anne Philips, Jürgen Innos, and Eero Vasar. 2019. "Increased Sensitivity to Psychostimulants and GABAergic Drugs in Lsamp-Deficient Mice." *Pharmacology, Biochemistry, and Behavior* 183 (August): 87–97. https://doi.org/10.1016/j.pbb.2019.05.010.

III. **Bregin, Aleksandr**, Maria Kaare, Toomas Jagomäe, Karina Karis, Katyayani Singh, Karita Laugus, Jürgen Innos, et al. 2020. "Expression and Impact of Lsamp Neural Adhesion Molecule in the Serotonergic Neurotransmission System." *Pharmacology, Biochemistry, and Behavior* 198 (November): 173017. https://doi.org/10.1016/j.pbb.2020.173017.

IV. Singh, Katyayani, Kersti Lilleväli, Scott F. Gilbert, **Aleksandr Bregin**, Jane Narvik, Mohan Jayaram, Märt Rahi, et al. 2018. "The Combined Impact of IgLON Family Proteins Lsamp and Neurotrimin on Developing Neurons and Behavioral Profiles in Mouse." *Brain Research Bulletin* 140 (June): 5–18. https://doi.org/10.1016/j.brainresbull.2018.03.013.

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I. The author participated in the behavioral experiments and was involved in interpreting the data, participated in writing.
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IV. The author participated in the behavioral experiments, was involved in interpreting the data, participated in writing and correspondence.

# ABBREVIATIONS

| | |
|---|---|
| 3-MT | 3-methoxytyramine |
| 5-HIAA | 5-hydroxyindoleacetic acid |
| 5-HT | serotonin |
| 5-HT2C | serotonin receptor type-2C |
| Alpra | alprazolam |
| AMPA | alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| Amph | amphetamine |
| ANOVA | analysis of variance |
| BLA | basolateral amygdala |
| BST | bed nucleus of stria terminalis |
| BZ | benzodiazepine |
| CeA | central nucleus of amygdala |
| CRF | corticotropin-releasing factor |
| CS | conditioned stimulus |
| D str | dorsal striatum |
| DA | dopamine |
| dB | decibel |
| Diaz | diazepam |
| DOPAC | 3,4-dihydroxyphenylacetic acid |
| DR | dorsal raphe |
| ECF | extracellular fluid |
| EPM | elevated plus maze |
| Esc | escitalopram |
| Eth | ethanol |
| GABA | gamma-aminobutyric acid |
| GABAA | GABA type-A |
| Gabra | gamma-aminobutyric acid receptor subunit alpha-2 |
| GPI | glycosylphosphatidylinositol |
| Hip | hippocampus |
| HPLC | high performance liquid chromatography |
| Hprt-1 | hypoxanthine-guanine phosphoribosyltransferase |
| HVA | homovanillic acid |
| i.p. | intraperitoneal |
| Ig | immunoglobulin |
| Keta | ketamine |
| LacZ | Lactose operon Z, synthesises beta-galactosidase |
| LORR | loss of righting reflex |
| Lsamp | limbic system-associated membrane protein |
| lx | lux |
| mA | milliAmpere |
| Maoa | monoamine oxidase A |
| Maob | monoamine oxidase B |

| | |
|---|---|
| MDD | major depressive disorder |
| MK-801 | uncompetitive antagonist of the N-Methyl-D-aspartate receptor, Dizocilpine |
| NA | noradrenaline |
| NaCl | sodium-chloride |
| Negr1 | neuronal growth regulator 1 |
| NMDA | N-Methyl-D-aspartic acid |
| NMN | normetanephrine |
| Ntm | neurotrimin |
| OF | open-field |
| Opcml | opioid-binding cell adhesion molecule |
| Pento | pentobarbital |
| RRR | regain of righting reflex |
| RT-qPCR | real-time quantitative polymerase chain reaction |
| SE | standard error |
| SEM | standard error of the mean |
| SERT | serotonin transporter |
| SIH | stress-induced hyperthermia |
| Slc6a4 | solute carrier family 6 member 4, a serotonin transporter |
| SSRI | selective serotonin reuptake inhibitor |
| T1 | baseline temperature for SIH |
| T2 | temperature measurement 10 minutes after T1 |
| Temp | temporal cortex |
| Tph2 | tryptophan hydroxylase 2 |
| TS | tail suspension |
| US | unconditioned stimulus |
| V str | ventral striatum |
| WM | water maze |
| ΔCT | difference in cycle threshold between the target genes and the housekeeper gene Hprt-1 |

# 1. INTRODUCTION

The IgLON family members – Lsamp, Opcml, Ntm, Negr1 and IgLON5 – are mainly clustered at pre- and postsynaptic sites in different brain regions (Zacco et al., 1990; Miyata et al., 2003). These glycoproteins carry three Ig domains and are anchored to neural and oligodendrocyte cell membranes by glycosylphosphatidylinositol (Salzer et al., 1998; Sharma et al., 2015).

IgLONs can form homodimers and heterodimers, which are called dimeric IgLONs or DIgLONs (Akeel et al., 2011; Lodge et al., 2000). Forming these dimers is important for promoting or inhibiting neurite outgrowth (Akeel et al., 2011; Gil et al., 2002; Reed et al., 2004). Also, intra-family interactions of IgLONs on the cell membrane surface can modify the context-dependent functional aspects of neural development, maintenance, and plasticity (Gil et al., 2002; Hashimoto et al., 2009; Akeel et al., 2011; Reed et al., 2004; Sanz et al., 2015; Szczurkowska, 2018). Among the five members in the IgLON superfamily, Lsamp and Ntm have been shown to have maximum affinity for trans-interactions (Reed et al., 2004).

It has also been shown that Lsamp, Ntm and Opcml harbour two alternative promoters (1a and 1b), leading to transcripts that encode proteins with alternative N-terminal sequences. Other members, Negr1 and IgLON5, instead have a single promoter (Pimenta et al., 2004; Vanaveski et al., 2017).

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

The first *Lsamp* gene knockout mouse line displayed no changes in sensory or motor development, was slightly hyperactive in novel environments, and showed a reduced level of anxiety in the elevated plus maze (Catania et al., 2008). This mouse line also exhibited a pronounced deficit in spatial memory acquisition in the water maze and poorly sustained hippocampal CA1 region long-term potentiation (Qiu et al., 2010). Moreover, the anxiolytic-like outcome

of *Lsamp* gene invalidation in Lsamp-deficient mice was accompanied by the significant modification of GABAergic activity (Innos et al., 2011).

The main aim of the present study is to extend the previous findings, showing the role of Lsamp in the regulation of emotional behaviour. This was done by investigating acute and chronic effects of different drugs on the behaviour, physiology and monoamine metabolism of Lsamp-deficient mice. As Lsamp is known to form dimers with other IgLONs, investigating one of its most significant binding partners, Ntm, and their interactions, was also an important part of this study.

# 2. REVIEW OF LITERATURE

## 2.1. General overview of the IgLON family of cell adhesion molecules

The IgLON gene family is a small group of genes belonging to the immunoglobulin (Ig) superfamily of cell adhesion molecules (Table 1). Five members of the IgLON family are limbic system associated membrane protein (Lsamp; IgLON3; (Levitt, 1984; Horton and Levitt, 1988; Pimenta et al., 1996), opioid-binding cell adhesion molecule (Opcml; IgLON1; Schofield et al., 1989), neurotrimin (Ntm; IgLON2; Struyk et al., 1995), neuronal growth regulator 1 (Negr1/Kilon; IgLON4; Funatsu et al., 1999) and IgLON5 (Grimwood et al., 2004; Sabater et al., 2014).

**Table 1**: IgLON superfamily genes in Homo sapiens and Mus musculus, their genomic location, protein size in amino acids and molecular mass in kiloDaltons.

| | **Lsamp** | **Ntm** | **Opcml** | **Negr1** | **IgLON5** |
|---|---|---|---|---|---|
| Genomic location (human) | 3q13.31 | 11q25 | 11q25 | 1p31.1 | 19q13.41 |
| Genomic location (mouse) | 16qB4 | 9qA4 | 9qA4 | 3qH4 | 7qB3 |
| Protein size (amino acids) | 338 | 344 | 337 | 348 | 336 |
| Molecular mass (kiloDaltons) | 64-68 | 65 | 46-51 | 46 | 36 |

High levels of intra-family homology were found in the IgLON superfamily. All the five IgLONs share the same structure, contain three C2-type Ig-like domains, and one conserved disulphide bond with each Ig domain through which they adhere to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Pimenta et al., 1996, Miyata et al., 2003). The sequences of the alternative promoters of Lsamp 1a and 1b transcripts were initially provided by Pimenta and Levitt (2004) and later a more detailed analysis was conducted by Vanaveski et al. (2017). Similar genomic structures were found in Ntm and Opcml with two alternative promoters 1a and 1b. Negr1 and IgLON5 possess uniform 5' regions, implying a single promoter (Vanaveski et al., 2017).

Functional studies have shown that each member of the IgLON family forms homo- and heterophilic dimers along the cell membrane as part of a larger signalling complex (Reed et al., 2004; McNamee et al., 2011). Ntm and IgLON5 contain one extra non-conserved cysteine inside the first 90 amino acids (C45 and C83 respectively), attributing to the ability to form covalent dimers (Struyk et al., 1995). A recent study demonstrates that all IgLONs perform basic homodimeric interactions via their first Ig-domain – more specifically, Ntm and Negr1 have the capacity to uphold homo- and hetero-dimers via trans-interactions over different cells or a synaptic cleft (Ranaivoson et al., 2019).

In the mouse brain Lsamp, Opcml and Negr1 are expressed both in neurons and oligodendrocytes (Sharma et al., 2015). Ntm is expressed specifically in neurons, Opcml and Lsamp were also shown to be expressed in astrocytes (Sugimoto et al., 2010; Zhang et al., 2014; Sharma et al., 2015). IgLON5 and Ntm are expressed in microglia (Zhang et al., 2014). Among all IgLONs, only Ntm is expressed in endothelial cells (Petryszak et al., 2014).

The neuroanatomical distribution of Lsamp, Ntm, Opcml and Negr1 has been shown to be extremely diverse throughout the brain, with co-expression at a few sites (Philips et al., 2015; Gil et al., 2002; Hachisuka et al., 2000; Schafer et al., 2005). The timing of their first mRNA expression in the developing nervous system of mice has also been almost identical, starting from E12.5-15 (Philips et al., 2015; Struyk et al., 1995; Hachisuka et al., 2000, Jagomäe et al, 2021).

The distinct expression and differential function of IgLON cell adhesion molecules suggest their vital contribution to the development of neural circuits (Gil et al., 1998; Funatsu et al., 1999; Miyata et al., 2003; Takamori et al., 2006). Both Lsamp and Ntm are involved in the establishment of neural circuits. An essential role for Lsamp has also been shown in the outgrowth of dopaminergic afferents from the midbrain to the lateral habenula (Keller et al., 1989; Pimenta et al., 1995; Mann et al., 1998; Struyk et al., 1995; Chen et al., 2001; Schmidt et al., 2014). One recent study indicated the significance of Negr1 for neurogenesis in the adult hippocampus (Noh et al., 2019).

Behavioural studies based on Lsamp-deficient mice reveal the importance of Lsamp in the coordination of complex emotional and social behaviours, in hippocampal plasticity and during adaptation in dynamic environments (Innos et al., 2011, 2012, 2013a, 2013b; Philips et al., 2015; Qiu et al., 2010; Heinla et al., 2015). Recent studies with Negr1-deficient mice showed anxiety and depression-like behaviour due to impaired neurogenesis in the dentate gyrus of the hippocampus (Noh et al., 2019). Besides that, Szczurkowska et al. (2018) showed autistic-like core behaviour in Negr1-deficient mice.

## 2.2. Characterisation of functional role of Lsamp and Ntm genes

The first described IgLON family member, the *Lsamp* gene, is located on chromosome 3 in humans and on chromosome 16 in mice (Table 1). Its product, the Lsamp protein is expressed in cortical and subcortical limbic-associated regions of the developing and adult brain (Cote et al., 1995; Cote et al., 1996; Horton and Levitt, 1988; Levitt, 1984; Pimenta et al., 1996; Reinoso et al., 1996; Zacco et al., 1990). Lsamp is a 64- to 68-kDa heavily glycosylated protein, structurally characterised by three immunoglobulin domains (Pimenta et al., 1996). The Lsamp protein is expressed on the surface of somata and proximal dendrites of neurons (Zacco et al., 1990), where it integrates via a GPI anchor (Pimenta et al., 1995). The amino acid sequence of Lsamp is highly

conserved among species. The protein exhibits 99% homology between rodent and human (Pimenta et al., 1996a) and there is a close correlation between Lsamp mRNA and protein distribution patterns in rat (Levitt, 1984; Pimenta et al., 1996b; Reinoso et al., 1996; Zacco et al., 1990), monkey (Cote et al., 1995; Cote et al., 1996), and human (Prensa et al., 1999; Prensa et al., 2003), indicating strong phylogenetic conservation of protein structure and associated functional properties. Lsamp immunoreactivity in mice is developmentally present within 24–36 hours after neurons undergo their final mitosis on embryonic days E15–E19 (Horton and Levitt, 1988). Functional and biochemical studies have revealed that Lsamp can promote or inhibit neurite outgrowth depending on counter partners (Mann et al., 1998, Gil et al., 2002), more specifically, experimental manipulations of Lsamp *in vitro* result in altered axon targeting and neurite growth (Eagleson et al., 2003; Keller et al., 1989; Mann et al., 1998; Pimenta et al., 1995; Zhukareva et al., 1997). No observable anatomical alterations have been identified in the brain of the Lsamp-deficient mouse line (Catania et al., 2008).

The mRNA expression of Lsamp in the developing and adult rat brain can be observed in the hippocampus, amygdala, ventral and dorsal striatum, the preoptic and hypothalamic areas, somatosensory, motor cortex, thalamic nuclei, midbrain, hindbrain, superior and inferior colliculus, dorsal and ventral cochlear nuclei, nucleus of lateral lemniscus, superior olive and spinal trigeminal nucleus (Pimenta et al., 1996b; Reinoso et al.1996). In the cat, Lsamp expression can be observed in the caudate nucleus and substantia nigra (Chesselet et al., 1990). The basal ganglia, hippocampus and amygdaloid area (Cote et al., 1995; 1996) are also labelled with Lsamp in the primate's brain.

One previous study confirmed the significant variety in the twin promoter activity of Lsamp 1a and 1b in the mouse brain (Philips et al., 2015). Lsamp 1a promoters are highly expressed in classical limbic structures, like the hippocampal formation, temporal cortex, amygdaloid area, ventral striatum, olfactory tubercle, limbic cortex, cingulate cortex, insular cortex, anterior thalamus, anterior hypothalamus and preoptic areas. In contrast, the Lsamp 1b promoters are strongly expressed in the sensory pathways like the brainstem, the sensory nucleus in the thalamus, layers IV and VI of the cortex and sensory areas like the visual, auditory and somatosensory cortex. Lsamp 1b promoters are also expressed in regions regulating stress and arousal, like the mammillary bodies, the paraventricular nucleus of the hypothalamus and regions important in olfactory and gustatory regulation like the insular and piriform cortices (Philips et al., 2015).

Among all the five members of the IgLON superfamily, Lsamp and Ntm have been shown to have maximum affinity for trans-interactions (Reed et al., 2004), which can suggest a strong functional interaction between these IgLONs. The *Ntm* gene is located on chromosome 11 in humans and on chromosome 9 in mice (Table 1). In the genome Ntm is positioned closely with another member of the IgLON family – Opcml.

Ntm expression was first described in the developing rat brain (Struyk et al., 1995), which seems to be restricted to post-mitotic neurons. Ntm is highly expressed in somatosensory areas between E18 to P10 in the developing thalamus, cortical subplate, V and VI laminae of the forebrain cortex, pontine nucleus, internal granule cells and Purkinje cells of the cerebellum. Expression in these neurons suggests Ntm involvement in the development of these neural projections. Lsamp and Ntm are expressed in a complementary pattern in the brain with similar co-expressions, including the sensory-motor cortex, entorhinal cortex, hippocampus, amygdala, thalamus (ventral posteromedial, lateral geniculate nucleus and lateral dorsal nuclei), piriform cortex, cerebellum, brain stem nuclei, spinal cord, and dorsal root ganglia (Philips et al., 2015; Struyk et al., 1995; Gil et al., 2002). In cultured hippocampal neurons, the co-expression of Lsamp and Ntm has also been shown at the level of single neurons (Gil et al., 2002).

Taking the other functional and morphological indices, it appears that, despite close interactions between Lsamp and Ntm, there are less studies of the Ntm gene, considering its functional role in the brain.

## 2.3. Impact of Lsamp and Ntm in the regulation of behaviour and implications for human disorders

The first evidence confirming the *Lsamp* gene could play a role in the regulation of emotional behaviour came from a study where male Wistar rats were selected according to their exploratory behaviour in the elevated plus-maze (EPM) model of anxiety. Animals with lower exploratory activity, thus displaying increased anxiety, had elevated levels of the Lsamp transcript in the periaqueductal grey (Nelovkov et al., 2003). In the same rats, an increase in *Lsamp* gene expression was also noticed in the amygdala, but not in the frontal cortex (Nelovkov et al., 2006). Exposure of rats to cat odour, another model of anxiety in rodents, also increased the expression of the Lsamp transcript in the amygdala (Kõks et al., 2004). These findings were extended by Alttoa et al. (2010), demonstrating that the transcript for Lsamp was more expressed in the raphe, hippocampus and frontal cortex of rats displaying reduced exploratory activity in the motility box. Lamprecht et al. (2009) established that fear conditioning caused changes in the Lsamp transcript expression in the amygdala of rats. Altogether, rodent studies indicate that the increased level of the Lsamp transcript in several brain areas is related to increased trait anxiety (Alttoa et al., 2010; Nelovkov et al., 2003; Nelovkov et al., 2006), acute fear reaction (Kõks et al., 2004) and fear conditioning (Lamprecht et al., 2009). The first *Lsamp* gene knockout mouse line, generated by means of deleting exon 2 in the gene and by crossing the mice into C57/BL6J background, displayed no changes in sensory and motor development, was slightly hyperactive in novel environments, performed more open arm entries and head-dips and spent more time on open arms in the elevated plus maze (Catania et al., 2008). Furthermore, it

exhibited a pronounced deficit in spatial memory acquisition in the water maze and poorly sustained hippocampal CA1 region long-term potentiation (Qiu et al., 2010). The anxiolytic-like effect of *Lsamp* gene invalidation in mice was accompanied by the significant modification of GABAergic activity (Innos et al., 2011). Behavioural studies based on Lsamp-deficient mice reveal the importance of Lsamp in the coordination of complex emotional and social behaviours, in hippocampal plasticity and adaptation to dynamic environments (Innos et al., 2011, 2012, 2013a, 2013b; Philips et al., 2015; Qiu et al., 2010; Heinla et al., 2015).

Diazepam had no effect on the frequency of open arm entries and head-dips in Lsamp-deficient mice, mostly because untreated Lsamp-deficient mice showed similar values to diazepam-treated wild-type animals (Innos et al., 2011). However, administration of diazepam increased the time Lsamp-deficient mice spent in the open arms by almost three times, indicating a strong stimulating effect of this drug. Based on the gene expression data, it was proposed that the anxiolytic-like phenotype and reaction to diazepam in Lsamp-deficient mice is related to altered proportional balance between *Gabra1* and *Gabra2* genes (Innos et al., 2011). There was a significant decrease in the *Gabra1* gene in the temporal cortex and thus there was significantly more transcript encoding the α2 subunit of GABAA receptors, related to the stimulating effect of diazepam, in the temporal cortex of Lsamp-deficient mice (Innos et al., 2011).

Lsamp-deficient mice displayed lower expression levels of the dopamine transporter in the mesencephalon and had a blunted response to the locomotor effect of amphetamine at higher dose levels compared to wild-type littermates (Innos et al., 2013). Also, in the conditioned place preference test, amphetamine induced place preference in wild-type mice, but not in Lsamp-deficient mice. This indicates that the partial loss of sensitivity to amphetamine in Lsamp-deficient mice is probably not confined to locomotor effects, but rather is systemic, also affecting reward-related mechanisms (Innos et al., 2013). Monoamine measurements showed that the level of 5-HT was lower and the turnover of 5-HT higher in Lsamp-deficient mice, which means that in transgenic mice amphetamine raised the general level and lowered the turnover of 5-HT to a greater extent than in wild-type mice. It has been suggested that Lsamp-deficient mice seem to have an increased endogenous serotonin tone that may explain their lower anxiety and a decrease in agonistic behaviour and aggression (Innos et al., 2013).

Human data link the *Lsamp* gene not only with anxiety, but also a wider spectrum of psychiatric disorders: polymorphisms in the human *Lsamp* gene have been associated with panic disorder (Koido et al., 2006) and male completed suicide (Must et al., 2008). Also, a relation between gene polymorphisms of the *Lsamp* gene and major depressive disorder (MDD) (Koido et al., 2012) as well as schizophrenia (Koido et al., 2014; Chen et al., 2017) has been established. Furthermore, the levels of Lsamp protein have been found to be increased by approximately 20% in the postmortem frontal cortex in both

patients with schizophrenia and bipolar disorder (Behan et al., 2009). Karis et al. (2018) established that significantly increased levels of the Ntm 1b isoform transcript were found in the dorso-lateral prefrontal cortex of post-mortem brains of schizophrenia patients. Besides that, *Opcml*, a close partner of Ntm, turned out to be a susceptibility gene for schizophrenia in both the European (O'Donovan et al., 2008) and Thai population (Panichareon et al., 2012).

Accumulating evidence also suggests the function of *Opcml*, *Lsamp* and *Negr1* as tumour-suppressor genes in a wide range of carcinomas, e.g. epithelial ovarian tumours, leukemias, osteosarcomas, breast cancers, colorectal cancers, ovarian cancers, renal carcinomas, cerebellar pilocytic astrocytoma, gliomas etc. (Sellar et al., 2003; Ntougkos et al., 2005; Reed et al., 2007; Cui et al., 2008; Barøy et al., 2014; Kim et al., 2014; Coccaro et al., 2015; Zanini et al., 2017; Dong et al., 2018). On the contrary, Ntm has been shown to have increased expression in tumours where Lsamp has higher expression levels (Ntougkos et al., 2005; Pascal et al., 2009), indicating that Ntm displays an opposite impact on tumourigenesis.

## 2.4. Concluding remarks

IgLONs belong to the second most enriched adhesion molecule family in neurons and oligodendrocytes (Sharma et al., 2015). In the rodent brain IgLONs have been found on developing neurons and glia, playing a role in neural circuit establishment (Mann et al., 1998; Funatsu et al., 1999; Miyata et al., 2003). The timing of IgLON expression and their varied distribution in the brain indicate their significant role in creating diversified neuronal circuitry via intra-family interactions (Reed et al., 2004; Schwarz et al., 2009). Genetic variation and expression studies suggest that IgLONs are involved in several psychiatric and disease conditions in humans (Pan et al., 2010; Koido et al., 2014; Genovese et al., 2015; Hyde et al., 2016; Karis et al., 2018). Animal studies indicate roles of Lsamp and Ntm in controlling the development and activity of neural circuits and their underlying behaviours (Lee et al., 2012; Philips et al., 2015).

Based on the existing evidence there is a plan to extend the existing knowledge on the functional interactions between Lsamp and Ntm in the regulation of brain functions due to the fact that these two IgLONs have been shown to have maximum affinity for trans-interactions (Reed et al., 2004). On the other hand, Lsamp and Ntm seem to have an opposite impact in tumourigenesis. Therefore, due to scarce information, the evidence relating to the interplay between Lsamp and Ntm should be expanded into the area of behavioural regulation. Lsamp- and Ntm-double-deficient mice are a valid animal model for exploring this particular question.

The pharmacological characterisation of Lsamp-deficient mice revealed significant alterations in the function of GABA- and dopaminergic systems (Innos et al., 2011; 2013). In order to extend this knowledge, two pharmacological studies have been conducted. Firstly various GABA modulating drugs

have been applied in behavioural studies to further explore the changes in the GABAergic system of Lsamp-deficient mice. Secondly, alterations in the dopamine system in Lsamp-deficient mice have been further investigated in locomotor activity studies by using various drugs indirectly affecting dopaminergic neurotransmission, e.g. ketamine, MK-801, morphine, cocaine.

There is also a clear indication that the activity of the serotonin system is affected in Lsamp-deficient mice (Innos et al., 2013). This has been a reason to explore the alterations in the serotonergic system using morphological, biochemical, pharmacological and behavioural approaches using Lsamp-deficient mice.

Altogether, we expect that all the different approaches proposed above may give us a better vision of the role of Lsamp in the control of emotional behaviour with potential indications for neuropsychiatric disorders.

# 3. AIMS OF THE STUDY

The general aim of the present study was to characterise the pharmacological, biochemical and behavioural profiles of Lsamp-, Ntm- and Lsamp/Ntm-deficient mice. More specific aims of the present study were as follows:

1. To provide an initial behavioural characterisation of Ntm-deficient mice;
2. To further explore the impact of the mutational deletion of the Lsamp protein on the dopaminergic and GABAergic neurotransmission systems by using pharmacology combined with behavioural profiling;
3. To expand our understanding of the impact of deleting the Lsamp protein on the serotonergic neurotransmission systems by using a pharmacological approach;
4. To study the combined impact of two IgLONs on behaviour by comparatively studying the mice groups deficient for either Lsamp, Ntm or both of these genes.

# 4. MATERIALS AND METHODS

## 4.1. Experimental Animals

### 4.1.1. Living conditions (Papers I, II, III, IV)

Breeding and housing of the mice was conducted at the animal facility of the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. Mice were group-housed in standard laboratory cages measuring 42.5 (L) × 26.6 (W) × 15.5 (H) cm, 6–8 animals per cage in the animal colony at 22 ± 1ºC, under a 12:12 h light/dark cycle (lights off at 19:00 h). A 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia) was used in each cage and changed every week. No other enrichment was used besides nesting material. Water and food pellets (R70, Lactamin AB, Sweden) were available ad libitum. Male Lsamp−/−, Ntm−/−, Lsamp−/−Ntm−/− mice with mixed genetic background [(129S6/SvEvTac × C57BL/6N) × (129S6/SvEvTac × C57BL/6N)] were used in this study. Their wild-type littermate mice were used as control animals.

As Bl6 and 129Sv mice are different in their behavior and all our animals are a mix of the two lines, thus can vary in their behavior depending on which line dominates, within this thesis “wild-type” will always mean that the animal has a mixed genetic background of the Bl6 and 129Sv lines.

### 4.1.2. Lsamp-deficient mice (Papers II, III)

The scheme for generation of Lsamp-deficient (Lsamp−/−) mice with a LacZ transgene has been described in detail by Innos et al. (2011). Briefly, exon 1b of the murine *Lsamp* gene was replaced by an in-frame NLS-LacZ-NEO cassette resulting in the disruption of all functional Lsamp transcripts.

### 4.1.3. Ntm-deficient mice (Paper I)

The Ntm-gene heterozygous mutant strain (032496-UCD B6;129S5-Ntmtm1Lex/Mmucd) was obtained from the Mutant Mouse Regional Resource Centre at UC Davis (https://www.mmrrc.org/catalog/sds.php? mmrrc_id=32496). Briefly, the strategy for the creation of Ntm-deficient (Ntm−/−) mice was analogous to that of Lsamp-deficient mice, as exon 1b was deleted, leading to the disruption of all functional Ntm transcripts.

### 4.1.4. Lsamp/Ntm double-deficient mice and their single mutant littermates (Paper IV)

For generation double deficient (Lsamp/Ntm−/−) mice this scheme was followed: double heterozygous mice for Lsamp and Ntm (Lsamp+/−/Ntm+/−) were generated first by crossing Lsamp−/− and Ntm−/− mice in F2 strain background [(129S5/SvEvBrd × C57BL/6N) × (129S5/SvEvBrd ×

C57BL/6N)]. Further crossing the pair of the obtained double heterozygous mice (Lsamp+/−Ntm+/−) gave the entire spectrum of genotypes (including Lsamp+/+Ntm+/+, Lsamp+/+Ntm−/−, Lsamp−/− Ntm+/+, Lsamp−/− Ntm−/−).

## 4.2. Behavioural testing

### 4.2.1. General principles of behavioural testing

Behavioural testing was performed between 9:00 to 17:00 h with light intensity usually under 30 lx. Mice were let to habituate to the experimental room and the lighting conditions therein for 1 h before each experiment. To reduce the number of used animals, mice were used in several experiments with at least a four-day interval between experiments. The behavioural experiments with genotypes Lsamp−/−, Ntm−/− and Lsamp−/−Ntm−/− were performed with male mice between 8 to 16 weeks of age. Mice were first tested in sensitivity to previous experimental experience; stressful tests involving injections or electric shocks were performed last.

**Table 2.** All behavioral experiments and genotypes tested.

| **Behavioral experiments** | **Animal genotypes tested** |
|---|---|
| Reflex tests | Wild-type, Ntm+/−, Ntm−/− |
| Hot plate test | Wild-type, Ntm+/−, Ntm−/− |
| Locomotor activity test | Wild-type, Ntm+/−, Ntm−/−, Lsamp−/−, Lsamp−/−Ntm−/− |
| Elevated plus-maze | Wild-type, Ntm+/−, Ntm−/−, Lsamp−/−, Lsamp−/−Ntm−/− |
| Light-dark box test | Wild-type, Ntm+/−, Ntm−/− |
| Hyponeophagia test | Wild-type, Ntm+/−, Ntm−/− |
| Active avoidance test | Wild-type, Ntm+/−, Ntm−/− |
| Morris water maze test | Wild-type, Ntm+/−, Ntm−/−, Lsamp−/−, Lsamp−/−Ntm−/− |
| Fear conditioning test | Wild-type, Ntm+/−, Ntm−/− |
| Marble burying test | Wild-type, Ntm+/−, Ntm−/− |
| Nesting behaviour test | Wild-type, Ntm+/−, Ntm−/− |
| Social interaction test | Wild-type, Ntm+/−, Ntm−/− |
| Loss and regain of righting reflex test | Wild-type, Lsamp−/− |
| Stress-induced hyperthermia (SIH) test | Wild-type, Lsamp−/− |
| Tail suspension test | Wild-type, Lsamp−/− |

All animal procedures in this study were performed in accordance with the European Communities Directive (86/609/EEC) and permit (No. 29, April 28, 2014) from the Estonian National Board of Animal Experiments.

### 4.2.2. The order of behavioural tests (Paper I)

**Table 3.** The order of behavioral tests for Paper I.

| Batch | Behavioural experiments | Number of animals |
|---|---|---|
| 1 | 1. Elevated plus maze<br>2. Motility box<br>3. Motility box with amphetamine | 15-17 |
| 2 | 1. Light-dark box<br>2. Hyponeophagia<br>3. Active avoidance | 8 |
| 3 | 1. Reflex tests<br>2. Morris water maze<br>3. Active avoidance | 15-17 |
| 4 | 1. Reflex tests<br>2. Marble burying<br>3. Fear conditioning | 8 |
| 5 | 1. Nesting behaviour<br>2. Loss / regain of righting reflex with ethanol | 8 |
| 6 | 1. Social interaction<br>2. Loss / regain of righting reflex with ethanol | 15-17 |
| 7 | 1. Hot plate | 8 |

For some of the experiments, only a part of the batch was used. Group sizes were kept as even as possible; slight fluctuations were caused by excluding some mice for technical reasons (e.g. for avoiding electric shocks by climbing). No mice were excluded on the basis of being "outliers".

### 4.2.3. Reflex tests (Paper I)

Sensory testing was performed to rule out robust deficits in vision, hearing, olfaction and pain sensitivity. Forepaw reach test (also called "visual placing test"), estimating vision, and ear twitch test, estimating hearing, were performed as described earlier. In the reach test, a mouse was held by its tail at a height of 15 cm from a table surface. As the mouse was gradually lowered, extension of its forepaws for a "soft landing" was observed. In the ear twitch test, ear twitching reflex in response to a pen click was observed. The buried food finding test, measuring olfactory abilities, was carried out as described by Radyushkin et al. (2009). Starting two days prior to testing, each day mice received several pieces of chocolate cookies within 24 h. Then, mice were deprived of food for 12 h before testing, with water ad libitum. For testing, mice were placed individually into clear cages measuring 42.5 (L) × 26.6 (W) × 15.5 (H) cm in which a piece of chocolate cookie was hidden under a 1.5 cm standard bedding in the left corner of the cage. The mouse was positioned in the right corner at the opposite end of

the cage, and the food-finding time, i.e. the time from the moment the mouse was placed into the cage to the time it located the cookie piece and initiated burrowing, was recorded. A clean cage and new bedding was used for each trial.

### 4.2.4. Hot plate test (Paper I)

This test was carried out to assess pain sensitivity. The mouse was confined by a Plexiglas cylinder (diameter 15 cm, height 20 cm) to a plate that was heated to 53°C. The latency to show hind paw response (licking or shaking) was measured in seconds. The cut-off time for the experiment was 60 seconds.

### 4.2.5. Locomotor activity test (Papers I, II, III, IV)

The open field test of individual mice was measured in a lit room (ca 200 lx) for 30 min in sound proof photoelectric motility boxes [measuring 44.8 × 44.8 × 45 (H) cm] made of transparent Plexiglas and connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). Before each experiment mice were let to habituate with the experimental room for 1 h. The floor of the boxes was cleaned with 5% of ethanol and dried thoroughly after each experiment. The computer registered the distance travelled, the number of rearings, and the time spent in the central part of the box.

### 4.2.6. Elevated plus-maze (Papers I, II, III, IV)

The elevated plus maze (EPM) test was carried out as described by Innos et al. (2011) to assess anxiety-related behaviours. In short, the apparatus consisted of two opposite open (17.5 × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. The entire plus maze apparatus was elevated to a height of 30 cm and placed in a dim room (15 lx in open arms). Testing began by placing the animal on the central platform (5 × 5 cm) of the maze, facing a closed arm. An arm entry was counted only when all four limbs were within a given arm. The standard 5 min test duration was employed and the sessions were video-recorded. The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse. The following parameters were recorded by an experienced observer, blind to the experimental group: latency to enter an open arm; number of entries into open arms; number of entries into closed arms; time spent on close arms; total number of head-dippings and number of unprotected head-dippings, defined as head-dippings made on open arms.

### 4.2.7. Light-dark box test (Paper I)

The light-dark box (TSE, Technical & Scientific Equipment GmbH, Germany) consisted of a quadratic arena made out of dark Plexiglas on three sides and transparent Plexiglas at the front, measuring 30 × 30 × 24 (H) cm. Running

from front to back of the arena and situated at its midline was a dark Plexiglas wall, containing an opening 3.5 (W) × 10.0 (H) cm, allowing the mouse to transfer from one compartment of the arena to the other. The wall divided the arena into a lit chamber (ca 200 lx) and a dark chamber (ca 10 lx, with a lid). The Plexiglas arena was surrounded by a soundproof chamber. The apparatus was located in a quiet, dimly (ca 5 lx) illuminated room. The animal was placed in the dark chamber, facing away from the opening, and released. During a 20 min trial, the latency to enter the lit chamber, time spent in the lit chamber, and the numbers of transitions were measured.

### 4.2.8. Hyponeophagia test (Paper I)

The experiment was carried out in a brightly lit (ca 400 lx) room. The mice, food deprived for 24 h, were taken from their home cage and placed singly in a translucent plastic box measuring 22 (L) × 16 (W) × 14 (H) cm filled with a single layer of food pellets (Lactamin AB, Sweden; weighing 1.5–3.5 g) to a depth of ca 1 cm. To avoid social transmission of behaviour, mice that had already been tested were placed in a separate box. The latency to start eating was measured from the time a mouse was placed in the box. Eating was defined as eating for at least 3 s consecutively. A cut-off score of 180 s was used.

### 4.2.9. Active avoidance test (Paper I)

The active avoidance test was carried out in a rectangular two-way automated shuttle-box (TSE, Technical and Scientific Equipment GmbH, Germany), consisting of two identical chambers, both measuring 15 (W) × 30 (D) × 24 (H) cm, connected by an opening measuring 3.5 (W) × 10 (H) cm. For administering electric shocks, the box had a grid floor measuring 30 × 30 cm comprising 29 stainless steel rods (Ø = 4 mm, inter-rod centre-to-centre distance = 10 mm). The box was surrounded by a soundproof chamber. The apparatus was located in a quiet, dimly illuminated (10 lx) room. Light bulbs above the testing chambers, attached to the ceiling of the surrounding soundproof chamber, provided illumination and served as the light stimulus. Mice were placed in the right chamber, facing the wall, and submitted to an active avoidance test for three consecutive days, 25 consecutive trials a day. The test started with a habituation time of 10 s during which the illumination in both chambers was ca 5 lx. The conditioned stimulus (CS) was a 10 kHz tone with a maximum duration of 20 s accompanied by illuminating the target chamber (creating an illumination level of ca 10 lx in the “dark” chamber and ca 150 lx in the “lit” target chamber). The unconditioned stimulus (US; 0,3 mA electrical foot-shock for 5 s) was switched on 5 s after the CS and was followed by a stronger US (0.6 mA foot-shock for a maximum of 10 s) in case the mouse failed to move to the target compartment. The interval between trials was 10 s. The floor of the testing apparatus was cleaned with 5% ethanol and dried thoroughly after each mouse.

### 4.2.10. Morris water maze test (Paper I and IV)

The water maze consisted of a circular pool (diameter 150 cm), escape platform (16 cm in diameter), video camera and a computer with software (TSE, Technical & Scientific Equipment GmbH, Germany). The pool (depth 50 cm) was filled with tap water (22°C, to a depth of 40 cm) that was made opaque by adding a small amount of non-toxic white putty. The escape platform was positioned in the centre of the Southwest quadrant (Q2), 20 cm from the wall. The water level was 1 cm above the platform, making it invisible. In each trial, the animals were put into the water, facing the wall, at pseudo-randomly assigned starting positions (East, North, South, West). The acquisition phase of the experiment consisted of a series of 16 training trials (four trials per day for four consecutive days with the interval between trials ca 1 h). The mice were allowed to search for the platform for a maximum of 60 s, at which time the mice were gently guided to the platform by means of a metal sieve. The mice remained on the platform for ca 15 s. Posters and furniture around the maze served as visual cues. During testing, the room was dimly lit with diffused white light (20 lx). Distance travelled during the trial, latency to find the submerged platform and swim velocity were registered. Average values per day were used, which were obtained by collapsing the data from four trials for each animal. On day 5 the platform was removed for a probe trial. Mice were placed into the water in the Northeast position (Q4) and were allowed to swim for 60 s. Time spent in all four quadrants (Q1, Q2, Q3,Q4) was measured, with time spent in the target quadrant (Q2) where the platform had been located serving as an indicator of spatial memory.

### 4.2.11. Fear conditioning test (Paper I)

In this conditioning test a simple association between a conditioned stimulus (10 kHz tone, 90 dB, CS) and an unconditioned aversive stimulus (0.5 mA, 2 s electric foot-shock, US) is established. The study was performed by means of a computer-controlled fear conditioning system (TSE, Technical and Scientific Equipment GmbH, Germany), according to the method described by Paylor et al. (1994), with some modifications. The apparatus was located in a quiet, dimly illuminated (10 lx) room. The conditioning was conducted in a transparent Plexiglas chamber measuring 15 (W) × 30 (D) × 24 (H) cm with a stainless steel rod floor through which electric foot-shocks could be administered. The test chamber was placed inside a sound-attenuated chamber and was constantly illuminated (ca 100 lx). Mice were observed through a window in the front wall of the sound-attenuated chamber. Animals were placed in the conditioning context for 120 s and were then exposed to a CS for 30 s. The CS was terminated by a US. 120 s later another CS–US pairing was presented. The mouse was removed from the chamber 15–30 s later and returned to its home cage. The mice were tested for contextual memory 24 h later by placing them back into the test chamber for 5 min with no CS applied. Total time of freezing and the number of

rearings were recorded automatically. Four hours later the mouse was tested for freezing behaviour to the auditory CS. Testing was performed in a different Plexiglas chamber measuring 30 × 30 × 24 (H) cm the floor of which was covered with white cardboard and the walls of which were covered with black paper. Duration of the test was 6 min: 3 min without the tone (pre-CS phase) and 3 min with the tone (CS phase). Again, freezing time and rearings were registered.

### 4.2.12. Marble burying test (Paper I)

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

### 4.2.13. Nesting behaviour test (Paper I)

Two hours before the dark phase of the lighting cycle (17:00) the mice were individually housed in Plexiglas cages measuring 22 (L) × 16 (W) × 14 (H) cm with aspen bedding. Four cotton pads with a diameter of 5 cm and weighing 0.6 g were placed in the cage, one pad into each corner. The nests were scored 24 h later by using a 5-point rating scale described by Deacon (2006).

### 4.2.14. Social interaction test (Paper I)

The social interaction test was carried out as described previously (Innos et al., 2011) with some modifications. Two male mice (one wild-type or −/− mouse and a wild-type age- and weight-matched partner) were simultaneously placed in an empty housing cage measuring 22 (L) × 16 (W) × 14 (H) cm with a cover made of transparent Plexiglas. The illumination level of the testing arena was 25 lx. Mice were videotaped for 10 min. The videotapes were later scored by a trained observer. The following measurements were registered for each mouse: episodes of aggressive behaviour (attacks, biting, chasing, rattling the tail), anogenital sniffing ofthe other mouse, sniffing the body of the other mouse, digging episodes and digging time, grooming episodes and grooming time, and rearings and rearing time.

### 4.2.15. Locomotor activity test with d-amphetamine (Papers I and IV)

Wild-type, heterozygous and −/− mice were randomly assigned to groups that received an intraperitoneal (i.p.) injection of either saline or 5 mg/kg of d-amphetamine. Mice were held in small individual cages measuring 22 (L) × 16 (W) × 14 (H) cm for 30 min post-injection, after which they were placed individually for 30 min in photoelectric motility boxes measuring 44.8 × 44.8 × 45 (H) cm (illumination level ca 200 lx), connected to a computer (TSE,

Technical & Scientific Equipment GmbH, Germany). The floor of the testing apparatus was cleaned with 5% ethanol and dried thoroughly after each mouse. The computer registered the distance travelled, the number of rearings and time spent in the central part of the box.

In the other experiment the effects of d-amphetamine on the locomotor activity were checked in the open field test using 3–4 months-old Lsamp−/− Ntm−/− double-deficient animals and their wild-type littermates. On day 1 all mice received an i.p. injection of saline for baseline measurement and on day 5 all mice received an i.p. injection of 2.5 mg/kg of d-amphetamine dissolved in saline 30 min before testing in the motility box.

### 4.2.16. Loss and regain of righting reflex test (Paper II)

The mice were given an i.p. injection of ethanol (3.5 g/kg), alprazolam (3 mg/kg), diazepam (15 mg/kg), pentobarbital (30 or 45 mg/kg) or ketamine (150 mg/kg), placed in supine position in a V-shaped cardboard trough and tested for the ability to right themselves. It was considered that the animal had lost the righting reflex if it could not right itself on all four paws within 30 s and re-gained the righting reflex if it could fully right itself three times within 30 s. The onset of drug induced sedation (the latency to the loss of righting reflex, LORR) and the latency to regain the righting reflex (RRR) were measured.

### 4.2.17. Stress-induced hyperthermia (SIH) test (Paper II)

The SIH procedure was carried out as described in Vinkers et al. (2012), with minor modifications. Animals, prehandled for 5 min a day for 3 days before the experimental day to decrease handling-related SIH response, were injected i.p. with a vehicle or drug (diazepam, TP003 or SL651498) 60 min before the first temperature measurement (T1). The temperature was again measured 10 min later (T2), representing stress-induced body temperature. The stress-induced hyperthermia response was calculated by subtracting T1 from T2. The body temperature of mice was measured by inserting a thermistor probe 2 cm deep into the rectum. Digital temperature recordings were obtained with an accuracy of 0.1 °C. The probe, dipped into vaseline before inserting, was held in the rectum until a stable rectal temperature had been obtained for 20 s.

### 4.2.18. Tail suspension test (Paper III)

Mice were suspended for 6 min from the edge of a shelf 60 cm above a tabletop by adhesive tape, placed approximately 1 cm from the tip of the tail. The duration of immobility, the number of immobility episodes (an episode defined as hanging passively and being motionless for at least 3 s), and the number of short immobility episodes lasting 1–2 s were scored during the last 4 min of the recorded videos by an observer blind to the genotype.

## 4.3. Drugs and treatment (Papers I, II, III, IV)

Amphetamine (d-amphetamine, Sigma-Aldrich, USA) and cocaine (cocaine hydrochloride, Oriola Oy, Espoo, Finland) were administered 15 min; morphine (morphine sulphate, Mundipharma) and MK-801 (RBI, Natick, MA, USA) 30 min before testing. Diazepam and alprazolam (Grindex, Latvia) were diluted in a 0.9% NaCl solution (B. Braun Melsungen AG, Germany) with the help of a few drops of Tween 80 (Sigma-Aldrich) and injected 30 min before the study. Ethanol was injected 20 min prior to testing. Pentobarbital sodium salt (Sigma-Aldrich) and ketamine hydrochloride (Vetoquinol Biowet Sp. Z.o.o.) were administered 30 min before testing. TP003 and SL651498 (Axon Medchem) were administered 60 min before testing. The control groups received the vehicle solution (0.9% NaCl solution). All drugs were injected i.p. at a volume of 10 ml/kg.

In regard to the escitalopram study, all mice were age-matched with littermates and were tested at 3–4 months of age. Lsamp−/− and wild-type mice were randomly divided into groups that received an i.p. injection of either saline or 10 mg/kg of escitalopram for 18 consecutive days. Escitalopram (Sigma-Aldrich, USA) was freshly prepared in a sterile pyrogen free 0.9% NaCl solution (B. Braun Melsungen AG, Germany). Body weight was measured three weeks and one week before administration of escitalopram and on days 3, 8, 12, 14, 16 and 18 during the period of injection. Behavioural changes were evaluated in the elevated plus maze (day 14), tail suspension (day 16) and open field (day 18) tests. Two separate groups of Lsamp−/− (n = 8) and wild-type (n = 8) mice, not exposed to the behavioural studies, were used for qPCR gene expression studies.

## 4.4. Measurement of monoamines (Paper III)

In the escitalopram study, all mice were decapitated immediately after the last behavioural test. The brains were dissected into five parts – the raphe nuclei (including both dorsal and median groups of the raphe nuclei), dorsal striatum (caudate putamen), ventral striatum (including the nucleus accumbens and the bed nucleus of stria terminalis), temporal lobe (including the amygdaloid nuclei) and hippocampus, after which they were immediately frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas (Franklin and Paxinos, 1997). The levels of monoamines – serotonin (5-HT), noradrenaline (NA) and dopamine (DA) – and their metabolites – normetanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5- HIAA), and 3-methoxytyramine (3-MT) – were assessed by high performance liquid chromatography (HPLC) with electrochemical detection.

Gene expression was determined by two-step RT-qPCR (qPCR). The total RNA was extracted from each tissue sample by using the Trizol reagent

(Invitrogen), according to the manufacturer's protocol. First strand cDNA was synthesised by using Random Hexamer (Applied Biosystems) and Super-Script™ IV Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. Predesigned Taqman Gene Expression Assays (Applied Biosystems) were used for the measurement of the expression of monoamine oxidase A (Maoa; assay number: Mm00558004_m1), monoamine oxidase B (Maob, Mm00555412_m1) and serotonin transporter (Slc6a4, Mm00439391_m1). The same assays have been previously used by Hansson et al. (2014). TaqMan Universal PCR Master Mix was used according to the manufacturer's protocol as a reaction buffer. Two micrograms of RNA were used in the 20 µl end-reaction for cDNA synthesis from brain tissues. Each reaction mix was divided into 10 µl quadruplicates. ABI Prism 7900HT Sequence Detection System with ABI Prism 7900 SDS 2.4.2 software (Applied Biosystems) was used for qPCR detection. The analysis of qRT-PCR data was performed as described previously by Raud et al. (2009). Briefly, 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 genes (*Maoa*, *Maob* and *Slc6a4*) and the housekeeper gene *Hprt-1* (VIC-MGB).

## 4.5. Data presentation and Statistical Analysis (Papers I, II, III, IV)

The statistical analysis was performed with Statistica V12 (Statsoft Inc., Oklahoma, USA), R and the Graphpad Prism 5 software (Graph Pad, San Diego, CA). The analysis of qRT-PCR data was performed as described previously by Philips et al. (2015). The Shapiro-Wilk test was used to establish the normal distribution of data. Statistical analysis was performed using unpaired Student's t-test, one-way or two-way analysis for variance (ANOVA) followed by Newman-Keuls *post hoc* test for parametric analysis. Chi-square, Mann-Whitney U-test or Wilcoxon Rank Sum test (W) for non-parametric data analysis. Reported correlations were calculated using Spearman's rank-order method. Specific tests used per experiment have been specified in the figure legends. Values of $p < 0.05$ were considered significant and experimental values represent the mean ± SEM.

# 5. RESULTS

## 5.1. Deficit in emotional learning in neurotrimin knockout mice (Paper I)

### 5.1.1. Body weight, basic reflexes and viability (Paper I)

At 2.5 months of age, there were no differences in body weight between Ntm+/+, Ntm+/− and Ntm−/− animals (Table 4). Also, no differences were observed in the forepaw reach test, estimating vision, and ear twitch test, estimating hearing. The buried food finding test showed that invalidation of the *Ntm* gene does not abolish olfactory abilities and the hot plate test failed to reveal any differences between the three genotype groups in pain sensitivity (Table 4). Knockouts were viable both in utero and postnatally as the analysis of 150 litters revealed an expected Mendelian ratio of +/+, +/− and −/− genotypes.

**Table 4**. Overview of statistically insignificant differences between the genotypes. Results are presented as means ± SEM. Abbreviations: g = grams; % = percentage of animals that displayed the reflex/ability; s = seconds; cm/s = centimetres per second; min = minutes

| **Parameter** | **N (+/+, +/–, –/–)** | **Ntm+/+** | **Ntm+/–** | **Ntm–/–** | **Statistics** |
|---|---|---|---|---|---|
| Body weight (g) | 24, 23, 24 | 20.6±0.3 | 20.6±0.3 | 19.9±0.3 | F(2,68) = 2.12, p = 0.13 |
| Forepaw reach (%) | 8, 8, 8 | 100.00 | 100.00 | 100.00 | |
| Ear twitch test (%) | 8, 8, 8 | 100.00 | 100.00 | 100.00 | |
| Buried food finding (%) | 8, 8, 8 | 100.00 | 87.50 | 100.00 | |
| Hind paw response (s) | 8, 8, 8 | 17.7±1.4 | 17.8±1.3 | 17±1.1 | F(2,21) = 0.11, p = 0.89 |
| Elevated plus maze | 15, 17, 13 | | | | |
| Latency to open arm (s) | | 227±28 | 237±25 | 198±32 | F(2,42) = 0.48, p = 0.62 |
| Open arm entries | | 0.67±0.32 | 0.53±0.23 | 0.85±0.32 | F(2,42) = 0.3, p = 0.74 |
| Closed arm entries | | 7.9±0.9 | 9.4±0.7 | 9.6±1.1 | F(2,42) = 1.1, p = 0.34 |
| Time in open arms (s) | | 3.8±2.2 | 3.5±1.8 | 4.8±1.9 | F(2,42) = 0.11, p = 0.9 |
| Total head-dippings | | 2.3±0.5 | 2.3±0.5 | 2.8±0.7 | F(2,42) = 0.35, p = 0.71 |
| Unprotected head-dippings | | 0.07±0.07 | 0.24±0.11 | 0.31±0.21 | F(2,42) = 0.86, p = 0.43 |
| Light-dark box | 8, 8, 8 | | | | |
| Visits to bright side | | 25.1±3.5 | 26.3±3.8 | 34.6±3.1 | F(2,21) = 2.11, p = 0.15 |
| Time in bright side (s) | | 329±46 | 354±42 | 366±28 | F(2,21) = 0.22, p = 0.81 |
| Latency to bright side (s) | | 35.7±14.4 | 22.8±6.9 | 38.7±12.9 | F(2,21) = 0.6, p = 0.56 |
| Hyponeophagia | 8, 8, 8 | | | | |
| Latency to start eating (s) | | 156.7±29.2 | 173.8±31. | 115.7±19.9 | F(2,21) = 1.35, p = 0.28 |
| Morris water maze | 13, 11, 13 | | | | |
| Swim speed, day 1 (cm/s) | | 18±1.2 | 16±0.5 | 16.1±1.2 | F(2,34) = 1.11, p = 0.34 |
| Swim speed, day 5 (cm/s) | | 20.5±1.2 | 19±1.3 | 18.2±0.9 | F(2,34) = 1.10, p = 0.34 |
| Number of marbles buried | 8, 8, 8 | 8.3±0.8 | 8.38±1.3 | 9.1±1.35 | F(2,21) = 0.15, p = 0.86 |
| Nesting score | 8, 8, 8 | 3.6±0.5 | 3.3±0.5 | 3.5±0.6 | F(2,20) = 0.1, p = 0.9 |
| Social interaction | 15, -, 16 | | | | |
| Anogenital sniffing (s) | | 11.8±3.5 | - | 7.9±2.9 | F(1,29) = 1.5, p = 0.24 |
| Sniffing of other body parts (s) | | 31.7±4.7 | - | 20.5±2.9 | F(1,29) = 3.9, p = 0.06 |
| LORR / RRR with ethanol | 23, 20, 21 | | | | |
| Loss of righting reflex (min) | | 2.54±0.13 | 2.17±0.12 | 2.41±0.13 | F(2,61) = 1,72, p = 0.19 |
| Regain of righting reflex (min) | | 79.4±7.4 | 70.7±8.6 | 97.8±14.2 | F(2,61) = 1.55, p = 0.22 |

### 5.1.2. Locomotor activity test (Paper I)

There were no statistically significant differences between the genotype groups in the distance travelled (Ntm+/+ 77.9 ± 6.0; Ntm+/− 68.8 ± 5.9; Ntm−/− 85.3 ± 8.2; $F(2,41) = 1.55$, $p = 0.22$), in the number of rearings (Ntm+/+123 ± 22.7; Ntm+/− 84.1 ± 11.9; Ntm−/− 101.8 ± 34.3; $F(2,41) = 0.74$, $p = 0.49$) and in time spent in the central part of the box (Ntm+/+ 63.7 ± 14.6; Ntm+/− 64.2 ± 8.6; Ntm−/− 95.8 ± 16.4; $F(2,41) = 1.82$, $p = 0.17$) (Fig. 1).

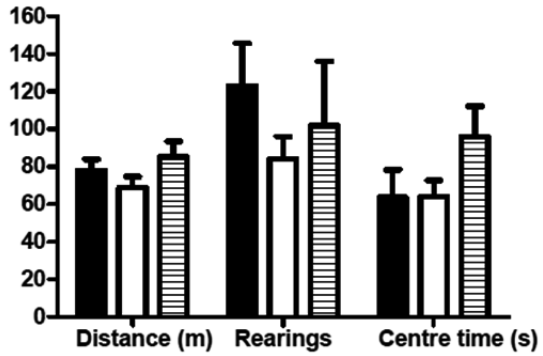



**Figure 1**. Distance travelled (in meters), the number of rearings performed, and time spent (in seconds) in the central part of the box in the locomotor activity test. Black columns = Ntm+/+ (N=15); white columns = Ntm+/− (N=16): striped columns = Ntm−/− (N=13). m – meters; s – seconds.

### 5.1.3. Elevated plus maze test (Paper I)

There were no statistically significant differences between the genotype groups in any of the parameters measured (Table 4).

### 5.1.4. Light-dark box test (Paper I)

No statistically significant differences between the genotype groups were detected in this test (Table 4).

### 5.1.5. Hyponeophagia test (Paper I)

The latency to start eating was similar in all the groups (Table 4).

### 5.1.6. Active avoidance test (Paper I)

All the genotypes showed progress on days 1–3, but Ntm+/+ mice and Ntm+/− mice had a much steeper learning curve than Ntm−/− mice (Fig. 2). The number of correct responses was affected by genotype [$F(2,58) = 6.6$, $p = 0.003$], day [$F(2,116) = 62.0$, $p = 0.0000$], and genotype × day interaction [$F(4,116) = 2.55$,

p = 0.04]. *Post hoc* comparison revealed that on day 3 both Ntm+/+ (p = 0.002) and Ntm+/− (p = 0.0002) mice had significantly more correct responses compared to Ntm−/− mice (Fig. 2).

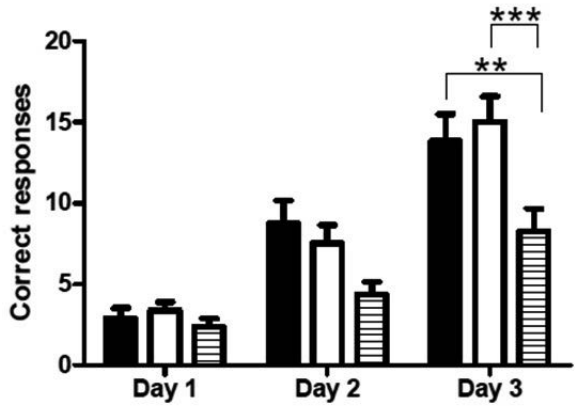



**Figure 2**. The learning curve (days 1–3) in the active avoidance test, showing the number of corrected responses on each day. Black columns – Ntm+/+ (N = 19); white columns – Ntm+/− (N = 23); striped columns – Ntm−/− (N = 19). **p < 0.01, ***p < 0.001, compared to Ntm−/− animals.

### 5.1.7. Morris water maze test (Paper I)

No significant differences between Ntm+/+, Ntm+/− and Ntm−/− mice were observed in any of the parameters measured. The latency to find the platform was dependent on day [F(3,102) = 59.5, p = 0.0000], but not genotype [F(2,34) = 0.92, p = 0.41] or genotype × day interaction [F(6,102) = 0.22, p = 0.97] (Fig. 3). There were no differences in swimming speed between the genotypes neither on day 1 nor on day 5 (Table 4), no thigmotaxis was observed and floating was minimal. In the probe trial on day 5 all the genotypes clearly preferred the target quadrant over the other three quadrants and displayed no differences between the groups. The number of platform crossings showed that the search strategy used by the groups was also similar in its effectiveness (Fig. 3).

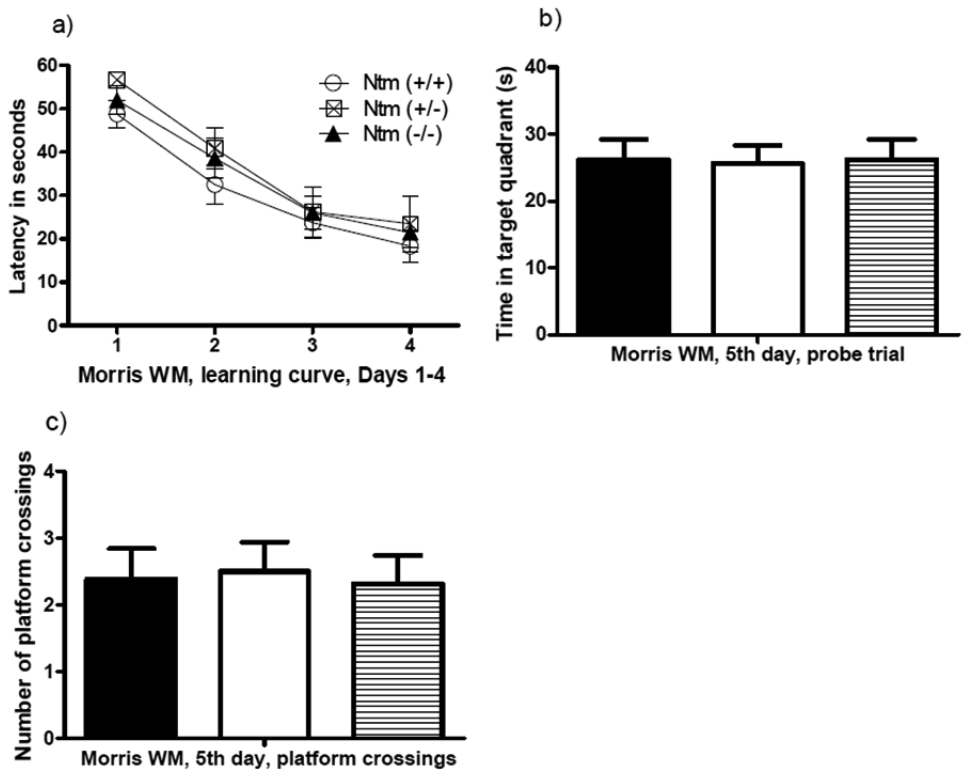



**Figure 3**. The learning curve (a) in the Morris water maze, showing the time (in seconds) to reach the submerged platform on days 1–4. Average values per day, obtained by collapsing data from 4 trials for each animal, are presented. In (b) and (c), time spent in the target quadrant and the number of platform crossings during the probe trial on day 5 are presented, respectively. Black columns – Ntm+/+ (N = 13); white columns – Ntm+/− (N = 11); striped columns – Ntm−/− (N = 13). WM – water maze.

### 5.1.8. Fear conditioning test (Paper I)

No freezing behaviour was evident in the pre-conditioning phase in any of the genotypes. In the contextual part, there was no difference between the groups in freezing time (Ntm+/+ 37.6 ± 16.5 s; Ntm+/− 42.1 ± 9 s; Ntm−/− 22.2 ± 5.4 s; $F(2,21) = 1.2$, $p = 0.32$). In the cued part, freezing time (no stimulus: Ntm+/+ 5.7 ± 5.7 s; Ntm+/− 23.4 ± 12.1 s; Ntm−/− 6.3 ± 4.6 s; sound stimulus: Ntm+/+ 39.8 ± 19.9 s; Ntm+/− 60.6 ± 24.9 s; Ntm−/− 31 ± 16.2 s) was dependent only on exposure to CS [$F(1,21) = 12.8$, $p = 0.002$], but not genotype [$F(2,21) = 0.87$, $p = 0.43$] or genotype × exposure to CS interaction [$F(2,21) = 0.19$, $p = 0.83$]. The number of rearings (no stimulus: Ntm+/+ 13.3 ± 3.5; Ntm+/− 8.9 ± 2.7; Ntm−/− 12.7 ± 5; sound stimulus: Ntm+/+ 11.4 ± 4.5; Ntm+/− 13.1 ± 10.5; Ntm−/− 9.9 ± 4.4) was not dependent on genotype [$F(2,21) = 0.02$, $p = 0.98$], exposure to CS [$F(1,21) = 0.001$, $p = 0.98$] or genotype × exposure to CS interaction [$F(2,21) = 0.27$, $p = 0.76$].

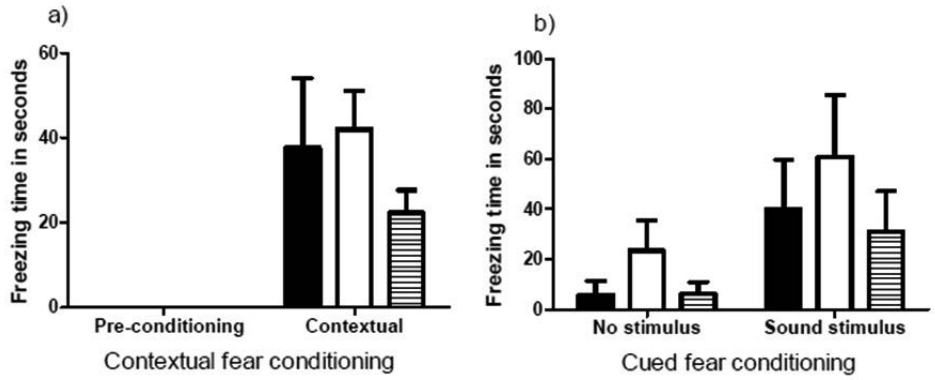



**Figure 4**. Results of the contextual (a) and cued (b) fear conditioning experiment. N = 8 in every group. Black columns – Ntm+/+; white columns – Ntm+/−; striped columns – Ntm−/−.

### 5.1.9. Marble burying test (Paper I)

There was no difference between the genotypes in the number of marbles buried (Table 4).

### 5.1.10. Nesting behaviour test (Paper I)

There were no differences between the nest scores (Table 4).

### 5.1.11. Social interaction test (Paper I)

Of the 15 Ntm+/+ and 16 Ntm−/− mice tested, only two Ntm+/+ mice and one Ntm−/− mouse displayed a very brief episode of aggressive behaviour. There was no difference between the genotypes in the duration of anogenital sniffing and in the duration of sniffing of other body parts (Table 4).

### 5.1.12. Locomotor activity test with 5 mg/kg of d-amphetamine (Paper I)

Distance travelled was affected by treatment [$F(1,37) = 279.3$, $p = 0.0000$] and genotype × treatment interaction [$F(2,37) = 3.85$, $p = 0.03$], but not genotype [$F(2,37) = 1.7$, $p = 0.2$]. *Post hoc* analysis showed that 5 mg/kg of amphetamine increased distance travelled significantly in all genotypes compared to respective saline groups ($p = 0.0001$ in all groups), but induced a much stronger activating response in Ntm+/+ mice compared to Ntm+/− ($p = 0.02$) and Ntm−/− mice ($p = 0.009$) (Fig. 5).

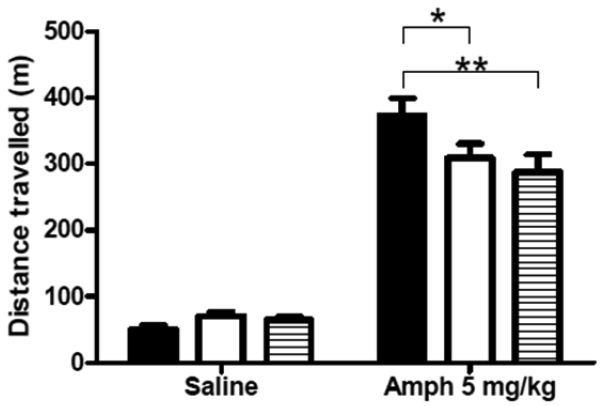



**Figure 5**. Results of the locomotor activity test with 5mg/kg amphetamine. N = 7-9 in every group. Black columns – Ntm+/+; white columns – Ntm+/−; striped columns – Ntm−/− . *p < 0.05, **p < 0.01.

### 5.1.13. Loss and regain of righting reflex test with 4 g/kg of ethanol (Paper I)

There were no differences between the genotypes in the latency to the loss of righting reflex and in the latency to the regain of righting reflex (Table 4).

## 5.2. Increased sensitivity to GABAergic drugs in Lsamp-deficient mice (Paper II)

### 5.2.1. Effects of diazepam in the elevated plus maze test (Paper II)

For closed arm entries: genotype: $F(1,39) = 0.36$, $p = 0.55$; treatment: $F(3,39) = 1.1$, $p = 0.36$; genotype × treatment: $F(3,39) = 1.1$, $p = 0.36$. For open arm entries: genotype: $F(1,39) = 3.88$, $p = 0.06$; treatment: $F(3,39) = 1.56$, $p = 0.21$; genotype × treatment: $F(3,39) = 0.99$, $p = 0.41$. For latency to open arm: genotype: $F(1,39) = 8.22$, $p = 0.0067$; treatment: $F(3,39) = 1.32$, $p = 0.28$; genotype × treatment: $F(3,39) = 0.39$, $p = 0.76$. Nevertheless, diazepam seems to decrease the time to enter into the open arm. For open arm time: genotype: $F(1,39) = 0.67$, $p = 0.42$; treatment: $F(3,39) = 1.78$, $p = 0.17$; genotype × treatment: $F(3,39) = 1.14$, $p = 0.34$. For protected head dips: genotype: $F(1,39) = 0.25$, $p = 0.62$; treatment: $F(3,39) = 0.93$, $p = 0.43$; genotype × treatment: $F(3,39) = 0.40$, $p = 0.76$. For unprotected head dips: genotype: $F(1,39) = 0.48$, $p = 0.49$; treatment: $F(3,39) = 0.10$, $p = 0.96$; genotype × treatment: $F(3,39) = 0.49$, $p = 0.69$ (Fig. 6a–f).

Thus no statistical differences between the genotypes in the elevated plus maze test with the administration of diazepam were identified. None of the

measured parameters showed statistical significance between the treatment and genotype groups. No *post hoc* differences were identified in any of the tests (Figure 6).

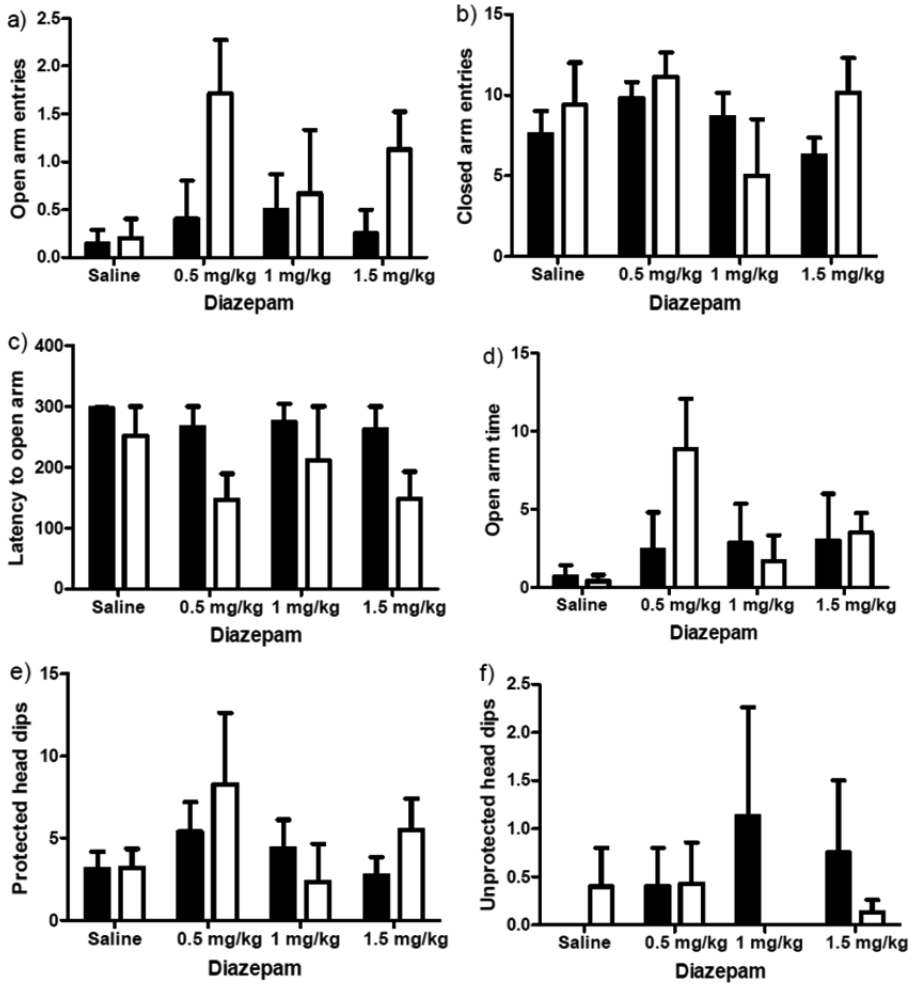



**Figure 6**. The elevated plus maze test with 0.5 mg/kg, 1 mg/kg and 1.5 mg/kg of diazepam. Six parameters – the number of open arm entries (a), the number of closed arm entries (b), the latency in seconds to enter an open arm (c), time in seconds spent on open arms (d), the number of protected head dips (e) and the number of unprotected head dips (f) – were calculated. N = 6–7 in every genotype × treatment group. Black columns – , white columns – Lsamp–/–.

### 5.2.2. Effects of diazepam in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,61) = 0.7$, $p = 0.41$; treatment: $F(2,61) = 4.1$, $p = 0.02$; genotype × treatment: $F(2,61) = 0.46$, $p = 0.64$. For the number of rearings: genotype: $F(1,61) = 0.41$, $p = 0.52$; treatment: $F(2,61) = 2.291$, $p = 0.11$; genotype × treatment: $F(2,61) = 0.13$, $p = 0.88$. For corner visits: genotype: $F(1,61) = 1.76$, $p = 0.19$; treatment: $F(2,61) = 3.94$, $p = 0.024$; genotype × treatment: $F(2,61) = 0.31$, $p = 0.73$. For the time spent in centre: genotype: $F(1,61) = 4.67$, $p = 0.035$; treatment: $F(2,61) = 0.21$, $p = 0.81$; genotype × treatment: $F(2,61) = 0.55$, $p = 0.58$. (Fig. 7a–d).

Thus no statistically significant differences between the genotypes in the locomotor activity test with the administration of diazepam were identified. None of the measured parameters showed statistical significance between the treatment and genotype groups. No *post hoc* differences were identified in any of the tests (Figure 7).

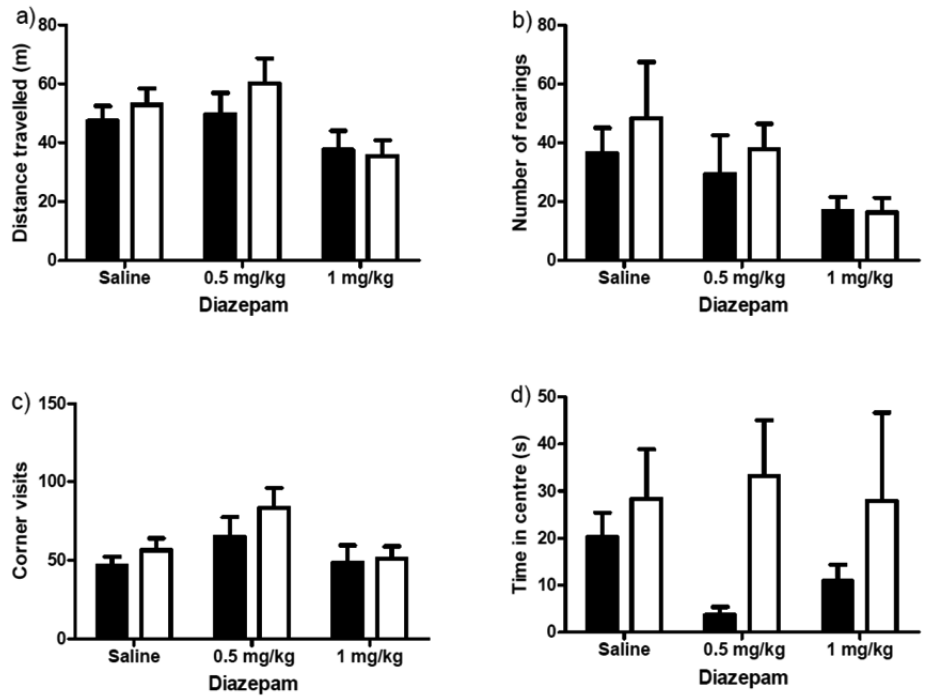



**Figure 7**. The locomotor activity test with 0.5 and 1.0 mg/kg of diazepam. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 8–9 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–.

### 5.2.3. Effects of diazepam in the loss and regain of righting reflex test (Paper II)

After the administration of 15 mg/kg of diazepam, Lsamp−/− mice lost the righting reflex significantly faster than their wild-type littermates [F(1,27) = 14.1, p = 0.00085]. However, the time to regain the righting reflex was only moderately longer in Lsamp−/− mice and the difference compared to wild-type animals was not significant [F(1,27) = 1.75, p = 0.2] (Fig. 8a–b).

### 5.2.4. Effects of ethanol in the loss and regain of righting reflex test (Paper II)

After the administration of 3.5 g/kg of ethanol, there was no difference in the latency to loss of righting reflex (LORR) between the genotypes [F(1,27) = 0.98, p = 0.33]. However, the time to regain the righting reflex (RRR) was significantly longer in Lsamp−/− animals [F(1,27) = 4.41, p = 0.045] (Fig. 8a–b).

### 5.2.5. Effects of pentobarbital in the loss and regain of righting reflex test (Paper II)

After the administration of 45 mg/kg of pentobarbital, Lsamp−/− mice lost the righting reflex much faster than wild-type animals [F(1,30) = 15.1, p = 0.00052]. However, there was no difference in the time to regain the righting reflex [F(1,30) = 0.69, p = 0.41] (Fig. 8a–b).

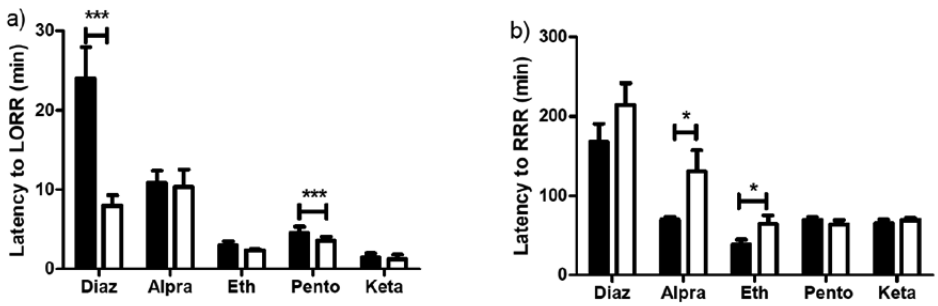



**Figure 8**. Loss (a) and regain (b) of righting reflex test with 15 mg/kg of diazepam (Diaz), 3 mg/kg of alprazolam (Alpra), 3.5 g/kg of ethanol (Eth), 45 mg/kg of pentobarbital (Pento) and 150 mg/kg of ketamine (Keta). *p < 0.05; ***p < 0.001 vs the other genotype. N = 14–15 in both genotype groups. Black columns – Lsamp+/+, white columns – Lsamp–/–.

### 5.2.6. Effects of diazepam in the stress-induced hyperthermia test (Paper II)

Baseline (60 min post-injection) body temperature T1 was significantly dependent on treatment: $F(3,40) = 6.72$, $p = 0.00089$, and tended to depend on genotype: $F(1,40) = 3.44$, $p = 0.071$, but not genotype × treatment interaction: $F(3,40) = 0.87$, $p = 0.47$. Compared to saline, 1.0 mg/kg of diazepam significantly decreased T1 temperature only in Lsamp−/− animals ($p = 0.047$), but not wild-type mice ($p = 0.11$). Stress-induced hyperthermia response (T2-T1) was not dependent on genotype: $F(1,40) = 0.1$, $p = 0.75$, treatment: $F(3,40) = 0.71$, $p = 0.55$ or genotype × dose interaction: $F(3,40) = 0.6$, $p = 0.62$ (Fig. 9a–b).

### 5.2.7. Effects of TP003 in the stress-induced hyperthermia test (Paper II)

Baseline (60 min post-injection) body temperature T1 was not affected by genotype: $F(1,71) = 2.36$, $p = 0.13$; treatment: $F(3,71) = 0.75$, $p = 0.53$; or genotype × treatment interaction: $F(3,71) = 0.19$, $p = 0.9$. Stress-induced hyperthermia response (T2-T1) was very significantly dependent on treatment: $F(3,71) = 9.61$, $p = 0.00002$, but not genotype [$F(1,71) = 1.14$, $p = 0.29$] or genotype × treatment interaction [$F(3,71) = 1.35$, $p = 0.26$]. *Post hoc* comparisons revealed that in wild-type mice, only the highest dose 3.0 mg/kg tended ($p = 0.067$) to decrease the SIH response, but in Lsamp−/− animals both 1.0 mg/kg ($p = 0.0015$) and 3.0 mg/kg ($p = 0.018$) significantly suppressed the SIH response compared to saline (Fig. 9c–d).

### 5.2.8. Effects of SL651498 in the stress-induced hyperthermia test (Paper II)

Baseline (60 min post-injection) body temperature T1 was not affected by genotype: $F(1,53) = 2.89$, $p = 0.095$; treatment: $F(3,53) = 0.2$, $p = 0.89$; or genotype × treatment interaction: $F(3,53) = 0.6$, $p = 0.62$. Stress-induced hyperthermia response (T2-T1) was also not affected by genotype: $F(1,53) = 0.12$, $p = 0.91$; treatment: $F(3,53) = 0.49$, $p = 0.69$; or genotype × treatment interaction: $F(3,53) = 0.72$, $p = 0.54$ (Fig. 9e–f).

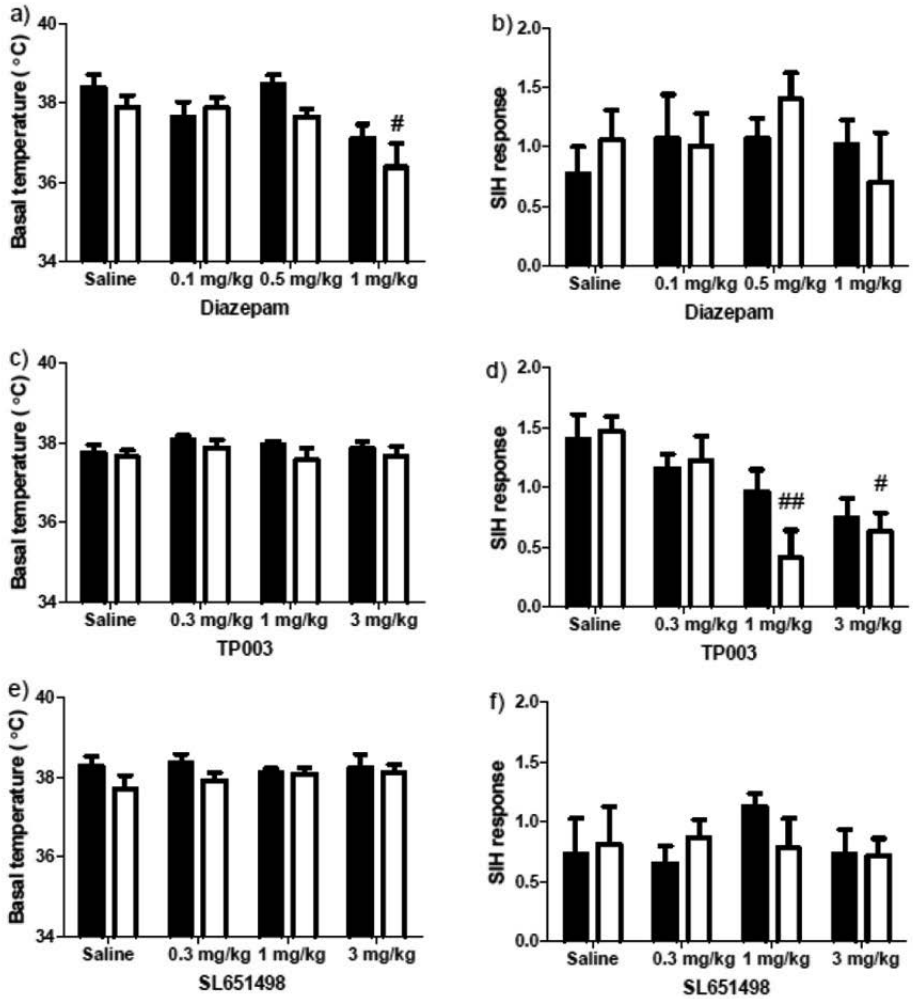



**Figure 9**. Stress-induced hyperthermia test with 0.1, 0.5 and 1.0 mg/kg of diazepam, 0.3, 1.0 and 3.0 mg/kg of TP003 and 0.3, 1.0 and 3.0 mg/kg of SL651498. Basal temperature (a, c, e) and SIH response (b, d, f) were measured for each drug. *$p < 0.05$, **$p < 0.01$ vs respective saline group. N = 9–11 in every genotype × treatment group. Black columns – Lsamp+/+, white columns – Lsamp–/–.

### 5.2.9. Effects of alprazolam in the elevated plus maze test (Paper II)

At dose level 0.15 mg/kg alprazolam seemed to have a strong muscle relaxation effect as 75% (6/8) of Lsamp−/− mice and 25% (2/8) of wild-type mice fell off the maze after wandering on the open arm; additionally, at a dose level 0.075 mg/kg 0% (0/8) of wild-type and 25% (2/8) of Lsamp−/− mice fell off the maze. As the fall may influence further behaviour, only one parameter, un-

affected by fall – latency to enter an open arm – was counted and it was dependent on genotype: $F(1,42) = 6.91$, $p = 0.012$; treatment: $F(2,42) = 16.69$, $p = 0.00000$; and genotype × treatment: $F(2,42) = 3.35$, $p = 0.045$. *Post hoc* tests showed that in Lsamp−/− mice the latency was shorter than in wild-type mice both at dose level 0.075 mg/kg ($p = 0.022$) and 0.15 mg/kg ($p = 0.023$) (Fig. 10).

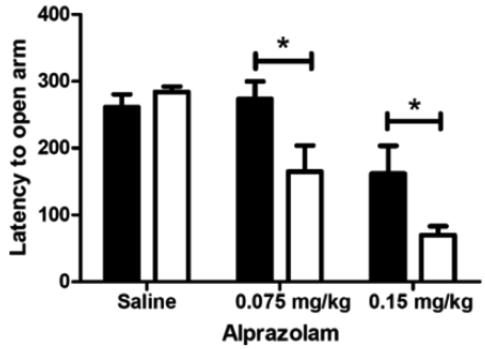



**Figure 10**. The elevated plus maze test with 0.075 mg/kg and 0.15 mg/kg of alprazolam. Because of a large number of falls from the maze in the Lsamp–/– group, only one parameter, unaffected by falls – the latency in seconds to enter an open arm – was calculated. N = 7–8 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–. *$p < 0.05$ vs the other genotype at the same dose level.

### 5.2.10. Effects of alprazolam in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,56) = 1.87$, $p = 0.18$; treatment: $F(1,56) = 6.12$, $p = 0.016$; genotype × treatment: $F(2,56) = 0.1$, $p = 0.75$. For the number of rearings: genotype: $F(1,56) = 0.21$, $p = 0.65$; treatment: $F(1,56) = 5.32$, $p = 0.025$; genotype × treatment: $F(1,56) = 0.47$, $p = 0.49$. For corner visits: genotype: $F(1,56) = 3.58$, $p = 0.064$; treatment: $F(1,56) = 0.27$, $p = 0.61$; genotype × treatment: $F(1,56) = 0.14$, $p = 0.71$. For the time spent in centre: genotype: $F(1,56) = 0.43$, $p = 0.52$; treatment: $F(1,56) = 0.29$, $p = 0.59$; genotype × treatment: $F(1,56) = 0.81$, $p = 0.37$ (Fig. 11a–d).

Thus no statistically significant differences between the genotypes in the locomotor activity test with the administration of alprazolam were identified. None of the measured parameters showed statistical significance between the treatment and genotype groups. No *post hoc* differences were identified in any of the tests (Figure 11).

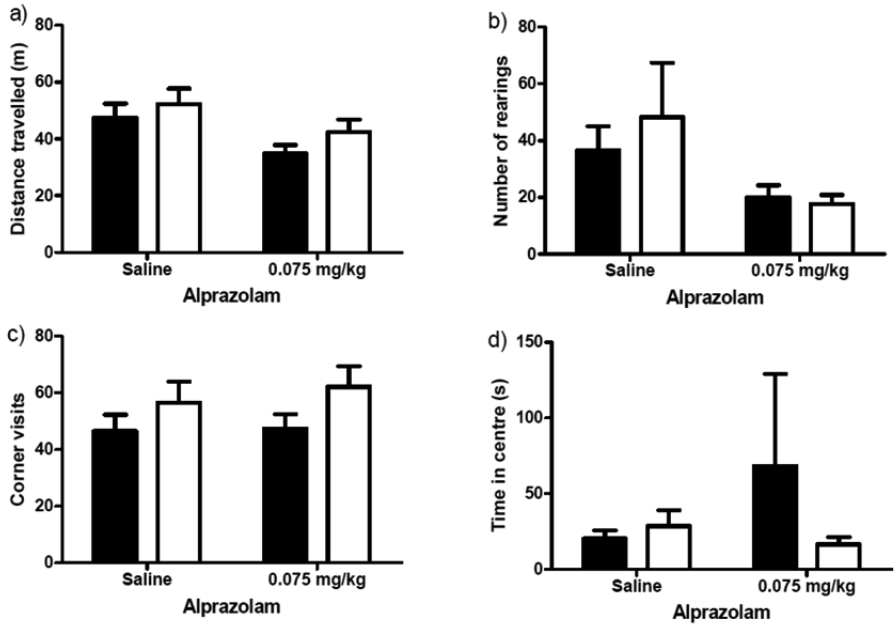



**Figure 11**. The locomotor activity test with 0.075 mg/kg of alprazolam. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 9–10 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–.

### 5.2.11. Effects of alprazolam in the loss and regain of righting reflex test (Paper II)

After the administration of 3 mg/kg of alprazolam, there was no difference in the latency to LORR between the genotypes [$F(1,10) = 0.038$, $p = 0.86$]. However, the time to regain the righting reflex was significantly longer in Lsamp−/− animals [$F(1,10) = 5.14$, $p = 0.047$] (Fig. 8).

### 5.2.12. Effects of ethanol in the elevated plus maze test (Paper II)

At dose level 2 g/kg 40% (4/10) of Lsamp−/− mice and only 10% (1/10) of wild-type mice fell off the maze. No falls were registered in the saline groups and at dose levels 0.5 g/kg and 1 g/kg one mouse from each dose × genotype group fell off the maze. Therefore, only one parameter, unaffected by falls – latency to open arm – was analysed with all the doses (saline, 0.5, 1 and 2 g/kg); all the other parameters were calculated for three doses (saline, 0.5 and 1 g/kg).

For closed arm entries: genotype: $F(1,48) = 0.49$, $p = 0.49$; treatment: $F(2,48) = 0.7$, $p = 0.5$; genotype × treatment: $F(2,48) = 0.2$, $p = 0.82$. For open arm entries: genotype: $F(1,48) = 16.5$, $p = 0.00018$; treatment: $F(2, 48) = 0.71$, $p = 0.5$; genotype × treatment: $F(2,48) = 0.6$, $p = 0.55$. For latency to open arm: genotype: $F(1,71) = 24.19$, $p = 0.00001$; treatment: $F(3,71) = 0.017$, $p = 1.00$;

genotype × treatment: F(3,71) = 0.45, p = 0.72. No *post hoc* differences. For open arm time: genotype: F(1,48) = 13.37, p = 0.00063; treatment: F(2,48) = 0.31, p = 0.74; genotype × treatment: F(2,48) = 0.072, p = 0.93. For protected head dips: genotype: F(1,48) = 0.66, p = 0.42; treatment: F(2,48) = 1.4, p = 0.26; genotype × treatment: F(2,48) = 0.96, p = 0.39. For unprotected head dips: genotype: F(1,48) = 14.94, p = 0.00033; treatment: F(2,48) = 0.52, p = 0.6; genotype × treatment: F(2,48) = 0.26, p = 0.77 (Fig. 12a–f).

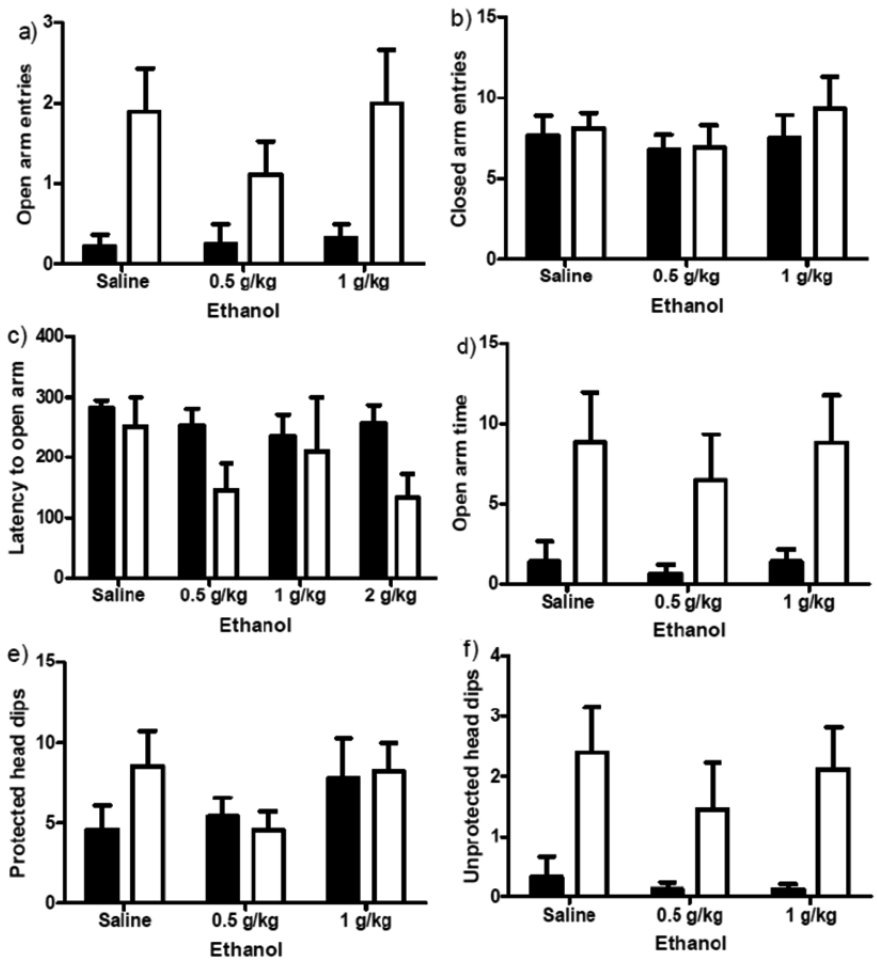



**Figure 12**. The elevated plus maze test with 0.5 g/kg, 1 g/kg and 2 g/kg of ethanol. Six parameters – the number of open arm entries (a), the number of closed arm entries (b), the latency in seconds to enter an open arm (c), time in seconds spent on open arms (d), the number of protected head dips (e) and the number of unprotected head dips (f) – were calculated. Because of an unequal number of falls in the genotype groups induced by the largest dose, only one parameter, unaffected by falls – the latency to enter an open arm – was calculated for all the doses. All the other parameters were calculated for three smaller doses. N = 8–10 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–.

Thus no differences between the genotypes in the elevated plus maze test with administration of ethanol were identified. None of the measured parameters showed statistical significance between the treatment and genotype groups. No *post hoc* differences were identified in any of the tests.

### 5.2.13. Effects of ethanol in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,85) = 7.04$, $p = 0.0095$; treatment: $F(3,85) = 9.13$, $p = 0.00003$; genotype × treatment: $F(3,85) = 1.17$, $p = 0.33$. Compared to saline, 1.0 g/kg of ethanol increased the distance significantly in wild-type mice ($p = 0.04$) and almost significantly ($p = 0.054$) in Lsamp−/− mice. However, compared to 1.0 g/kg, the largest (2.0 g/kg) dose decreased locomotor activity significantly only in Lsamp−/− mice ($p = 0.0026$), but not wild-type mice ($p = 0.21$). For the number of rearings: genotype: $F(1,85) = 1.28$, $p = 0.26$; treatment: $F(3,85) = 4.95$, $p = 0.0033$; genotype × treatment: $F(3,85) = 0.94$, $p = 0.43$. No *post hoc* differences. For corner visits: genotype: $F(1,85) = 3.43$, $p = 0.068$; treatment: $F(3,85) = 6.14$, $p = 0.00079$; genotype × treatment: $F(3,85) = 1.16$, $p = 0.33$. Compared to saline, 1.0 g/kg increased the number of corner visits significantly in both wild-type ($p = 0.038$) and Lsamp−/− mice ($p = 0.04$). For time spent in centre: genotype: $F(1,85) = 0.92$, $p = 0.34$; treatment: $F(3,85) = 0.54$, $p = 0.66$; genotype × treatment: $F(3,85) = 0.35$, $p = 0.79$ (Fig. 13a–d).

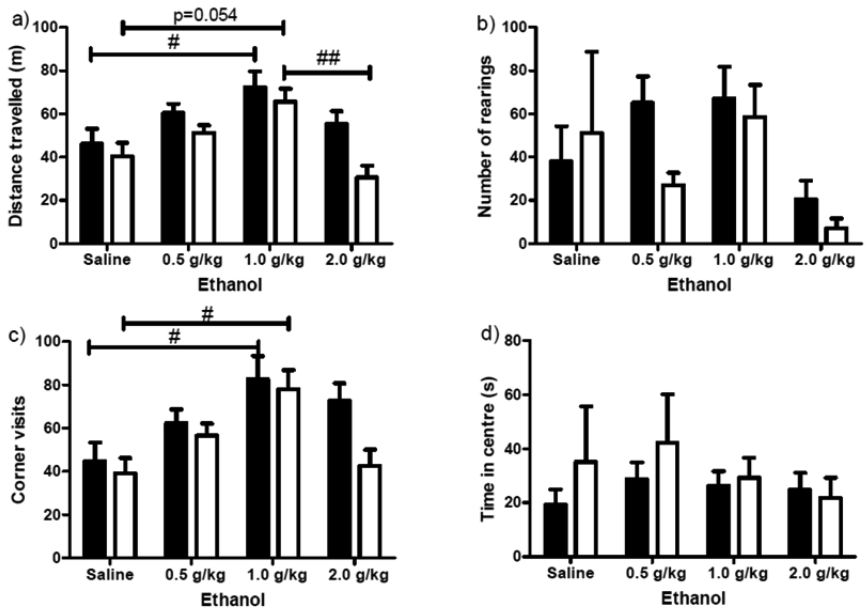



**Figure 13**. The locomotor activity tests with 0.5, 1.0 and 2.0 g/kg of ethanol. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 13–14 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–. #$p < 0.05$; ##$p < 0.01$ vs respective saline group.

## 5.3. Increased sensitivity to psychostimulants in Lsamp-deficient mice (Paper II)

### 5.3.1. Effects of cocaine in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,62) = 10.2$, $p = 0.0022$; treatment: $F(3,62) = 9.96$, $p = 0.00002$; genotype × treatment: $F(3,62) = 2.05$, $p = 0.12$. Compared to saline, 20 mg/kg of cocaine increased the distance significantly ($p = 0.00017$) in Lsamp−/− mice, but not in wild-type mice ($p = 0.28$). For the number of rearings: genotype: $F(1,62) = 0.39$, $p = 0.53$; treatment: $F(3,62) = 1.28$, $p = 0.29$; genotype × treatment: $F(3,62) = 1.57$, $p = 0.21$. For corner visits: genotype: $F(1,62) = 9.36$, $p = 0.0033$; treatment: $F(3,62) = 6.94$, $p = 0.00042$; genotype × treatment: $F(3,62) = 3.25$, $p = 0.028$. At the largest dose level 20 mg/kg Lsamp−/− mice performed significantly ($p = 0.00062$) more corner entries than wild-type mice. For the time spent in centre: genotype: $F(1,62) = 4.95$, $p = 0.03$; treatment: $F(3,62) = 1.28$, $p = 0.29$; genotype × treatment: $F(3,62) = 1.38$, $p = 0.26$. No *post hoc* differences (Fig. 14a–d).

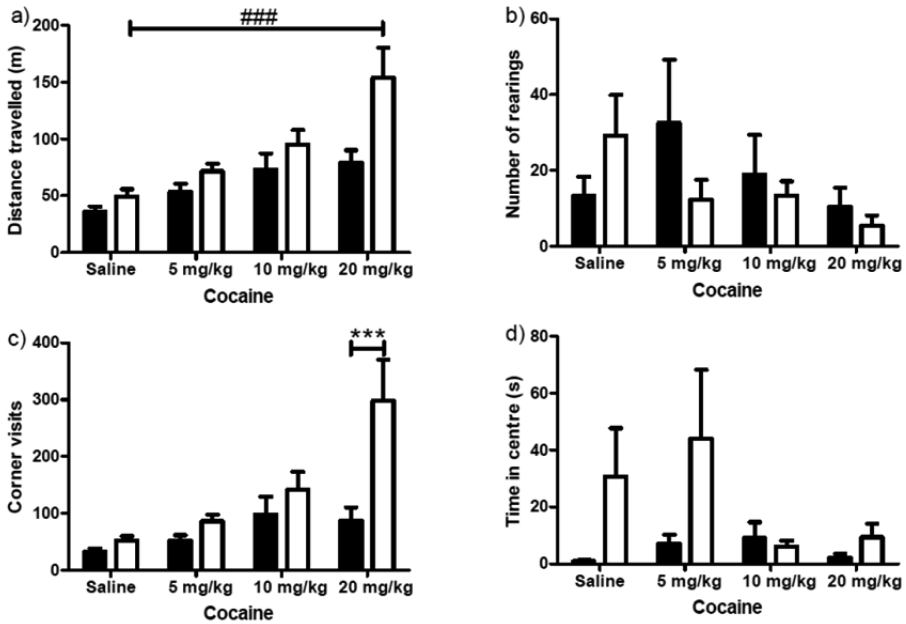



**Figure 14**. The locomotor activity test with 5, 10 and 20 mg/kg of cocaine. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 9–10 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns: Lsamp–/–. ***$p < 0.001$ vs another genotype at the same dose level. ###$p < 0.001$ vs saline group; ***$p < 0.001$ vs another genotype.

### 5.3.2. Effects of morphine in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,70) = 7.1$, $p = 0.0094$; treatment: $F(3,70) = 2.73$, $p = 0.05$; genotype × treatment: $F(3,70) = 2.08$, $p = 0.11$. Compared to saline, 20 mg/kg of morphine increased the distance significantly ($p = 0.015$) in Lsamp−/− mice, but not in wild-type mice ($p = 0.99$). For the number of rearings: genotype: $F(1,70) = 0.0027$, $p = 0.96$; treatment: $F(3,70) = 1.16$, $p = 0.33$; genotype × treatment: $F(3,70) = 0.71$, $p = 0.55$. For corner visits: genotype: $F(1,70) = 7.95$, $p = 0.0063$; treatment: $F(3,70) = 2.94$, $p = 0.039$; genotype × treatment: $F(3,70) = 2.8$, $p = 0.046$. At the largest dose level 20 mg/kg Lsamp−/− mice performed significantly ($p = 0.0059$) more corner entries than wild-type mice. For the time spent in centre: genotype: $F(1,70) = 5.33$, $p = 0.024$; treatment: $F(3,70) = 0.45$, $p = 0.72$; genotype × treatment: $F(3,70) = 0.98$, $p = 0.41$. No *post hoc* differences (Fig. 15a–d).

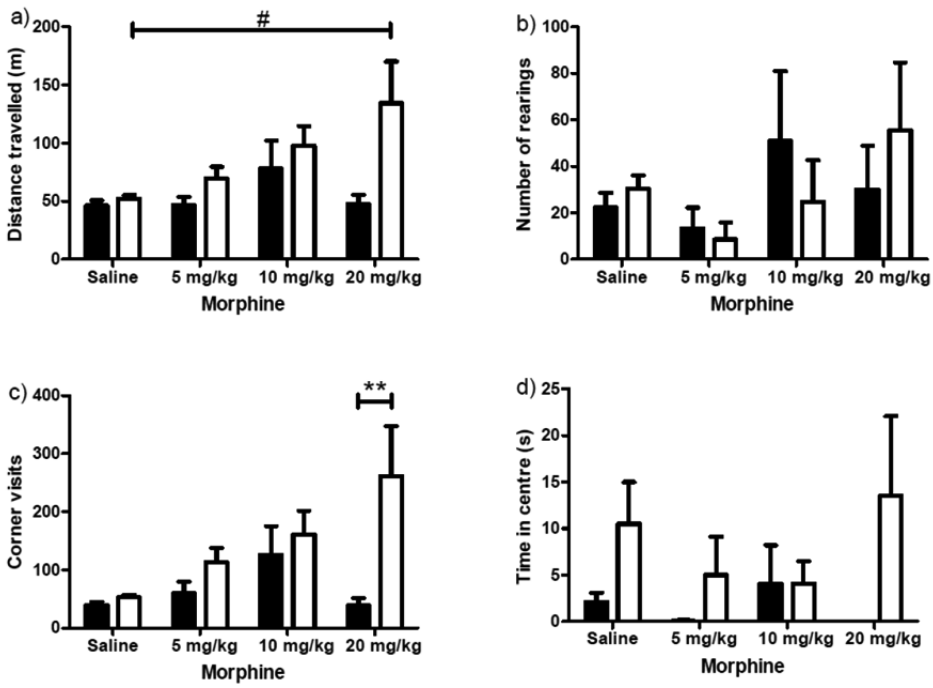



**Figure 15**. The locomotor activity tests with 5, 10 and 20 mg/kg of morphine. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 9–10 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–. **$p < 0.01$ vs another genotype at the same dose level. #$p < 0.05$ vs saline group.

### 5.3.3. Effects of MK-801 in the locomotor activity test (Paper II)

For distance travelled: genotype: $F(1,70) = 1.53$, $p = 0.22$; treatment: $F(3,70) = 9.84$, $p = 0.00002$; genotype × treatment: $F(3,70) = 0.29$, $p = 0.83$. No *post hoc* differences. For the number of rearings: genotype: $F(1,70) = 0.65$, $p = 0.42$; treatment: $F(3,70) = 11.2$, $p = 0.00000$; genotype × treatment: $F(3,70) = 0.12$, $p = 0.95$. No *post hoc* differences. For the corner visits: genotype: $F(1,70) = 6.08$, $p = 0.016$; treatment: $F(3,70) = 4.97$, $p = 0.0035$; genotype × treatment: $F(3,70) = 0.59$, $p = 0.62$. No *post hoc* differences. For the time spent in centre: genotype: $F(1,70) = 0.02$, $p = 0.89$; treatment: $F(3,70) = 3.77$, $p = 0.014$; genotype × treatment: $F(3,70) = 0.96$, $p = 0.42$. No *post hoc* differences (Fig. 16a–d).

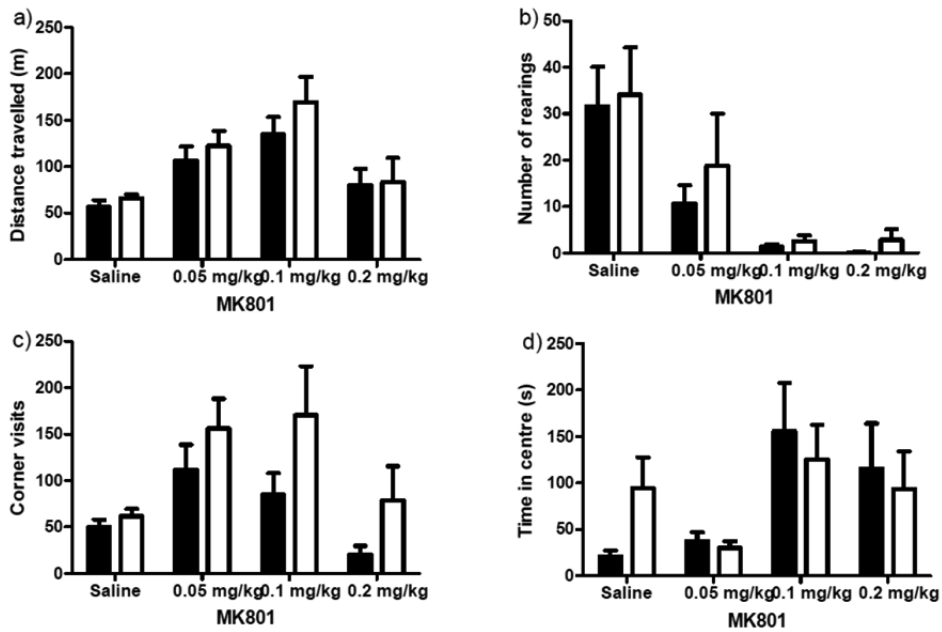



**Figure 16**. The locomotor activity test with 0.05, 0.1 and 0.2 mg/kg of MK-801. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. N = 9 in all genotype × treatment groups. Black columns – Lsamp+/+, white columns – Lsamp–/–.

### 5.3.4. Effects of ketamine in the loss and regain of righting reflex test (Paper II)

After the administration of 150 mg/kg of ketamine, there was no difference in the latency to lose [$F(1,30) = 1.13$, $p = 0.3$] or regain [$F(1,30) = 0.57$, $p = 0.46$] the righting reflex between Lsamp−/− and Lsamp +/+ mice (Fig. 8a–b).

## 5.4. Impact of Lsamp neural adhesion molecule in the serotonergic neurotransmission system (Paper III)

### 5.4.1. Effects of chronic administration of escitalopram on the body weight of mice (Paper III)

The body weight of Lsamp−/− and Lsamp+/+ mice was measured three weeks before beginning of the administration of escitalopram and continued during the whole experiment at eight distinct time-points (Fig. 17). Slight reduction in body weight can be seen at day 16, measured 2 days after the beginning of the behavioural investigations indicating a response to stress derived from the behavioural experiments. A two-way repeated-measures ANOVA was used to assess the changes over time, and differences between the groups (genotype × treatment). In multivariate tests the Wilks's Lambda was non-significant for all interactions. The body weight of saline-injected Lsamp−/− mice was significantly reduced compared to their wild-type littermates (main genotype effect $F = 7.675$; $p = 0.013$; Fig. 17, left). There was also a significant time-effect (main time effect $F = 18.095$, $p < 0.0001$), but no interaction (time × genotype $F = 1.166$; $p = 0.216$). Under escitalopram treatment, no significant differences could be observed between the body weights of Lsamp−/− and Lsamp+/+ mice (main genotype effect $F = 0.109$; $p = 0.745$; Fig. 17, right). There was a significant time-effect (main time effect $F = 330.157$, $p < 0.0001$), but no interaction (time × genotype $F = 1.765$; $p = 0.185$).

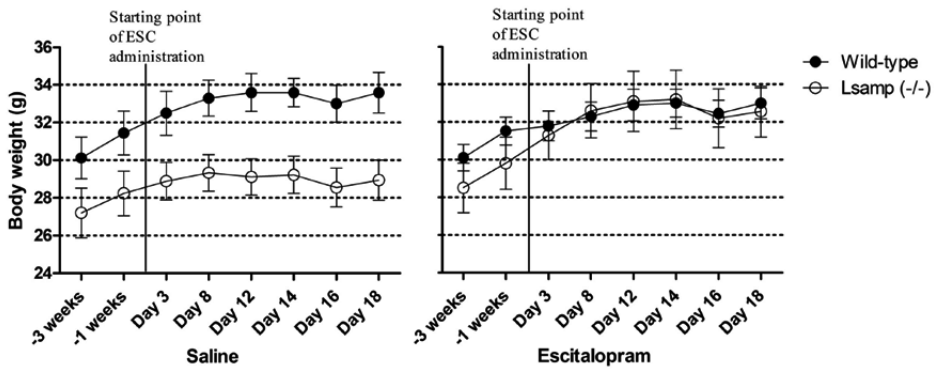



**Figure 17**. Body weight changes. The body weight was first measured three weeks before the first administration of escitalopram, the behavioural tests were performed on day 14 (elevated plus maze), day 16 (tail suspension test) and day 18 (open field test). After the last test, the mice were immediately decapitated and the brains dissected for the measurement of monoamines and their metabolites. N = 9 in the Lsamp−/− the saline group, N = 10 in all other groups. Error bars represent ± SEM; ESC – escitalopram, g – gram.

### 5.4.2. Effects of chronic administration of escitalopram on the behaviour of mice (Paper III)

#### 5.4.2.1. Effects of escitalopram in the elevated plus maze test

To study the effects of chronic escitalopram treatment on anxiety-like behaviours, we tested mice in the elevated plus maze.

The number of closed arm entries, reflecting locomotor activity, was heavily dependent on genotype [$F(1,35) = 49.8$; $p < 0.00001$], treatment [$F(1,35) = 17.5$; $p = 0.00018$] and genotype × treatment interaction [$F(1,35)=14.6$; $p = 0.00052$]. *Post hoc* comparisons with the Tukey HSD test revealed that in Lsamp−/− mice, escitalopram administration significantly increased the number of closed arm entries ($p < 0.001$) both compared to Lsamp−/− mice receiving saline and to Lsamp+/+ mice receiving escitalopram (Fig. 18a).

Open arm entries, which reflect the level of anxiety, were dependent on genotype [$F(1,35) = 7.5$; $p = 0.01$], but not on treatment [$F(1,35) = 0.011$; $p = 0.92$ or genotype × treatment interaction [$F(1,35) = 0.33$; $p = 0.57$] (Fig. 18b).

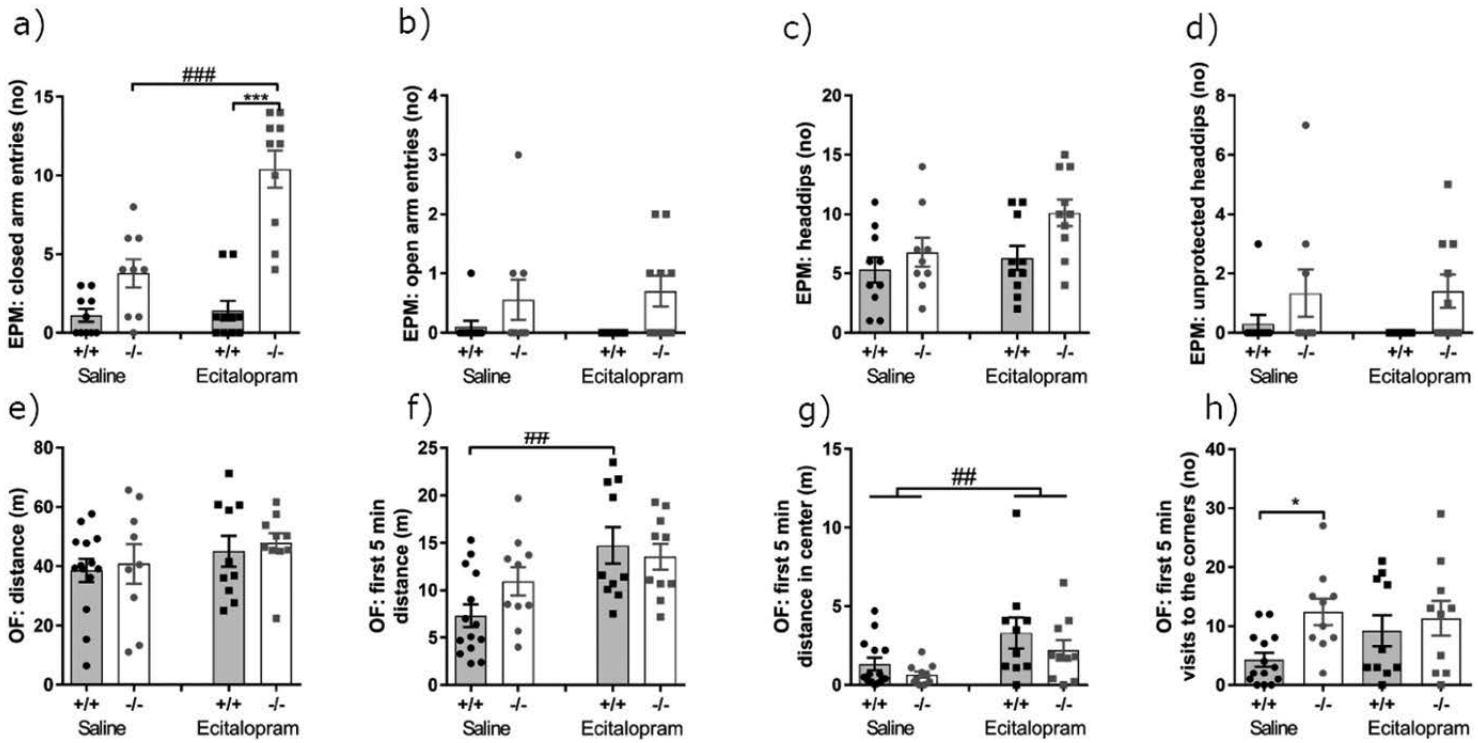
The latency to enter an open arm was also dependent on genotype [$F(1,35) = 10.1$; $p = 0.003$], but not on escitalopram treatment [$F(1,35) = 0.16$; $p = 0.7$] or genotype × treatment interaction [$F(1,35) = 0.93$; $p = 0.34$].

The time spent on open arms depended on genotype [$F(1,35) = 4.5$; $p = 0.04$], but not on treatment [$F(1,35) = 0.002$; $p = 0.97$] or genotype × treatment interaction [$F(1,35) = 0.43$; $p = 0.52$].

For protected head dips, a significant genotype main effect [$F(1,35) = 5.7$; $p = 0.023$] was established, but the effects of treatment [$F(1,35) = 3.8$; $p = 0.059$] and genotype × treatment interaction [$F(1,35) = 1.1$; $p = 0.3$] were not significant (Fig. 18c).

Unprotected head dips were dependent on genotype [$F(1,35) = 6.1$; $p = 0.018$], but not escitalopram treatment [$F(1,35) = 0.056$; $p = 0.81$] or genotype × treatment interaction [$F(1,35) = 0.14$; $p = 0.71$] (Fig. 18d).

The percentage of time that mice spent on the open arms tended to be higher in Lsamp−/− mice [$F(1,34) = 3.99$; $p = 0.054$], but neither treatment [$F(1,34) = 0.013$; $p = 0.91$] nor treatment × genotype interaction [$F(1,34) = 0.32$; $p = 0.58$] were significant. Similarly, another measure for anxiety-like behaviour, the percentage of open arm entries (of all arm entries) was also not affected by genotype [$F(1,34) = 2.30$; $p = 0.14$], treatment [$F(1,34) = 1.21$; $p = 0.28$] or their interaction [$F(1,34) = 0.00$; $p = 1.00$].


a)
EPM: closed arm entries (no)
###
***
b)
EPM: open arm entries (no)
c)
EPM: headdips (no)
d)
EPM: unprotected headdips (no)
e)
OF: distance (m)
f)
OF: first 5 min distance (m)
##
g)
OF: first 5 min distance in center (m)
##
h)
OF: first 5 min visits to the corners (no)
*
+/+
-/-
Saline
Ecitalopram

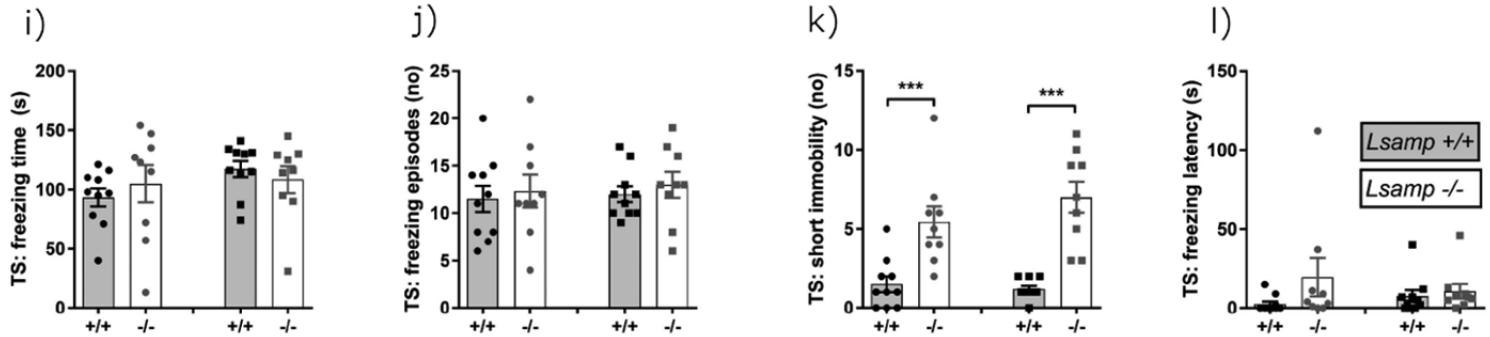



**Figure 18**. Behaviour of Lsamp+/+ and Lsamp−/− mice in the elevated plus maze, EPM (a-d), open field, (OF, e-h) and tail suspension (TS, i-l) tests. Chronic escitalopram had a significant impact on behaviour in the elevated plus maze, but not in the open field and tail suspension tests. In the elevated plus maze, escitalopram significantly increased general motor activity reflected by an increased number of closed arm entries (a) by Lsamp−/− mice; this effect was not present in their wild-type littermates. Only the genotype effect was present in the open arm entries (b), protected head dips (c) and unprotected head dips (d). In the open field test no statistical differences were found in the general distance during 30 min testing (e), the distance travelled during the first 5 min was significantly increased after administration of escitalopram only in wild-type mice (f); escitalopram had significant treatment effect to the distance travelled in the centre (g); Lsamp−/− mice in the saline group made more corner visits during the first 5 min of the open field test compared with their wild-type littermates (h). No significant effects were found in the tail suspension test related to freezing time (i), number of freezing episodes (j) and latency to freeze (l). Lsamp−/− mice displayed a specific behavioural pattern in the tail suspension test that was not dependent on the injected substance: a significantly higher number of short (1–2 s) episodes of immobility compared with their wild-type littermates (k). N = 9 in the Lsamp−/− the saline group, N = 10 in all other genotype x treatment groups. An exception was the open field test, where N = 14 in the Lsamp+/+ treatment group and N = 10 in all other genotype x treatment groups. *p < 0.05, ***p < 0.001 – genotype effects; ##p < 0.01, ###p < 0.001 – treatment effects.

### 5.4.2.2. Effects of escitalopram in the open field test

To assess the locomotor activity and anxiety-like phenotype of the mice, we conducted the open field test.

Distance travelled during the 30 min of testing, reflecting locomotor activity, was not dependent on genotype [$F(1,40) = 0.59$; $p = 0.45$], treatment [$F(1,40) = 1.19$; $p = 0.28$] or their interaction [$F(1,40) = 0.08$; $p = 0.78$] (Fig. 18e).

Time spent in the central square, reflecting anxiety levels, was also not dependent on genotype [$F(1,40) = 0.037$; $p = 0.85$], treatment [$F(1,40) = 0.78$; $p = 0.38$] or their interaction [$F(1,40) = 1.68$; $p = 0.2$].

For distance travelled in the central square, no significant main effects of genotype [$F(1,40) = 0.024$; $p = 0.88$], treatment [$F(1,40) = 1.68$; $p = 0.2$] or their interaction [$F(1,40) = 0.057$; $p = 0.81$] were established.

For distance covered in corners, which is another parameter of locomotor activity, a statistically significant genotype effect [$F(1,40) = 5.24$; $p = 0.028$] was evident, caused mainly by higher ambulation in the corners in the Lsamp−/− group receiving saline; however, distance in the corners was not dependent on treatment [$F(1,40) = 0.78$; $p = 0.38$] nor genotype × treatment interaction [$F(1,40) = 1.29$; $p = 0.26$].

As differences in anxiety-like behaviour are often most pronounced early in novel environments, we separately analysed the first 5 min of the open field behaviour.

The distance travelled during the first 5 min of testing was affected by treatment as escitalopram-treated mice covered longer distances [$F(1,40) = 11.20$; $p = 0.0018$] (Fig. 18f). This difference was caused by wild-type mice (*post hoc* $p = 0.0018$). The distance travelled during the first 5 min was not dependent on genotype [$F(1,40) = 0.66$; $p = 0.42$] or genotype × treatment interaction [$F(1,40) = 2.56$; $p = 0.12$].

Time spent in the central square during the first 5 min was not dependent on genotype [$F(1,40) = 2.38$; $p = 0.13$], treatment [$F(1,40) = 0.66$; $p = 0.42$] or their interaction [$F(1,40) = 0.39$; $p = 0.54$].

The distance travelled in the central square during the first 5 min was affected by treatment as escitalopram treated mice covered longer distances [$F(1,40) = 8.39$; $p = 0.006$] (Fig. 18g); *post hoc* comparison did not indicate significant differences in Lsamp−/− or wild-type mice separately. There was no main effect of genotype [$F(1,40) = 2.17$; $p = 0.15$] nor genotype × treatment interaction [$F(1,40) = 0.13$; $p = 0.72$] in distance travelled in the central square during the first 5 min.

Corner visits during the first 5 min were affected by genotype, as Lsamp−/− mice showed higher number of visits; the difference originated from saline-treated mice (*post hoc* $p = 0.047$) (Fig. 18h). There was no main effect of treatment on corner visits during the first 5 min [$F(1,40) = 0.74$; $p = 0.39$] nor genotype × treatment interaction [$F(1,40) = 1.86$; $p = 0.18$].

#### 5.4.2.3. Effects of escitalopram in the tail suspension test

To assess the depressive-like phenotype of the mice, we conducted the tail suspension test. Freezing time, reflecting behavioural despair, was not dependent on the genotype [$F(1,35) = 0.023$; $p = 0.88$], escitalopram treatment [$F(1,35) = 0.99$; $p = 0.32$] or their interaction [$F(1,35) = 1.461$; $p = 0.24$] (Fig. 18i). The number of freezing episodes was also not dependent on genotype [$F(1,35) = 0.033$; $p = 0.86$], escitalopram treatment [$F(1,35) = 0.002$; $p = 0.96$] or genotype × treatment interaction [$F(1,35) = 0.15$; $p = 0.70$] (Fig. 18j). Remarkably, Lsamp-deficient mice showed an increased number of short immobility episodes compared to the wild-type controls [$F(1,35) = 33.4$; $p < 0.00001$]. The number of short freezing episodes was neither affected by escitalopram treatment [$F(1,35) = 0.126$; $p = 0.72$] nor genotype × treatment interaction [$F(1,35)= 0.55$; $p = 0.47$] (Fig. 18k). The freezing latency was neither affected by genotype [$F(1,35) = 2.41$; $p = 0.13$], escitalopram treatment [$F(1,35) = 0.56$; $p = 0.46$] nor genotype × treatment interaction [$F(1,35) = 0.17$; $p = 0.68$] (Fig. 18l).

### 5.4.3. Effects of chronic administration of escitalopram on the level of monoamines in the mouse brain (Paper III)

#### 5.4.3.1. Effects of escitalopram on serotonin and its metabolite 5-HIAA

Serotonin (5-HT) and its metabolite 5-HIAA were measured in five brain regions. 5-HT turnover was calculated as the 5-HIAA/5-HT ratio. Remarkably, differences between the Lsamp−/− and control mice, most often in 5-HT turnover levels, were observable in all five brain regions studied.

In the raphe nuclei, 5-HT turnover was significantly higher in Lsamp-deficient mice receiving saline ($p = 0.034$) compared to the wild-type mice receiving saline. Escitalopram treatment reduced 5-HT turnover significantly ($p = 0.011$) only in Lsamp−/− mice (Fig. 19c, in raphe, but also other nuclei). Chronic escitalopram reduced the level of 5-HT significantly ($p = 0.032$) only in Lsamp−/− mice (Fig. 19a, in raphe). In Lsamp−/− mice receiving saline, 5-HIAA level was significantly ($p = 0.00048$) higher than in the corresponding wild-type group. Escitalopram reduced the level of 5-HIAA significantly ($p = 0.00053$) only in Lsamp−/− mice (Fig. 19b, in raphe).

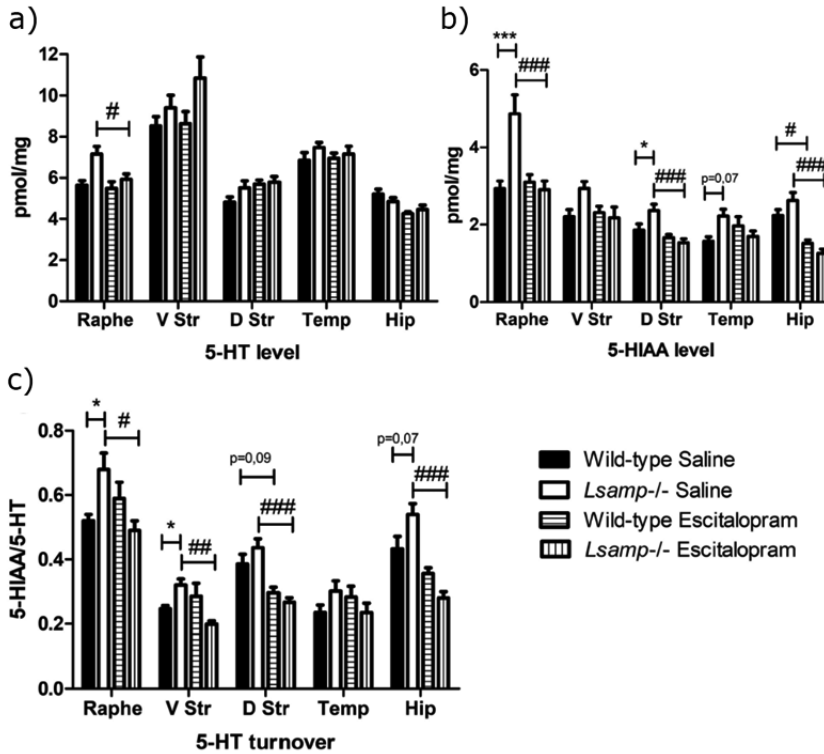



**Figure 19**. Effects of escitalopram on serotonin and its metabolism in the five brain regions of Lsamp+/+ and Lsamp−/− mice. 5-HT (a), 5-HIAA (b) and 5-HT turnover (5-HIAA/5-HT) (c) levels in the raphe, ventral striatum (V Str), dorsal striatum (D Str), temporal lobe including amygdala (Temp), and hippocampus (Hip). #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ – treatment effects; *$p < 0.05$; ***$p < 0.001$ – genotype effects.

In the ventral striatum, 5-HT turnover was significantly higher in Lsamp−/− mice receiving saline, than in the corresponding wild-type group ($p = 0.028$). Escitalopram administration reduced 5-HT turnover significantly ($p = 0.0026$) only in Lsamp−/− mice (Fig. 19c, V Str).

In the dorsal striatum, escitalopram decreased 5-HT turnover significantly ($p = 0.0002$) in Lsamp−/− mice, but in wild-type mice the turnover was not significantly different ($p = 0.09$) (Fig. 19c, D Str). Also, the level of 5-HIAA was significantly higher in Lsamp−/− mice receiving saline ($p = 0.042$) than in wild-type mice receiving saline. Escitalopram administration reduced the level of 5-HIAA statistically significantly ($p = 0.00052$) only in Lsamp−/− mice (Fig. 19b, D Str).

In the temporal lobe, there were no significant differences, however, the 5-HIAA level in Lsamp−/− mice receiving saline tended ($p = 0.074$) to be higher than in similarly treated wild-type mice (Fig. 19b, Temp).

In the hippocampus, the 5-HT turnover in Lsamp−/− mice receiving saline tended ($p = 0.066$) to be higher than in wild-type littermates treated similarly.

Escitalopram administration reduced 5-HT turnover significantly (p = 0.00016) only in Lsamp−/− mice (Fig. 19c, Hip). There was no difference between the 5-HIAA baseline levels, however, escitalopram reduced 5-HIAA levels in both groups and, notably, in Lsamp−/− mice to a greater extent (p = 0.00016) than in wild-type animals (p = 0.01) (Fig. 19b, Hip).

### 5.4.3.2. Effects of escitalopram on dopamine and its metabolites

Dopamine (DA) and its metabolites DOPAC and HVA were measured in five brain regions. (Fig. 20).

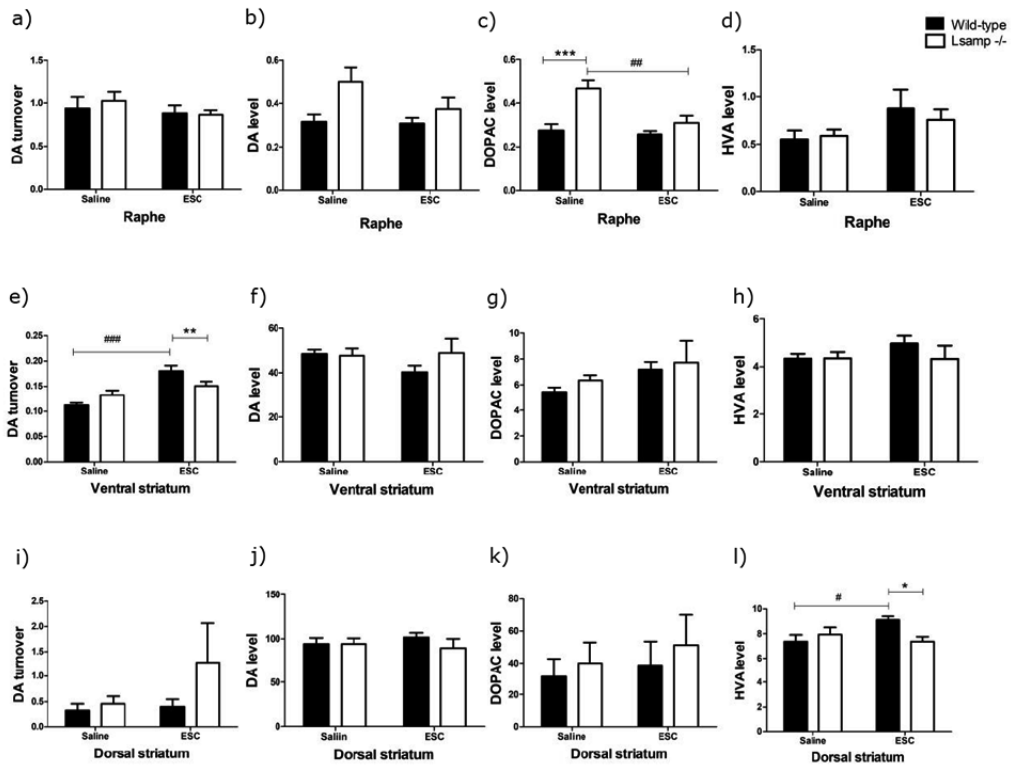



**Figure 20**. The levels of DA and its metabolites in three brain regions, including DA turnover. DA turnover was calculated as the (DOPAC+HVA)/DA ratio. In the raphe nuclei, in the saline group, Lsamp−/− mice had a significantly (p = 0.00046) higher level of DOPAC and escitalopram decreased the level of DOPAC significantly (p = 0.0042) only in Lsamp−/− mice. In the ventral striatum, escitalopram increased DA turnover only in wild-type mice (p = 0.00017) and in the escitalopram group wild-type mice had significantly (p = 0.0014) higher DA turnover than Lsamp−/− mice. In the dorsal striatum, escitalopram increased HVA level only in wild-type mice (p = 0.049) and in the escitalopram group wild-type mice had significantly (p = 0.049) higher HVA level than Lsamp−/− mice. No significant *post hoc* effects were observed in the temporal lobe or hippocampus (not shown).

#### 5.4.3.3. Effects of escitalopram on noradrenaline and its metabolite NMN

Noradrenaline (NA) and its metabolite NMN were measured in five brain regions. NA turnover was calculated as the NMN/NA ratio. No *post hoc* differences were evident in any of the brain regions (raphe nuclei, ventral striatum, dorsal striatum, temporal lobe, hippocampus).

## 5.5. Behavioural profiling indicated an interplay between Lsamp and Ntm (Paper IV)

### 5.5.1. Open field test (Paper IV)

The locomotor and exploratory activity were evaluated through the open field activity test using motility boxes. The distance travelled in the motility box during the 30 min testing was not dependent on genotype [$F(3,41) = 2,1$, $p = 0.1$]) (Fig. 21a). However, the number of rearings was dependent on genotype [$F(3,41) = 3.21$, $p < 0.05$]. Wild-type and Ntm−/− mice displayed approximately twice as much vertical activity as Lsamp−/− mice and Lsamp−/−Ntm−/− double deficient mice, while the *post hoc* comparisons remained at the tendency level (Fig. 21b). The distance travelled in the centre [$F(3,41) = 3.63$, $p < 0.05$] was significantly dependent on genotype – it was significantly longer in Ntm−/− mice, who demonstrated anxiolytic-like baseline behaviour in comparison with all the other three groups (Fig. 21c). For the effect of d-amphetamine on distance travelled in the motility boxes, repeated measures ANOVA revealed a significant treatment effect [$F(1,14) = 8.1$, $p = 0.013$], but the genotype effect [$F(1,14) = 1.26$, $p = 0.28$] was insignificant and genotype × treatment effect remained at a tendency level [$F(1,14) = 3.34$, $p = 0.089$].

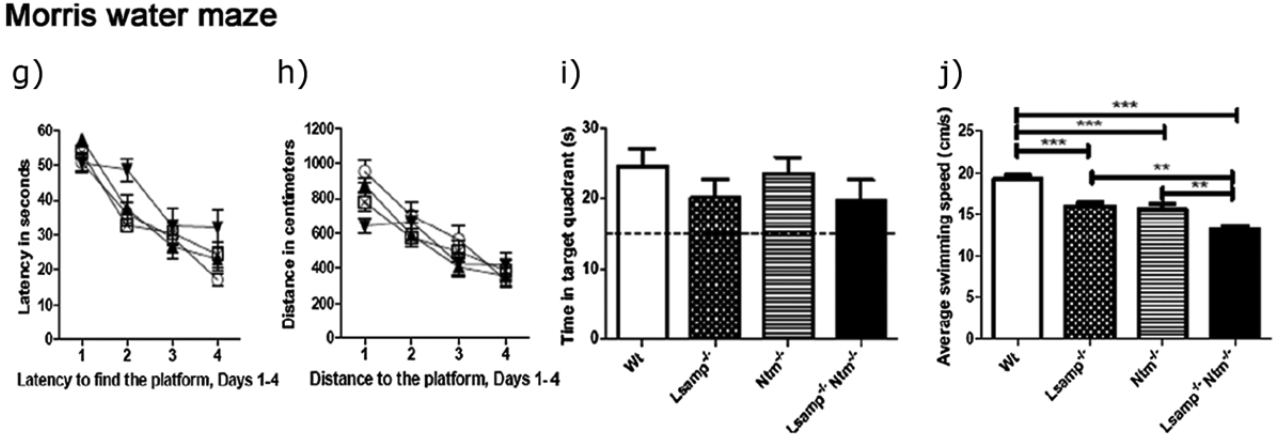



**Figure 21**. Behavioural analysis of Lsamp−/−, Ntm−/− and Lsamp−/− Ntm−/− mice in motility box (a–c), EPM (d–f) and Morris water maze (g–j). Distance travelled (a), the number of rearings performed (b), and distance travelled in the central part of the motility box (c) in the locomotor activity test in Lsamp−/− mice (N=11), Ntm−/− mice (N=10), Lsamp−/− Ntm−/− mice (N=10) and wild-type mice (N=14). Closed arm entries (d), open arm entries (e), and protected head dips (f) in the elevated plus maze in wild-type (N=11), Lsamp−/− (N=11), Ntm−/− (N=10) and Lsamp−/− Ntm−/− mice (N=10). The learning curve (g, h) shows the time (in s) and distance (in cm) to reach the submerged platform on days 1–4. Average values per day, obtained by collapsing data of four trials for each animal, are presented in the Morris water maze. Time spent in the target quadrant (i) and swimming speed (cm/s) (j) in Lsamp−/− mice (N=10), Ntm−/− mice (N=10), Lsamp-/- Ntm-/- mice (N=10) and wild-type mice (N=12) during the Morris water maze experiment. Data are presented as±SE (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test), (cm=centimetres; m=metres; s=seconds). Dotted line in (i) denotes chance level (15 s).

### 5.5.2. Light/dark box

Anxiety-like behaviours in mice were evaluated using the light/dark box. This test is based on spontaneous exploratory behaviour in a novel environment which contains a protected area (the dark compartment) and a naturally aversive unprotected area (the light compartment). None of the three anxiety-related parameters measured in the light/dark test — the number of visits to the light compartment, the time spent in the light compartment, and the latency to enter the light compartment — were dependent on genotype (data not shown).

### 5.5.3. Elevated plus-maze

The EPM is used to assess anxiety-like behaviour in mice. The number of entries mice made into the central platform of the EPM was not dependent on genotype [$F(3,38) = 2,43$, $p = 0.08$]. The time (s) that mice spent in the central platform of the EPM was also not dependent on genotype [$F(3,38) = 1,43$, $p = 0.25$]. On average, wild-type mice spent 15 ± 4.4 s, Lsamp−/− mice 12.7 ± 2.7 s, Ntm−/− mice 21.2 ± 3.6 s and Lsamp−/−Ntm−/− mice 22 ± 5 s in the central platform of the EPM. The number of closed arm entries was dependent of genotype [$F(3,38) = 4.05$, $p = 0.014$] and according to the *post hoc* analysis, wild-type mice performed significantly less closed arm entries than Lsamp−/−Ntm−/− mice ($p = 0.016$) (Fig. 21d). The number of open arm entries was also dependent on genotype [$F(3,38) = 4.79$, $p = 0.006$] and the *post hoc* analysis showed that Lsamp−/−Ntm−/− mice performed significantly more open arm entries than wild-type ($p = 0.008$) and Ntm−/− mice ($p = 0.026$) and the difference compared to Lsamp−/− mice verged on significance ($p = 0.06$) (Fig. 21e). Protected head dips were dependent on genotype [$F(3,38) = 2.9$, $p = 0.047$], but no *post hoc* differences were detected (Fig. 21f).

### 5.5.4. Morris water maze

The Morris water maze was used to study spatial learning and memory in mice (Jeffery and Morris, 1993). The mice have to rely on visual cues placed around the perimeter of the swimming arena to find the location of the submerged escape platform. Spatial learning is evaluated against repeated trials and the reference memory is analysed by preference for the platform area when the platform is absent. For the latency to find the submerged platform in days 1–4, there was a clear learning effect [$F(3,114) = 62.6$, $p < 0.0001$] and both genotype effect [$F(3,38) = 2.67$, $p = 0.061$] and the genotype × day interaction [$F(9,114) = 1.91$, $p = 0.058$] approached significance with Lsamp−/−Ntm−/− mice tending to display longer latencies on days 2 and 4 (Fig. 21g–h). However, this should not be interpreted as a learning deficiency as there were clear differences in swimming speed between the genotype groups (see below). Therefore, the distance to the platform is a more objective parameter for expressing the learning curve. The distance was likewise very significantly dependent on day [$F(3,114) = 46.19$, $p < 0.0001$], but not genotype [$F(3,38) = 1.67$, $p = 0.19$].

Genotype × day interaction was significant [$F(9,114) = 2.14$, $p = 0.031$], but *post hoc* comparisons revealed differences between the groups only on the first day (most notably, wild-type vs Lsamp−/−Ntm−/− mice, $p = 0.004$), which reflects differences in swimming speed. The probe trial on day 5 confirmed the lack of learning deficiency in transgenic mice groups as all the genotypes clearly preferred the target quadrant (Fig. 7I) and there were no differences between the groups [$F(3,38) = 0.87$, $p = 0.47$]. Swimming speed was very significantly dependent on genotype [$F(3,38) = 24.19$, $p < 0.0001$]; both single deficient groups swam slower than wild-type mice and the simultaneous deletion of *Lsamp* and *Ntm* genes further aggravated the swimming speed deficiency compared to single deficient mice (Fig. 21j).

# 6. DISCUSSION

## 6.1. Initial phenotype of Ntm deficient mice (Paper I)

This study gives an initial characterisation of the phenotype of Ntm deficient mice. It is evident that these mice have normal body weight, no gross vision, hearing or olfaction deficiencies and no alterations in pain sensitivity. Ntm and Lsamp are co-localised in a few brain regions and act as heterophilic dimers, so we expected to find at least some overlap in the phenotypes of Lsamp−/− and Ntm−/− mice as in colocalisation regions the deletion of either partner should result in the lack of functional heterodimers. However, Ntm−/− mice had much less differences compared to wild-type mice than Lsamp−/− mice in studies by Innos et al. (2011, 2012, 2013). Unlike Lsamp−/− mice, Ntm−/− mice had no differences in anxiety, social interaction and locomotor activity, displayed normal exploratory activity, barbering behaviour and swimming speed, and showed no altered sensitivity to the sedative effect of ethanol. As for overlapping changes in phenotype, similarly to Lsamp−/− mice, Ntm−/− mice had lower sensitivity to the locomotor stimulating effect of amphetamine, but the magnitude of this difference was much smaller than in Lsamp−/− animals. The results of the three learning experiments indicate that invalidation of the *Ntm* gene may result in inferior performance only in cognitively challenging emotional learning tasks (active avoidance), but not in simple emotional learning tasks (fear conditioning) or learning paradigms involving mainly hippocampus (spatial navigation in the Morris water maze). The learning deficiency revealed in active avoidance is indirectly in line with studies associating Ntm with cognitive function and intelligence (Liu et al., 2007; Pan et al., 2011). Table 5 gives a comparison of the behavioural phenotypes of Ntm−/− and Lsamp−/−mice, underlining the difference of these two knockout mouse models on the behavioural level.

**Table 5**. Comparison of the phenotypes of Lsamp–/– and Ntm–/– mice. Only experiments conducted with both models have been reported [2,10,12,27]. = no change; ↑ moderate increase; ↑↑ strong increase; ↓ moderate decrease; ↓↓ strong decrease. *These experiments have been conducted with two different Lsamp knockout models (University of Tartu / University of Vanderbilt).

| **Parameter** | **Lsamp−/− mice** | **Ntm−/− mice** |
|---|---|---|
| Body weight | = / =* | = |
| Vision, hearing, olfaction, pain sensitivity | = | = |
| Locomotor activity | ↑ / ↑* | = |
| Anxiety | ↓ / ↓* | = |
| Barbering behaviour | ↓↓ | = |
| Social interaction | ↓↓ | = |
| Swimming speed | ↓↓ | = |
| Spatial memory (Morris water maze) | = / ↓* | = |
| Emotional learning (active avoidance) | = | ↓↓ |
| Nest building | = | = |
| Obsessive-compulsive (marble burying) | = | = |
| Sensitivity to amphetamine | ↓↓ | ↓ |
| Sensitivity to ethanol | ↑↑ | = |

With caution, a few conclusions can be drawn based on this study. First, deletion of the *Ntm* gene has a much smaller effect on the behaviour of mice than the deletion of the Lsamp gene, indicating that, at least on the level of the nervous system, Lsamp plays a more central role than Ntm. Second, except for the decreased sensitivity to amphetamine, there were no overlaps in the phenotypes of Ntm−/− and Lsamp−/− mice, indicating that although being interaction partners in certain brain regions, both Ntm and Lsamp have separate roles, probably both spatially and temporally. Despite interaction in *in vitro* experiments, the actual regional overlap of these proteins in the brain is limited and their role seems to be complementary. Third, as Ntm+/− mice were less sensitive to amphetamine (similarly to Ntm−/− mice), but performed well in the active avoidance task (differently from Ntm−/− animals), Ntm may modulate certain aspects of behaviour in a gene-dose dependent manner.

## 6.2. Increased sensitivity to GABAergic drugs and psychostimulants in Lsamp-deficient mice (Paper II)

We have previously shown decreased sensitivity to the locomotor activating effect of amphetamine, increased serotonin turnover and decreased level of dopamine transporter mRNA in Lsamp−/− mice alongside with extensive changes in behaviour, such as slight hyperactivity in novel environments, decreased aggressiveness, lack of whisker trimming and decreased swimming speed (Innos et al., 2011, 2012, 2013), indicating that the deletion of the *Lsamp*

gene induces extensive changes in several major neurotransmitter systems. Here, we proceeded to test the effects of morphine, MK-801 and cocaine on the locomotor behaviour of Lsamp−/− mice. Furthermore, the findings that Lsamp−/− mice were hypersensitive to alprazolam, displayed decreased anxiety, a shift in the balance of GABAA receptor α1 and α2 subunits (Innos et al., 2011), and had increased serotonergic tone (Innos et al., 2013), alongside with the fact that both serotonergic and non-serotonergic neurons of the dorsal raphe nucleus receive GABAA receptor subtype-specific regulatory input (Corteen et al., 2015), prompted us to study the sensitivity of Lsamp−/− mice to benzodiazepines (BZs) and other GABAA receptor modulators in more detail.

First, we found that although Lsamp−/− mice are significantly less sensitive to amphetamine (Innos et al., 2013), their sensitivity to the locomotor activating effects of other stimulants, cocaine and morphine, has been increased. Second, we found that Lsamp−/− mice display increased sensitivity to the anxiolytic, hypnotic and (as evidenced by falls from the plus maze) possibly also muscle relaxation effects of BZs, especially alprazolam, and other modulators of the GABAergic system (ethanol and pentobarbital). Table 6 provides a very general overview of the strength of the effects observed in the behavioural pharmacology test battery used in this study in Lsamp−/− mice.

**Table 6**. An overview of the sensitivity of male Lsamp–/– mice to the effects of psychostimulants, benzodiazepines and other drugs tested in this study, compared to wild-type littermates. ↑↑ strongly increased sensitivity; ↑ moderately increased sensitivity; = no change in sensitivity.

| Substance | Effect | Effect strength |
|---|---|---|
| Cocaine | Locomotor activating | ↑ |
| Morphine | Locomotor activating | ↑ |
| MK-801 | Locomotor activating | = |
| | Stereotypy-inducing | = |
| Ketamine | Sedative | = |
| Diazepam | Anxiolytic | = |
| | Muscle relaxant | = |
| | Sedative | ↑ |
| Alprazolam | Anxiolytic | ↑↑ |
| | Muscle relaxant | ↑↑ |
| | Sedative | ↑↑ |
| Ethanol | Anxiolytic | = |
| | Sedative | ↑↑ |
| TP003 (GABAA non-specific) | Anxiolytic | ↑ |
| SL651498 (GABAA α2 and α3) | Anxiolytic | = |
| Pentobarbital | Sedative | ↑↑ |

Increased sensitivity to the sedative/hypnotic effect of BZs in Lsamp−/− mice in the LORR tests most likely reflects upregulation of α1 subunits of GABAA receptor as sedation is mediated by α1 subunits (Clayton et al., 2007). Anxiolytic-like effects are mediated by α2 and α3, and as more recently demonstrated (Behlke et al., 2016), also α5 subunits.

With SL651498, a full agonist of only α2 and α3 subunits, we saw no differences in anxiety in the SIH test in Lsamp−/− mice compared to wild-type littermates, however, subunit-nonspecific agonists alprazolam and TP003 had, besides other effects, enhanced anxiolytic-like effect on Lsamp−/− mice, which led us to the hypothesis that besides α1 subunits, possibly α5 subunits are also upregulated in Lsamp−/− mice. This idea is supported by the fact that α5 subunits are thought to mediate muscle relaxation (Clayton et al., 2007) and the muscle relaxant effects of subunit-nonspecific GABA modulator alprazolam were enhanced in Lsamp−/− mice.

As for ethanol, this substance is known to possess many pharmacodynamic actions, but most importantly, ethanol is a GABAA receptor positive allosteric modulator and NMDA receptor negative allosteric modulator (Möykkynen and Korpi, 2012). Ethanol enhances the function of GABAA receptors, but the specific roles of each receptor subtype in ethanol induced behaviours remain to be elucidated. It is known, however, that knock-in mice with specific mutations making the α1 subunit of the GABAA receptor resistant to ethanol show quicker recovery from the motor-impairing effects of ethanol (Werner et al., 2006). Here, on the contrary, Lsamp−/− mice were more sensitive to the sedative dose of ethanol in the locomotor activity test, probably reflecting an upregulation of α1 subunits. In the LORR experiment, Lsamp−/− mice also displayed increased sensitivity to ethanol as evidenced by increased RRR time. To help clarify the possible mechanism behind this effect we performed the LORR test with pentobarbital, a GABAA receptor modulator, and ketamine, an antagonist of NMDA receptors that has no effect on GABA receptors. Pentobarbital at dose level 45 mg/kg induced the LORR effect (onset of sleep) faster in Lsamp−/− animals; combined with the pilot study, performed to find the most suitable dose, where 30 mg/kg of pentobarbital failed to induce sleep in 75% (6/8) of wild-type mice and only 41% (3/7) of Lsamp−/− mice, it can be concluded that male Lsamp−/− mice have increased sensitivity to the hypnotic effect of this lipophilic short-acting barbiturate. At clinically relevant concentrations pentobarbital acts as the potentiator of GABA, but at anaesthetic levels it can also act on calcium channels and directly opens GABAA receptor-associated chloride channels (Löscher and Rogawski, 2012). Bethmann et al. (2008) have also shown that the anti-epileptic effect of pentobarbital is heavily dependent on the expression pattern of GABAA receptor subunits.

Contributions of additional mechanisms such as blockade of AMPA/kainate receptors cannot be ruled out, but Kamiya et al. (1999) have suggested that the inhibition of AMPA receptors contributes little to the hypnotic action of the barbiturates. In our study, the NMDA receptor antagonist ketamine had a similar effect in the LORR test on Lsamp−/− and wild-type animals. Another

NMDA modulator, a non-competitive antagonist of NMDA receptors MK-801, also failed to induce activity- or stereotypy-related differences in the two genotype groups. We thus conclude that increased sensitivity of Lsamp-deficient mice to the hypnotic effects of both ethanol and pentobarbital is most likely related to altered expression patterns of GABAA receptor subunits.

## 6.3. The impact of Lsamp on the serotonergic neurotransmission (Paper III)

Limbic system associated membrane protein, Lsamp, is a neural adhesion protein implicated in the formation of fine connections in neuronal circuits, which can be paths for specific neurotransmission. Pharmacological studies with Lsamp-deficient mice have provided evidence that Lsamp is involved in GABA-ergic and dopaminergic neurotransmission (Innos et al., 2011; Innos et al., 2013a). The function of Lsamp as a guidance molecule for dopaminergic axons has been experimentally shown by Schmidt et al. (2014). We have previously shown that Lsamp also regulates the serotonergic system in the brain; Lsamp-deficient mice exhibit elevated serotonin turnover rates in several brain areas and changes in the baseline levels of serotonin (Innos et al., 2013a). With this study we aimed to further characterise the expression profile of Lsamp in the serotonergic nuclei and study the impact of pharmacological manipulations on the serotonergic system in these mice by using chronic administration of a selective serotonin reuptake inhibitor escitalopram.

Although accumulating data confirm expression of Lsamp in the serotonergic neurons (Alttoa et al., 2010; Bethea and Reddy, 2012; Huang et al., 2019), no data has been published on the isoform specific distribution of Lsamp expression within the raphe nuclei. Here, we show that Lsamp transcripts are expressed both in the dorsal and median raphe nuclei, whereas the signal is slightly more intense in the dorsal raphe. The expression of *Lsamp* gene promoters varies in distinct nuclei; while both Lsamp 1a and 1b promoters are active in the dorsal raphe (Bregin et al., 2020), most of the expression in the median raphe comes from 1b promoter and most of the Lsamp expression in the caudal subgroup of raphe nuclei derives from 1a promoter (Bregin et al., 2020).

Current data add important aspects to the previous knowledge indicating that the *Lsamp* gene is expressed in brain areas that receive input from raphe or send output towards raphe nuclei. Increased levels of the Lsamp transcript have been associated with lower activity and higher levels of anxiety or acute fear reaction. Furthermore, the genetic deletion of the *Lsamp* gene in mice resulted in behavioural disinhibition or reduced anxiety-like behaviour (Catania et al., 2008; Innos et al., 2011). Although no significant correlations between serotonin metabolism and behavioural outcomes were established in this study due to a small number of animals used, the updated Lsamp-specific staining in the mouse brain contributes supporting evidence to the hypothesis that the anxiety-

like phenotype in Lsamp-deficient mice could be regulated by changes in the serotonergic system.

Both dorsal and median raphe nuclei project to the dorsal and ventral striatum, but more significantly to the ventral striatum. Additionally, dorsal raphe nuclei have a stronger projection to the ventral striatum (Hassanzadeh and Behzadi, 2007). Serotonin from the dorsal raphe nucleus activates a subpopulation of neurons in the bed nucleus of the stria terminalis (BST) in mice (Marcinkiewcz et al., 2017) and a prominent projection from the BST, which controls anxiety-related behaviours, directly targets DR serotonergic neurons (Dorocic et al., 2014). Strong Lsamp expression on the protein (Levitt, 1984) and transcript level (Reinoso et al., 1996) has been shown in the ventral striatum (both in the nucleus accumbens and BST) and we have specified that most of the Lsamp expression in that area comes from the 1a promoter (Philips et al., 2015). It has been shown that 5-HT from the dorsal raphe nucleus enhances fear and anxiety and activates a subpopulation of corticotropin-releasing factor (CRF) neurons in the BST in mice (Marcinkiewcz et al., 2017). These results reveal an essential 5-HT-DRN → CRF-BST circuit governing fear and anxiety, and provide a potential mechanistic explanation for the clinical observation of early adverse events in response to SSRI treatment in some patients with anxiety disorders (Marcinkiewcz et al., 2017; Harada et al., 2008).

The serotonergic projection from DR also targets the basolateral nucleus of the amygdala (BLA) (Gao et al., 2002), which shows a strong Lsamp 1a isoform specific staining (Philips et al., 2015). Intense Lsamp 1b isoform specific staining has been shown in the central amygdala nucleus (Philips et al., 2015), which displays a major projection from the amygdala to the DR region (Dorocic et al., 2014). BLA and CeA, and the increase or decrease of connectivity between these nuclei has been shown to have an acute impact on anxiety-related behaviour (Tye et al., 2011).

Hippocampus receives serotonergic projections both from the DR (Kocsis et al., 2006) and median raphe nucleus (Cui et al., 2013). We have previously shown that the density of cells exhibiting 1b promoter activity is remarkably higher in the subgranular zone of the dentate gyrus in the hippocampal formation; 1a promoter, on the contrary, is selectively active in the pyramidal and granule cell layers (Heinla et al., 2015). Taken together, the brain areas that were selected for monoamine measurements in the current study are known to express *Lsamp* gene transcripts and display connectivity with the raphe nuclei.

We have previously shown that the average body weight of Lsamp deficient mice tends to be 5–10% lower compared to their wild-type littermates (Innos et al., 2011). Although it seems that escitalopram reduces the body-weight difference between genotypes, we did not detect statistically significant differences due to high variance of the data (Fig. 17). Nevertheless, the observation that 5-HT turnover was increased in most of the brain areas tested in Lsamp deficient mice fits well with the current view that increased 5-HT activity is linked to reduced body weight (Voigt and Fink, 2015); for instance, the anti-obesity drug lorcaserin is an agonist of 5-HT2C receptors (Shukla et al., 2015). The 5-HT

anti-obesity hypothesis is further supported by our finding that escitalopram-induced reduction of 5-HT turnover in Lsamp-deficient mice to the level of wild-type controls seems to normalise their body weight as well. Thus, it is important to follow up on these body weight changes carefully in future studies to determine the relevance of the phenotype.

Chronic injections have been shown to be a major source of stress for rodents, which can significantly alter their behaviour and related biochemical markers (Narvik et al., 2018). Nevertheless, even after a long period of injections the behavioural phenotype characteristic to Lsamp-deficient mice in our earlier studies (Innos et al., 2011, 2012) and in independently created Lsamp−/− mice (Catania et al., 2008), was still observable. Lsamp-deficient mice still displayed signs that could be interpreted as reduced anxiety, increased exploratory activity or behavioural disinhibition, entering more to both open and closed arms and making more head dips, both protected and unprotected (genotype effects, Fig. 18a, b, c).

Chronic escitalopram treatment significantly increased exploratory activity in both genotypes during the first five minutes in the open field (Fig. 18f). In the EPM, escitalopram significantly increased entries into the closed arms in Lsamp-deficient mice (Fig. 18a) which is indicative of protected exploration according to some investigators (Bourin et al., 2007).

It is interesting, however, that escitalopram did not normalise the number of entries to the closed arms, although it reversed the elevated 5-HT turnover. This indicates that behavioural disinhibition in Lsamp deficient mice does not stem from the serotonergic system and could be caused by changes in another modulatory system such as dopaminergic (Schmidt et al., 2014) or GABAergic (Bregin et al., 2019), which may have caused constitutive developmental alterations in the Lsamp-deficient brain. Lsamp-deficient mice displayed a specific behavioural pattern in the tail suspension test that was not dependent on the injected substance: a significantly higher number of short (1–2 s) episodes of immobility compared with their wild-type littermates (Fig. 18j). Based on current data we can only hypothesise about the meaning or physiological basis of short immobility episodes. Lsamp deficiency could be related with subtle psychomotor deficit that has also been seen in patients with microdeletion in the 3q13.31, locus that involves the *Lsamp* gene (Vuillaume et al., 2013). Still, the only motor deficit that has been detected in Lsamp-deficient mice is the reduction of swimming speed (Innos et al., 2012) that is amplified by the cold water (our unpublished data). Taking into account that Lsamp is expressed in the sensory nuclei and cortex areas (Philips et al., 2015) and is involved in the myelination process (Sharma et al., 2015), we hypothesise that Lsamp is involved in the sensorimotor coupling processes. Additionally, the possibility that short immobility episodes are the expression of mild epileptogenic activity needs to be tested in future studies.

Moreover, we present further evidence of elevated 5-HT turnover in the brains of Lsamp-deficient mice which is consistent with previous findings (Innos et al., 2013a, 2013b). Here, we show elevated levels of 5-hydro-

xyindoleacetic acid (5-HIAA), the major 5-HT metabolite, in the raphe area and in the dorsal striatum of Lsamp-deficient mice (Fig. 19b). Similar tendencies could be seen in other studied brain areas, however these did not reach statistical significance. Elevated 5-HT turnover in the brain is therefore characteristic for the pharmacologically non-manipulated Lsamp-deficient mice (saline group) after both acute and chronic injections. The extracellular fluid (ECF) levels of 5-HIAA have consistently been shown to decrease after acute SSRI administration (Carpenter et al., 2003). In the current experiments, this effect was detected as significant only in the hippocampus of wild-type mice (Fig. 19b). Due to high baseline 5-HIAA levels, however, the same effect was more prominent in mutant mice: escitalopram reduced 5-HIAA level in the raphe area, dorsal striatum and hippocampus of Lsamp deficient mice. Likewise, escitalopram reduced 5-HT turnover in Lsamp deficient mice in the raphe, ventral striatum, dorsal striatum and hippocampus.

In the current study, the reduction in serotonin turnover levels after SSRI treatment was accompanied with increased activity in the elevated plus-maze, reflecting reduced anxiety in Lsamp-deficient mice. From human studies we know that brain serotonin turnover is elevated in unmedicated patients with major depressive disorder (MDD) (Barton et al., 2008) and in patients with panic disorder (Esler et al., 2007). Moreover, a marked reduction in serotonin turnover in these patients following SSRI treatment and the accompanying improvement in symptoms suggest that high brain serotonin turnover may be an important biological substrate for both MDD and panic disorder (Esler et al., 2007; Barton et al., 2008). We detected a significant elevation in monoamine oxidase A (Maoa) transcript expression specifically in the raphe area, which is in line with the most prominent elevation of 5-HIAA in this area in Lsamp-deficient mice. Moreover, the levels of DOPAC, another product of Mao A, were also elevated only in the raphe of Lsamp-deficient mice (Fig. 20c). Both 5-HIAA and DOPAC were restored to the levels of wild-type mice by escitalopram treatment. These findings, together with the elevation of Maoa transcript in the raphe, suggest elevated activity of the SERT (serotonin transporter) in Lsamp-deficient mice. Although elevated brain 5-HT turnover is influenced by the 5-HTT (serotonin transporter) genotype in human patients (Barton et al., 2008), in the current study we did not detect any alterations in serotonin transporter (Slc6a4) mRNA expression in the raphe area of Lsamp-deficient mice. Nevertheless, the activity of SERT is most probably increased in Lsamp-deficient mice since the SERT blocker escitalopram reverses their 5-HT turnover. Our immunohistochemistry stainings show coexpression of Lsamp protein with serotonin transporter and tryptophan hydroxylase (Tph2) proteins, which is in line with recent data (Huang et al., 2019) showing the expression of Lsamp transcript in the same subgroups of cells in the dorsal raphe that express Slc6a4 and Tph2. Furthermore, our co-staining with synapsin reveals that Lsamp is localised in both pre- and postsynaptic terminals; the same results have been shown by using electron microscopy (Zacco et al., 1990), concordant with other earlier studies which suggest that Lsamp functions as a modulator for axon

guidance (Schmidt et al., 2014), molecular stability of synapses, the integrity of pre- and postsynaptic membranes, and synaptogenesis (Hashimoto et al., 2009; Um and Ko, 2017). Altogether, we hypothesise that Lsamp may have an impact on the properties of synaptic connections that could also influence the activity of serotonin transporter protein which leads to increased uptake of 5-HT. Measuring neurotransmitter levels from tissue homogenates, as performed in this study, is a reliable method for estimating monoamine turnover levels, however, microdialysis where the amounts of extracellular neurotransmitter release can be measured over time, would be needed to further understand whether the increased turnover is related with increased serotonergic transmission and how exactly the Lsamp modulates serotonergic neurotransmission. Furthermore, as a limitation, the present study was conducted with male mice only and the results need to be replicated with female animals.

## 6.4. The combined impact of IgLON family proteins Lsamp and Neurotrimin on behavioural profiles in mouse

Combining mutations within the IgLON family gave us a spectrum of phenotypes, which allowed us to show differential interactions between the two members of IgLONs for the performance of behaviour along with underlying neuronal development.The initial observation was the surprising discovery that Ntm/Lsamp double-knockout mice were not distinguishable from their wild-type, Lsamp−/− or Ntm−/− littermates. There were no gross vision or hearing deficiencies nor significant changes in body-weight in double-mutant mice. The behavioural profiling indicated an interplay between Lsamp and Ntm, which varied across specific tests. The phenotypes characteristic of the single mutant lines, such as reduced swimming speed in the Morris water maze and anxiolytic-like phenotype in the elevated plus maze, were magnified in Lsamp−/−Ntm−/− mice, indicating that in certain traits the deletion of both Lsamp and Ntm induces a more prominent phenotype than single-gene deficiency. However, the behavioural phenotypes apparent in single mutant lines could also stay the same or disappear in double-mutant mice.

### 6.4.1. Single and combined effects of Ntm/Lsamp-deficiency on the behaviour

There was no evidence of gross changes in the brain anatomy in Lsamp−/−, Ntm−/−or Lsamp−/−Ntm−/− mice. The general organisation of the cortex, hippocampus and other subcortical areas did not reveal gross changes (Fig. 6). However, behavioural testing of Lsamp−/−, Ntm−/−, and Lsamp−/−Ntm−/− double-deficient mutant mice exposed phenotypes indicating the combined operation of Lsamp and Ntm.

The current study revealed no significant differences in general locomotor activity between the four groups in the open field test; although Ntm−/− mice

spent more time and travelled a longer distance in the central area of the open field test. In the previous study (Mazitov et al., 2017) the same effect in Ntm−/− mice was observed as a tendency that did not meet statistical significance. Deletion of Lsamp alone had no effect on the exploratory activity in the centre of the open field, consistent with our previous results (Innos et al., 2011); furthermore, the significant exploratory effect of deleting Ntm alone disappeared if Lsamp was deleted as well (in Lsamp−/−Ntm−/− double-deficient mice, (Fig. 21c)). Again, one can hypothesise that Lsamp is an enhancer of the exploratory activity in the central area of the open field as increased time in the centre in Ntm−/− mice is apparent only in the presence of Lsamp. Rearings in the open field, reflecting vertical exploratory activity, were suppressed in Lsamp−/− mice and at normal level in Ntm−/− mice, confirming the findings of previous studies (Innos et al., 2011; Mazitov et al., 2017). Thus, the decreased frequency of rearings seen in Lsamp−/−Ntm−/− double deficient mice was apparently the effect of Lsamp deficiency (Fig. 21b). The decreased sensitivity to the locomotor stimulating effects of amphetamine in both Lsamp−/− and Ntm−/− animals has been previously demonstrated (Innos et al., 2013a, 2013b; Mazitov et al., 2017), whereas the magnitude of change was much larger in Lsamp−/− animals. This difference in amphetamine sensitivity was most prominent in young (2–3 months old) unhandled animals and tended to decrease in older and/or handled animals. The present results also show a tendency towards decreased sensitivity to amphetamine in Lsamp−/−Ntm−/− double deficient mice. However, because of the technical difficulties related to obtaining a sufficient number of double mutant mice we could not use young unhandled mice, and the experiment was performed with handled animals at 4 months of age. Keeping this shortcoming in mind, we can conclude that further deletion of the *Ntm* gene does not induce drastic changes in sensitivity to amphetamine as compared to Lsamp−/− animals.

The EPM was used to detect genotype-dependent changes in anxiety. Here it was shown that Lsamp−/−Ntm−/− double-deficient mice displayed a robust reduction in anxiety-like behaviour in the EPM compared to all the other genotypes. Lsamp−/−Ntm−/− double deficient mice performed more open arm entries and spent more time in the open arms than wild-type mice and single mutants (Fig. 21e). We propose that the prominent anxiolytic phenotype in Lsamp−/−Ntm−/− double deficient mice, which may reflect the magnification of reduced anxiety traits in single mutants, can be seen as a model of the architecture of a polygenic psychiatric disease: the effects of single genes behind maladaptive behavioural traits are magnified in the disturbance of multiple loci until the behavioural deviation meets diagnostic criteria. It has repeatedly been demonstrated that Lsamp−/− mice are more active and tend to spend more time on the open arm of the EPM (Catania et al., 2008; Innos et al., 2011, 2012). While we have not seen significant behavioural changes in the EPM in Ntm−/− mice, these transgenic mice tend to spend more time in the centre of the open field, representing another indication of reduced anxiety as the centre is normally avoided by wild-type mice (Bourin et al., 2007). It is not always clear

in the behavioural tests whether mice display reduced anxiety, exaggerated behavioural activation or increased locomotor activity (Catania et al., 2008, Innos et al., 2011). Taken together, these traits (for example, increased time in the open arm of the EPM and in the centre of the open field) reflect the multi-dimensional structure of anxiety-related behaviour in mice (Carola et al., 2002).

# CONCLUDING REMARKS AND SUGGESTIONS FOR FURTHER RESEARCH

Correct connections between neurons are essential for normal brain development and appropriate synapse formation. This morphological and physiological connectivity in the brain is thought to underlie appropriate neural conductivity and normal behaviours. Abnormal integrity of neural tracts, leading to disrupted functional brain connectivity, has been suggested to be an endophenotype for several psychiatric disorders, such as schizophrenia (Li et al., 2017). Certain cell membrane moieties, such as the IgLON proteins, are critical for such interactions between neural cells. We propose that the complementary involvement of both Lsamp and Ntm molecules are critical not only for intracellular adhesion, but also neurite differentiation, where they balance the speed and rate of neurite sprouting, as well as neurite elongation and branching. Based on current data, it is complicated to draw clear connections between the neuronal morphology and behavioural profile of the Lsamp-, Ntm- and double-knockout mouse lines. However, the findings that the deletional effect of one IgLON protein (Ntm) is dependent on the presence of another family member (Lsamp) can be seen both at the neuronal level (premature neuritogenesis) and at the behavioural level (increased activity in the centre of the open field). Future studies are needed to confirm if there is a direct causal relationship between alterations in the development seen in our mutant cell models and maladaptive risky behaviours in later life. However, our data provides evidence that neural adhesion molecules Lsamp and Ntm act synergistically, each forming the context for the work of the other in the regulation of neural circuit formation which manifests both at the level of neuronal morphology and behaviour.

Looking at the deletion of the *Lsamp* gene more specifically, it induces extensive dysbalance in several major transmitter systems, most notably serotonergic (Innos et al., 2013) and, as presented in this study, GABAergic, most likely mediated by upregulation of GABAA receptor α1 and α5 subunits. This finding and schizophrenia-like behaviours observed in Lsamp−/− mice are of special interest in the light that a deficit in GABA signalling is characteristic of schizophrenia in human patients as well (Benes and Berretta, 2001; Lewis et al., 2005). Furthermore, Lsamp−/− animals are hypersensitive to the psychostimulant effects of cocaine and morphine. Again, higher responsiveness to psychostimulants has been found in several animal models of schizophrenia (Lipina et al., 2010; Trossbach et al., 2016). It is intriguing, however, that the response to another dopaminergic stimulant, amphetamine, is drastically reduced in Lsamp−/− mice. In our further studies with the *Lsamp* gene deficient mouse line as a model of psychiatric diseases we hope to shed light on the question why several polymorphisms in the *Lsamp* gene make human subjects more susceptible to psychiatric diseases such as schizophrenia.

We demonstrated the distribution of Lsamp isoforms in the dorsal and median raphe nuclei and coexpression of Lsamp protein with the markers of

serotonergic neurons. As escitalopram restored the elevated 5-HT turnover in the brain areas of Lsamp-deficient mice to a level comparable with their wild-type littermates, it is likely that high 5-HT turnover in these mutants is mediated by the increased activity of SERT. We could not detect any changes in the baseline levels of *Slc6a4* gene expression in the raphe area of Lsamp-deficient mice, however, we found that Lsamp is specifically, but not exclusively co-expressed with the SERT protein, suggesting that Lsamp as a neural adhesion molecule may have an impact on the integrity of serotonergic synapses that could also influence the activity of SERT.

Significant upregulation of *Maoa* gene in the raphe area of Lsamp-deficient mice was detected in the same study, which can be a response to the higher activity of SERT proteins which transport more 5-HT from the synapse, leading to increased supply of 5-HT to Mao A. Indeed, additionally to 5-HIAA, we also showed a significant increase in DOPAC (another product of Mao A) only in the raphe area of Lsamp-deficient mice. These results suggest a molecular mechanism through which Lsamp acts as a modulator of serotonergic circuitry. Here we have shown that as a response to escitalopram, Lsamp-deficient mice display some alterations in behaviour and changes in brain biochemistry that are highly analogous to the changes described in psychiatric patients. Lsamp-deficient mice could be therefore used to model phenotypes relevant for psychiatric disorders.

# CONCLUSIONS

1. Ntm-deficient mice display none of the deviations in behaviour that have previously been shown in Lsamp-deficient mice, but differently from the latter, had a deficit in emotional learning in the active avoidance task. The only overlap was decreased sensitivity to the locomotor stimulating effect of amphetamine in both knockout models. This study was an essential step for designing further research on this topic.
2. Lsamp-deficient mice were more sensitive to the locomotor activating effects of cocaine and morphine, and hypersensitive to the sedative and muscle relaxant effects of GABA modulators, most likely reflecting enhanced function of α1 and α5 subunits of the GABAA receptor. No gross differences in sensitivity to NMDA receptor modulators were observed. Thus, as the lack of the *Lsamp* gene leads to widespread imbalances in major neurotransmitter systems and remarkable changes in the behavioural phenotype of the animals, Lsamp-deficient mice are a promising model for mimicking neuropsychiatric disorders.
3. Chronic administration of escitalopram significantly increased general activity in wild-type mice in the open field and protected exploration in Lsamp−/− mice in the elevated-plus maze. Elevated 5-HT turnover in the brains of Lsamp-deficient mice was reproduced in the saline treated group. Escitalopram restored the elevated 5-HT turnover of Lsamp-deficient mice to a level comparable with their wild-type littermates, suggesting that high 5-HT turnover in mutants is mediated by the increased activity of SERT, which is co-expressed with Lsamp in the raphe nuclei. We suggest that Lsamp may have an impact on the integrity of serotonergic synapses, which could be the neurochemical basis of the anxiety- and sociability-related phenotype in Lsamp-deficient mice.
4. Behavioural phenotyping indicated test-specific interactions between Lsamp and Ntm. The reduced swimming speed in the Morris water maze and increased activity in the elevated plus maze phenotypes from single-deletion lines were magnified in Lsamp−/− Ntm−/− mice. Evidence from behavioural experiments show mutual interactions between Lsamp and Ntm.

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# SUMMARY IN ESTONIAN

## Limbilise süsteemiga seotud membraanvalgu (Lsamp) väljalülitamisega seotud muutused hiirte emotsionaalses käitumises – lähenemisviis neuropsühhiaatriliste haiguste paremaks mõistmiseks

IgLONid on oluline glükoproteiinide perekond, mille liikmed vastutavad mitme ajuarenguga seotud funktsiooni eest. IgLONeid on kokku viis − Lsamp, Opcml, Ntm, Negr1 ja IgLON5. Igal IgLONil on kolm immunoglobuliindomeeni ning need valgud on enamasti ankurdatud neuronite pre- ja postsünaptilistele rakumembraanidele glükosüülfosfatidüülinositooli abil.

IgLONid võivad moodustada niinimetatud DIgLONeid − homo- ja heterodimeere, mis mängivad rolli näiteks neuriitide väljakasvu reguleerimisel. On näidatud, et viiest IgLONist kõige tugevam seos on Lsampi ja Ntm-i vahel.

Uuringud on samuti näidanud, et kolmel IgLONi liikmel − Lsampil, Ntm-il ja Opcml-il − on kaks eri promootorit N-terminuses (1a ja 1b), mis võimaldavad igal neist moodustada kaks transkripti. Ülejäänud kahel IgLONil − Negr1-l ja IgLON5-l − on vaid üks promootor ja transkript.

Esimesed tõendid sellest, et IgLONid võivad mõjutada emotsionaalset käitumist tulid Tartu Ülikoolis tehtud uuringust, kus Nelovkov jt. (2003) näitasid, et suurenenud ärevusega Wistar rottidel on Lsamp-transkriptide tasemed periakveduktaalses hallaines kõrgemad. Järgmiste aastate füsioloogilised ja käitumuslikud uuringud närilistel näitasid, et suurenenud Lsamp-transkriptide tasemed on seotud võimendatud ärevuse (Nelovkov jt., 2003; Nelovkov jt., 2006, Alttoa jt., 2009), akuutse hirmureaktsiooni (Kõks jt., 2004) ja õpitud hirmu käitumisega (Lamprecht jt., 2009).

Esimene Lsamp-puudulik hiireliin tehti Catania jt. (2008) poolt ning esmased käitumiskatsed näitasid, et nendel loomadel on metsikhiirtega võrreldes väiksem ärevustundlikkus ja halvem ruumiline mälu Morrise ujumistestis. Mõned aastad hiljem näitasid Innos jt. (2011), et neil loomadel on samuti oluliselt muutunud ka GABAergiline aktiivsus.

Käesoleva töö põhieesmärgiks oli edendada teadmisi Lsamp ja Ntm funktsioonidest. Selleks teostati farmakoloogilisi, biokeemilisi ja käitumuslikke uuringuid Lsamp-, Ntm- ja Lsamp-/Ntm-puudulikkusega hiirtel. Ülesanded oli alljärgnevad:

1. kirjeldada Ntm-puudulike hiirte käitumisprofiili;
2. sügavamalt uurida Lsamp-valgu kahjustamise mõju hiirte GABA- ja dopamiinergilisele süsteemile;
3. näidata Lsamp-valgu kahjustamise mõju serotonergilisele süsteemile;
4. uurida Lsamp- ja Ntm-väljalülitamise mõju Lsamp/Ntm-puudulikes hiirtes võrreldes üksikgeeni puudulike loomadega.

Käitumiskatsed Ntm-puudulike loomadega näitasid, et ainsaks kattuvaks fenotüübiks Lsamp-puudulike hiirtega oli vähenenud tundlikkus kaudse dopamiini agonisti d-amfetamiini suhtes. *Ntm* geeni väljalülitamisel oli tugevaim mõju hiirte emotsionaalsele õppimisele aktiivse vältimise katses.

Käitumuslikud ja farmakoloogilised katsed Lsamp-puudulike hiirtega näitasid loomade suurenenud tundlikkust kokaiini ja morfiini liikumisaktiivsust stimuleerivale toimele. Täpsemad uuringud GABAergiliste ainetega näitasid, et Lsamp-puudulikel hiirtel on ülitundlikkus GABA modulaatorite sedatiivse ja lihaseid lõõgastava toime suhtes.

Estsitalopraami krooniline manustamine ja sellele järgnenud käitumiskatsed kinnitasid Lsamp valgu olulisust serotonergilise süsteemi regulatsioonis. Metsiktüüpi loomade üldine aktiivsus ja Lsamp-puudulike hiirte uudistamisaktiivsus olid serotoniini tagasihaarde inhibiitori kroonilise manustamise järel oluliselt suurenenud. Samuti viis see ravim Lsamp-puudulike loomade muidu suurema serotoniini käibe ajustruktuurides metsiktüüpi hiirtega samale tasemele.

Rakukultuuri- ja käitumiskatsed Lsamp/Ntm-topelt puudulike hiirtega, kellel korraga puudusid nii Lsamp kui ka Ntm valk, viitavad Lsamp ja Ntm omavahelistele interaktsioonidele läbi teatavate fenotüübi-efektide võimendamise võrreldes üksik-mutantidega. Nimelt on Lsamp/Ntm-puudulikel loomadel veelgi vähenenud ujumiskiirus ja suurenenud liikumisaktiivsus võrreldes üksikmutantidega.

Kokkuvõtteks kinnitab käesolev töö, et Lsamp koostöös Ntm-iga osaleb käitumuslike ja emotsionaalsete kohanemisreaktsioonide formeerumises ja arengus läbi erinevate virgatsainesüsteemide moduleerimise. Nende kahe IgLONi interaktsiooni parem mõistmine võib omada tähtsust emotsionaalsete häirete alusmehhanismide selgitamisel.

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Bregin, Aleksandr, Maria Kaare, Toomas Jagomäe, Karina Karis, Katyayani Singh, Karita Laugus, Jürgen Innos, et al. 2020. "Expression and Impact of Lsamp Neural Adhesion Molecule in the Serotonergic Neurotransmission System." *Pharmacology, Biochemistry, and Behavior* 198 (November): 173017. https://doi.org/10.1016/j.pbb.2020.173017.

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