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Increased sensitivity to psychostimulants and GABAergic drugs in Lsamp-deficient mice

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A B S T R A C T

Lsamp, in combinations with other members of the IgLON family of cell adhesion molecules, promotes and inhibits neurite outgrowth and synapse formation during development. Mice lacking Lsamp gene display decreased social behaviour, hyperactivity; decreased anxiety level, alongside with altered balance in GABAA receptor α1 and α2 subunits; and decreased sensitivity to amphetamine, alongside with elevated serotonin function. In human studies, Lsamp has been associated with several psychiatric diseases, including schizophrenia, and suicide. Here, we provide a more thorough characterization of the pharmacological phenotype of Lsamp-deficient mice, including testing for sensitivity to morphine, cocaine, MK-801 and ketamine. More thorougly, sensitivity to GABA modulators (diazepam, alprazolam, ethanol, pentobarbital, TP003, and SL651498) was assessed. In brief, 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 Lsamp gene leads to widespread imbalances in major neurotransmitter systems in the brain accompanied by remarkable changes in behavioural phenotype as well, Lsamp-deficient mice are a promising model for mimicking psychiatric disorders.

1. Introduction

Limbic system associated membrane protein (LSAMP) is a cell adhesion molecule with three immunoglobulin (Ig) domains (Pimenta et al., 1996) expressed on the neuronal dendrites and somata (Zacco et al., 1990). Human and rodent LSAMP have 99% amino acid sequence identity (Pimenta et al., 1996), indicating strong phylogenetic conservation of the protein structure and associated functional properties. LSAMP belongs to the IgLON family of proteins with Ntm, Negr1 and Obcam and all four probably function predominantly as subunits of heterodimeric proteins (Reed et al., 2004), promoting or inhibiting neurite outgrowth depending on combinations (Gil et al., 2002; Mann et al., 1998). Lsamp regulates emotional and social behaviour by use of two alternative promoters: 1a is active in “classic” limbic structures such as hippocampus and amygdala, and 1b is mostly active in the thalamic sensory nuclei and cortical sensory areas, but also in areas that regulate stress and arousal (Philips et al., 2015). Sanz et al. (2015) showed that metalloproteinase-dependent shedding of IgLON family members regulates neurite outgrowth from mature cortical neurons; they suggest that such proteolytic cleavage of IgLON family members could have critical roles in specific targeting and synaptogenesis of cortical neurons. The importance of IgLONs has been underlined by Sharma et al. (2015) who demonstrated that most IgLON family members were enriched in neurons and oligodendrocytes, Lsamp being the second-most enriched adhesion molecule in neurons and oligodendrocytes.

In rodent studies, increased level of Lsamp transcript has been related with increased trait anxiety (Alttoa et al., 2010; Nelovkov et al., 2003, 2006), acute fear reaction (Kõks et al., 2004) and fear conditioning (Lamprecht et al., 2009). Lsamp gene deficiency has been associated with deviant social behaviour as evidenced by decreased agonistic behaviour and lack of whisker trimming (Innos et al., 2011), lower sensitivity to stressful or challenging environmental stimuli (Innos et al., 2012), increased activity in novel environments (Catania et al., 2008; Innos et al., 2011), deficit in spatial memory acquisition (Qiu et al., 2010), and decreased sensitivity to amphetamine alongside with elevated serotonin function and reduced activity of dopamine transporter (Innos et al., 2013). Furthermore, we have previously (Innos et al., 2011) shown decreased anxiety in Lsamp-deficient mice accompanied by a shift in the balance of GABAA receptor subunits α1 and α2 expression. We also discovered that Lsamp-deficient male mice are very sensitive to benzodiazepine (BZ) alprazolam as an anxiolytic dose 0.3 mg/kg had a severe sedative effect on Lsamp-deficient mice, rendering them unable to walk and swim properly (our unpublished pilot study).

Human data link LSAMP with a wide spectrum of psychiatric

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disorders: polymorphisms in the human LSAMP gene have been associated with panic disorder (Koido et al., 2006, 2012), male completed suicide (Must et al., 2008), major depressive disorder (Koido et al., 2012) and schizophrenia (Koido et al., 2014).

These findings, alongside with clear links between Lsamp and psychiatric disorders, prompted us to study the pharmacological phenotype of Lsamp-deficient mice, a potential animal model for psychiatric diseases, in more detail. First, we tested the locomotor activating effects of morphine, cocaine and MK-801 in the motility box, as enhanced reaction to psychostimulants and proneness to stereotypy has been observed in several animal models of schizophrenia-like behaviour (e.g. Lipina et al., 2010; Trossbach et al., 2016). Second, as deficit in GABA signaling is characteristic of schizophrenia (Benes and Berretta, 2001; Lewis et al., 2005), we tested the effects of benzodiazepines (BZs) on Lsamp-deficient mice in the elevated plus maze, an unconditioned anxiety test sensitive to the anxiolytic-like effect of BZs (Ennaceur and Chazot, 2016), locomotor activity test to examine whether the anxiety-related parameters and exploratory activity in the plus maze could be influenced by changes in locomotor activity, and loss of righting reflex (LORR) test to measure the sensitivity of animals to hypnotic doses of GABAA modulators. In these tests, we used two BZs (diazepam and alprazolam) and ethanol, acting at GABAA sites other than the BZ site, in doses ranging from anxiolytic to hypnotic. To clarify whether ethanol-induced LORR behaviour is mediated via GABAA or NMDA receptors, pentobarbital, a GABAA receptor modulator, and ketamine, an antagonist of NMDA receptors having no effect on GABA receptors, were also applied in the LORR test.

Finally, to clarify the involvement of anxiety-mediating GABAA receptor subunits, stress-induced hyperthermia (SIH) test – a "physiological" anxiety test (Olivier et al., 2002) – was performed: first with diazepam to validate the experimental design, and then with TP003, once considered GABAA α3 specific, but according to recent studies, having affinity for all the GABAA receptor subunits (Christian et al., 2015; de Lucas et al., 2015), and SL651498, full agonist of only α2 and α3 subunits (Licata et al., 2005).

2. Materials and methods

2.1. Animals

We used male (and in some experiments also female) wild-type (Lsamp+/+) mice and their homozygous Lsamp gene deficient littermates (Lsamp−/−), group-housed in standard laboratory cages (42.5 × 26.6 × 15.5 cm) 8 (in some instances 7 or 9) animals per cage in the animal colony at 22 ± 1 °C under a 12:12 h light/dark cycle (lights off at 19:00 h). A 2 cm thick 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. Tap water and food pellets (R70, Lactamin AB, Sweden) were available ad libitum. All experiments were performed with mice aged 2–3 months. All animal procedures in this study were performed in accordance with the European Communities Directive (86/609/EEC) and permit (No. 59, September 5, 2006) from the Estonian National Board of Animal Experiments. For more information on Lsamp-deficient mice and their creation, see our previous work (Innos et al., 2011).

2.2. Elevated plus maze test

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

2.3. Locomotor activity test

Locomotor activity of individual mice was measured for 30 min in sound-proof photoelectric motility boxes (44.8 × 44.8 × 45 cm) connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). The floor of the testing apparatus was cleaned with 5% ethanol and dried thoroughly after each mouse. Computer registered the distance travelled, the number of rearings, corner visits and time spent in the central part of the box. Heightened ratio of corner entries/distance travelled was construed as reflecting stereotypic behaviour, also clockwise and counterclockwise circling was checked individually to detect mice displaying stereotypic movement patterns.

2.4. Loss and regain of righting reflex test

The mice were given an intraperitoneal 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 itself. 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 regained 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 the regain of righting reflex (RRR) were measured.

2.5. Stress-induced hyperthermia (SIH) test

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 intraperitoneally with 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.

2.6. Drugs and treatment

Cocaine (cocaine hydrochloride, Oriola Oy, Espoo, Finland) was administered 15 min and morphine (morphine sulphate, Mundipharma) and MK-801 (RBI, Natick, MA, USA) 30 min before testing. Diazepam and alprazolam (Grindex, Latvia) were diluted in 0.9% NaCl solution (B. Braun Melsungen AG, Germany) with the help of a few drops of Tween 80 (Sigma) 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 vehicle (0.9% NaCl solution). All drugs were injected intraperitoneally (i.p.) at a volume of 10 ml/kg.

2.7. Data analysis

The pharmacological measurements in the motility box and plus

maze, and the results of the stress-induced hyperthermia test were analyzed by means of two-way ANOVA (genotype x treatment). The LORR tests were analyzed by means of one-way ANOVA. In all the experiments, $p < 0.05$ was considered statistically significant. Newman-Keuls post-hoc test was used. Statistical analyses were performed using Statistica V13 (Statsoft Inc., Oklahoma, USA). In dose curve experiments, in some cases the data sets for some parameters showed non-parametric distribution. For the sake of consistency we present all the results as analyzed by ANOVA followed by the Newman-Keuls post-hoc test, but double checked the non-parametric data sets with Kruskal-Wallis test. The *p*-values differed minimally compared to those obtained with ANOVA and none of the conclusions changed.

3. Results

3.1. Elevated plus maze test

3.1.1. Diazepam

Closed arm entries: genotype: F(1, 39) = 0.36, $p = 0.55$; treatment: F(3, 39) = 1.1, $p = 0.36$; genotype x treatment: F(3, 39) = 1.1, $p = 0.36$. Open arm entries: genotype: F(1, 39) = 3.88, $p = 0.06$; treatment: F(3, 39) = 1.56, $p = 0.21$; genotype x treatment: F(3, 39) = 0.99, $p = 0.41$. Latency to open arm: genotype: F(1, 39) = 8.22, $p = 0.0067$; treatment: F(3, 39) = 1.32, $p = 0.28$; genotype x treatment: F(3, 39) = 0.39, $p = 0.76$. No post-hoc differences. Open arm time: genotype: F(1, 39) = 0.67, $p = 0.42$; treatment: F(3, 39) = 1.78, $p = 0.17$; genotype x treatment: F(3, 39) = 1.14, $p = 0.34$. Protected head dips: genotype: F(1, 39) = 0.25, $p = 0.62$; treatment: F(3, 39) = 0.93, $p = 0.43$; genotype x treatment: F(3, 39) = 0.40, p = 0.76. Unprotected head dips: genotype: F(1, 39) = 0.48, $p = 0.49$; treatment: F(3, 39) = 0.10, $p = 0.96$; genotype x treatment: F(3, 39) = 0.49, $p = 0.69$ (Fig. 1a–f).

3.1.2. Alprazolam

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 of the maze after wandering on the open arm; additionally, at dose level 0.075 mg/kg 0% (0/8) of wild-type and 25% (2/8) of Lsamp−/− mice fell of the maze. As the fall may influence further behaviour, only one parameter, unaffected 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 x treatment: F(2, 42) = 3.35, $p = 0.045$. Post-hoc test 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. 2).

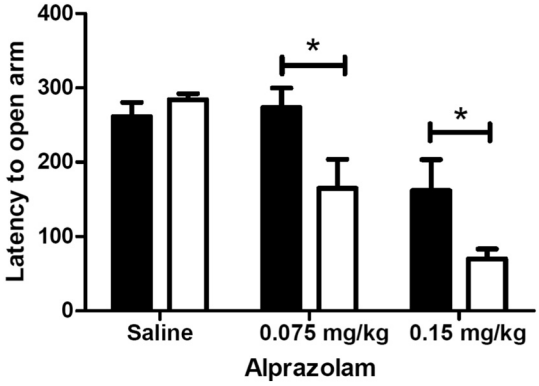

Fig. 2. The elevated plus maze test with 0.075 mg/kg and 0.15 mg/kg of alprazolam. Because of a large number falls from the maze in the Lsamp−/− group, only one parameter, unaffected by falls – the latency in seconds to enter an open arm – was calculated. Black columns: male wild-type, white columns: male Lsamp−/−. * $p<0.05$ vs another genotype at the same dose level. $N = 7–8$ in all genotype x treatment groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

3.1.3. Ethanol

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 x genotype group fell off the maze. Therefore, only one parameter, unaffected by falls – latency to open arm – was analyzed with all the doses (saline, 0.5, 1 and 2 g/kg); all the other parameters

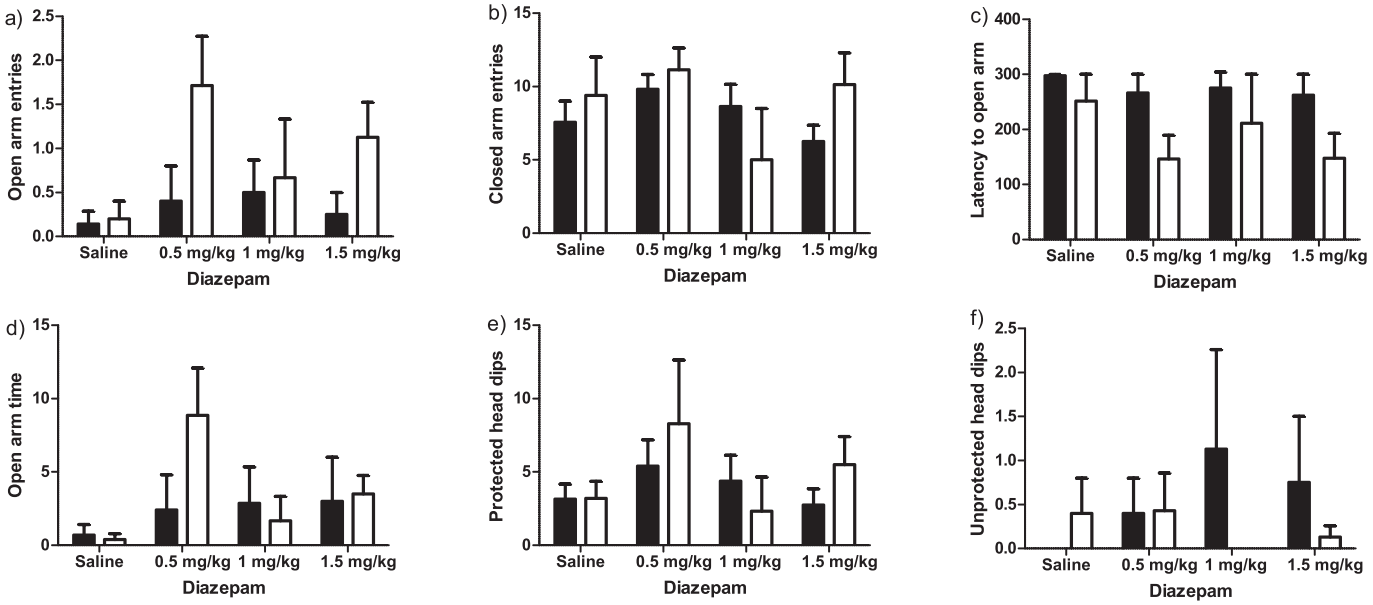

Fig. 1. The elevated plus maze test with 0.5 mg/kg, 1 mg/kg and 1.5 mg/kg of diazepam. Six parameters – the number of open arm entries (a), the number of closed arm entries (b), the latency in seconds to enter an open arm (c), time in seconds spent on open arms (d), the number of protected head dips (e) and the number of unprotected head dips (f) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. $N = 6–7$ in all genotype x treatment groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

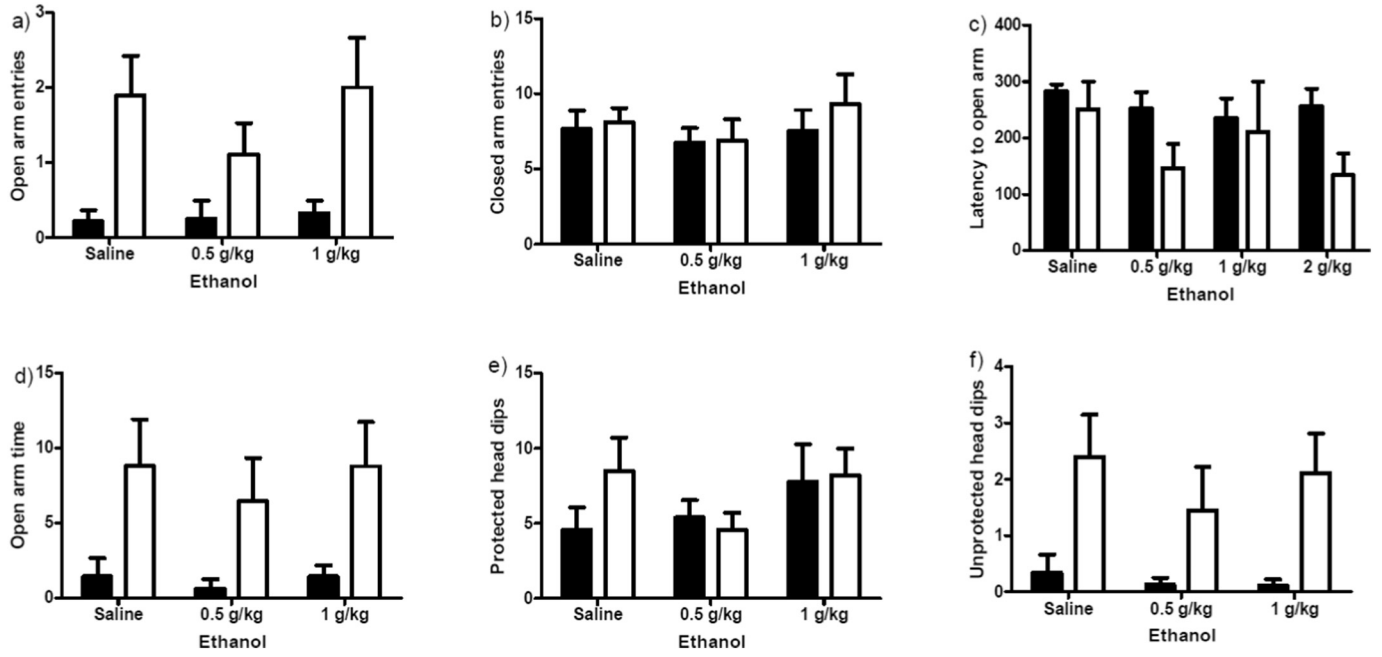

Fig. 3. The elevated plus maze test with 0.5 g/kg, 1 g/kg and 2 g/kg of ethanol. Six parameters – the number of open arm entries (a), the number of closed arm entries (b), the latency in seconds to enter an open arm (c), time in seconds spent on open arms (d), the number of protected head dips (e) and 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. Black columns: male wild-type, white columns: male Lsamp−/−. N = 8–10 in all genotype x treatment groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

were calculated for three doses (saline, 0.5 and 1 g/kg).

Closed arm entries: genotype: F(1, 48) = 0.49, p = 0.49; treatment: F(2, 48) = 0.7, p = 0.5; genotype x treatment: F(2, 48) = 0.2, p = 0.82. Open arm entries: genotype F(1, 48) = 16.5, p = 0.00018; treatment: F (2, 48) = 0.71, p = 0.5; genotype x treatment: F(2, 48) = 0.6, p = 0.55. Latency to open arm: genotype: F(1, 71) = 24.19, p = 0.00001; treatment: F(3, 71) = 0.017, p = 1.00; genotype x treatment: F(3, 71) = 0.45, p = 0.72. No post-hoc differences. Open arm time: genotype: F(1, 48) = 13.37, p = 0.00063; treatment: F(2, 48) = 0.31, p = 0.74; genotype x treatment: F(2, 48) = 0.072, p = 0.93. Protected head dips: genotype: F(1, 48) = 0.66, p = 0.42; treatment: F(2, 48) = 1.4, p = 0.26; genotype x treatment: F(2, 48) = 0.96, p = 0.39. Unprotected head dips: genotype: F(1, 48) = 14.94, p = 0.00033; treatment: F(2, 48) = 0.52, p = 0.6; genotype x treatment: F(2, 48) = 0.26, p = 0.77 (Fig. 3a–f).

3.2. Locomotor activity test

3.2.1. Cocaine

Distance travelled: genotype: F(1, 62) = 10.2, p = 0.0022; treatment: F(3, 62) = 9.96, p = 0.00002; genotype x 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). Number of rearings: genotype: F (1, 62) = 0.39, p = 0.53; treatment: F(3, 62) = 1.28, p = 0.29; genotype x treatment: F(3, 62) = 1.57, p = 0.21. Corner visits: genotype: F (1, 62) = 9.36, p = 0.0033; treatment: F(3, 62) = 6.94, p = 0.00042; genotype x 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. Time in centre: genotype: F(1, 62) = 4.95, p = 0.03; treatment: F(3, 62) = 1.28, p = 0.29; genotype x treatment: F(3, 62) = 1.38, p = 0.26. No post-hoc differences (Fig. 4a–d).

3.2.2. Morphine

Distance travelled: genotype: F(1, 70) = 7.1, p = 0.0094; treatment: F(3, 70) = 2.73, p = 0.05; genotype x 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). Number of rearings: genotype: F(1, 70) = 0.0027, p = 0.96; treatment: F(3, 70) = 1.16, p = 0.33; genotype x treatment: F (3, 70) = 0.71, p = 0.55. Corner visits: genotype: F(1, 70) = 7.95, p = 0.0063; treatment: F(3, 70) = 2.94, p = 0.039; genotype x 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. Time in centre: genotype: F(1, 70) = 5.33, p = 0.024; treatment: F(3, 70) = 0.45, p = 0.72; genotype x treatment: F(3, 70) = 0.98, p = 0.41. No post-hoc differences (Fig. 5a–d).

3.2.3. MK-801

Distance travelled: genotype: F(1, 70) = 1.53, p = 0.22; treatment: F(3, 70) = 9.84, p = 0.00002; genotype x treatment: F(3, 70) = 0.29, p = 0.83. No post-hoc differences. Number of rearings: genotype: F(1, 70) = 0.65, p = 0.42; treatment: F(3, 70) = 11.2, p = 0.00000; genotype x treatment: F(3, 70) = 0.12, p = 0.95. No post-hoc differences. Corner visits: genotype: F(1, 70) = 6.08, p = 0.016; treatment: F(3, 70) = 4.97, p = 0.0035; genotype x treatment: F(3, 70) = 0.59, p = 0.62. No post-hoc differences. Time in centre: genotype: F(1, 70) = 0.02, p = 0.89; treatment: F(3, 70) = 3.77, p = 0.014; genotype x treatment: F(3, 70) = 0.96, p = 0.42. No post-hoc differences (Fig. 6a–d).

3.2.4. Diazepam

Distance travelled: genotype: F(1, 61) = 0.7, p = 0.41; treatment: F (2, 61) = 4.1, p = 0.02; genotype x treatment: F(2, 61) = 0.46, p = 0.64. No post-hoc differences. Number of rearings: genotype: F(1, 61) = 0.41, p = 0.52; treatment: F(2, 61) = 2.291, p = 0.11; genotype x treatment: F(2, 61) = 0.13, p = 0.88. Corner visits: genotype: F(1, 61) = 1.76, p = 0.19; treatment: F(2, 61) = 3.94, p = 0.024; genotype

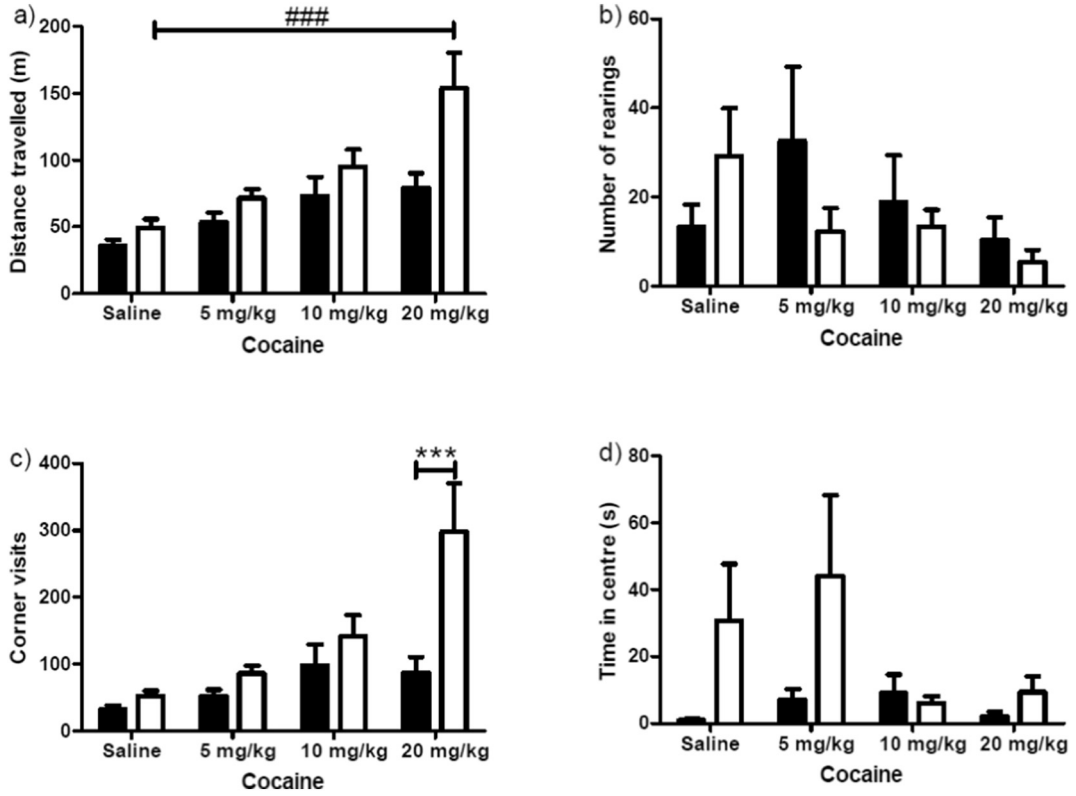

Fig. 4. The locomotor activity test with 5, 10 and 20 mg/kg of cocaine. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. *** $p^{<}0.001$ vs another genotype at the same dose level. ### $p^{<}0.001$ vs saline group. N = 9–10 in all genotype x treatment groups.

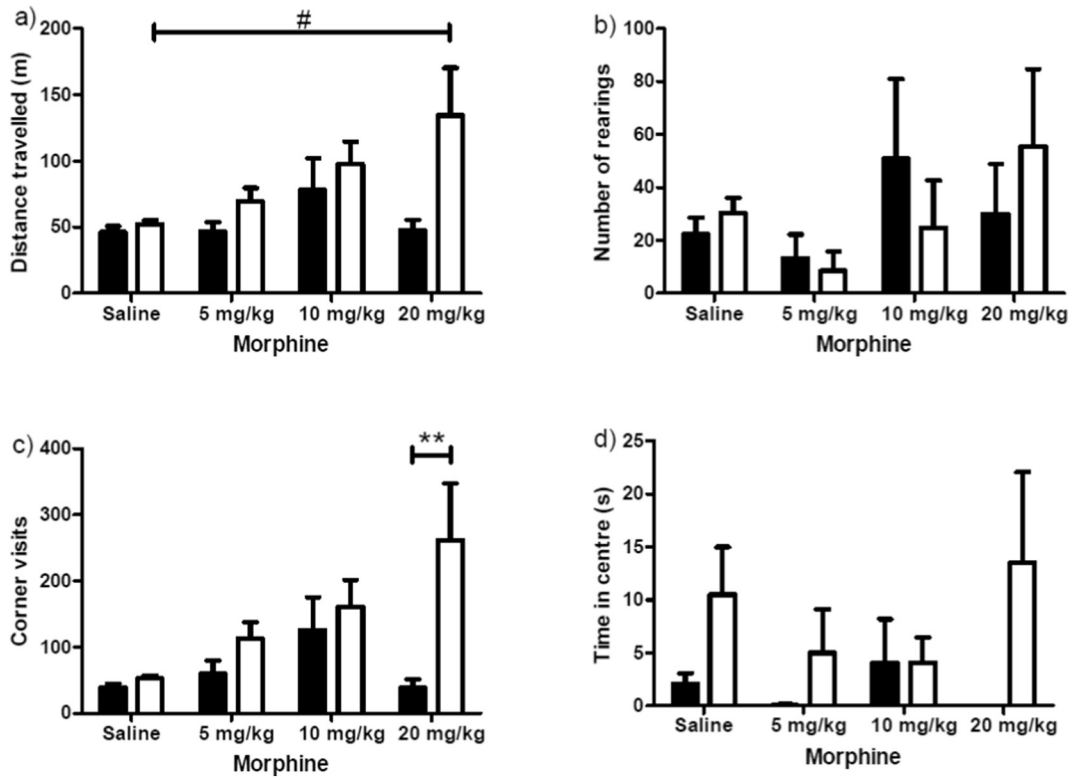

Fig. 5. The locomotor activity test with 5, 10 and 20 mg/kg of morphine. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. ** $p^{<}0.01$ vs another genotype at the same dose level. # $p^{<}0.05$ vs saline group. N = 9–10 in all genotype x treatment groups.

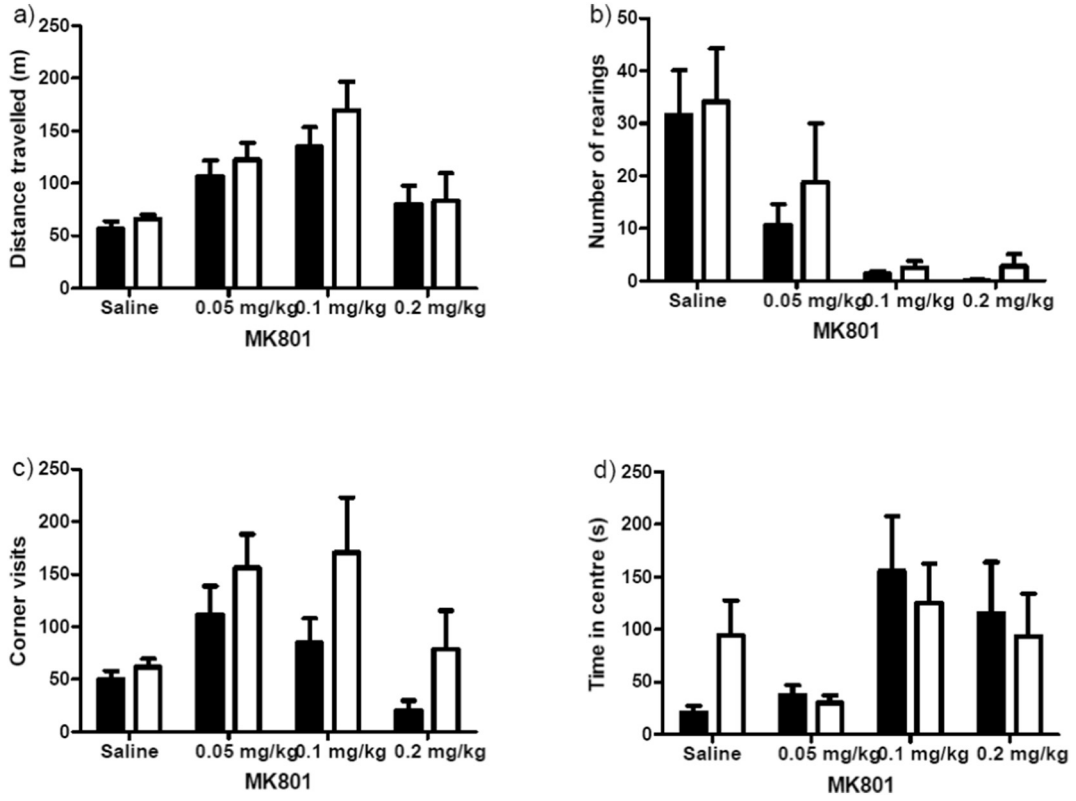

Fig. 6. The locomotor activity test with 0.05, 0.1 and 0.2 mg/kg of MK-801. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. N = 9 in all genotype x treatment groups.

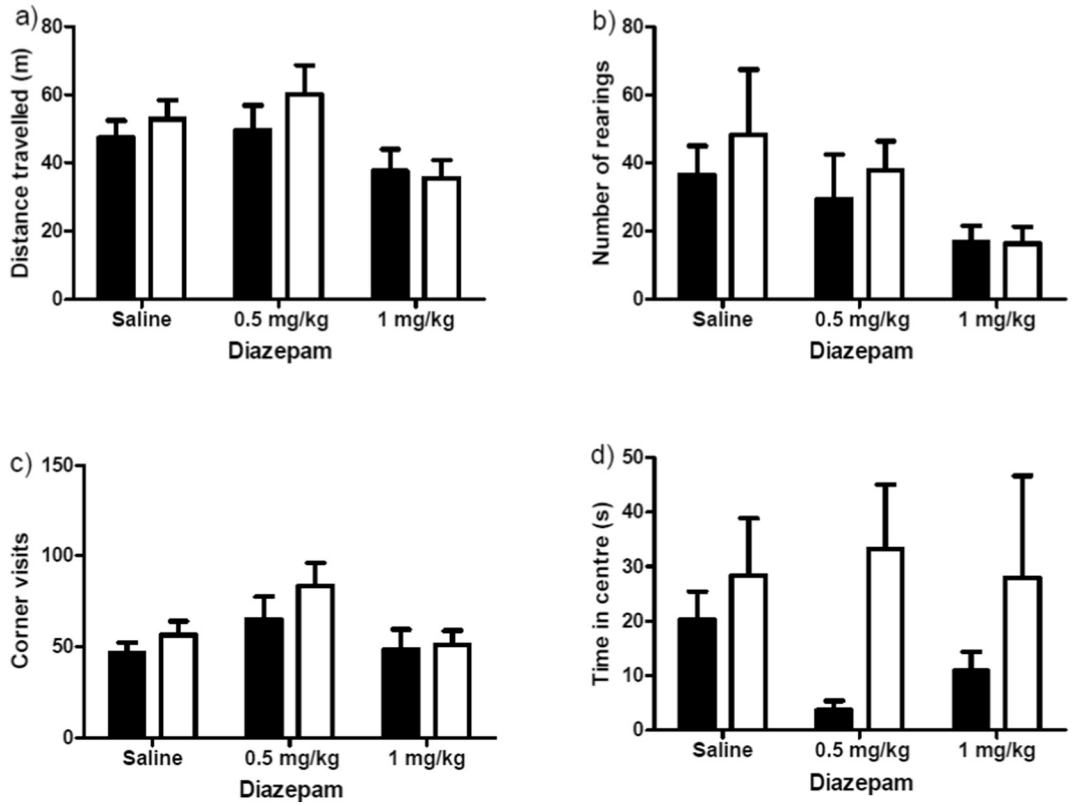

Fig. 7. The locomotor activity test with 0.5 and 1.0 mg/kg of diazepam. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. *N* = 8–9 in all genotype x treatment groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

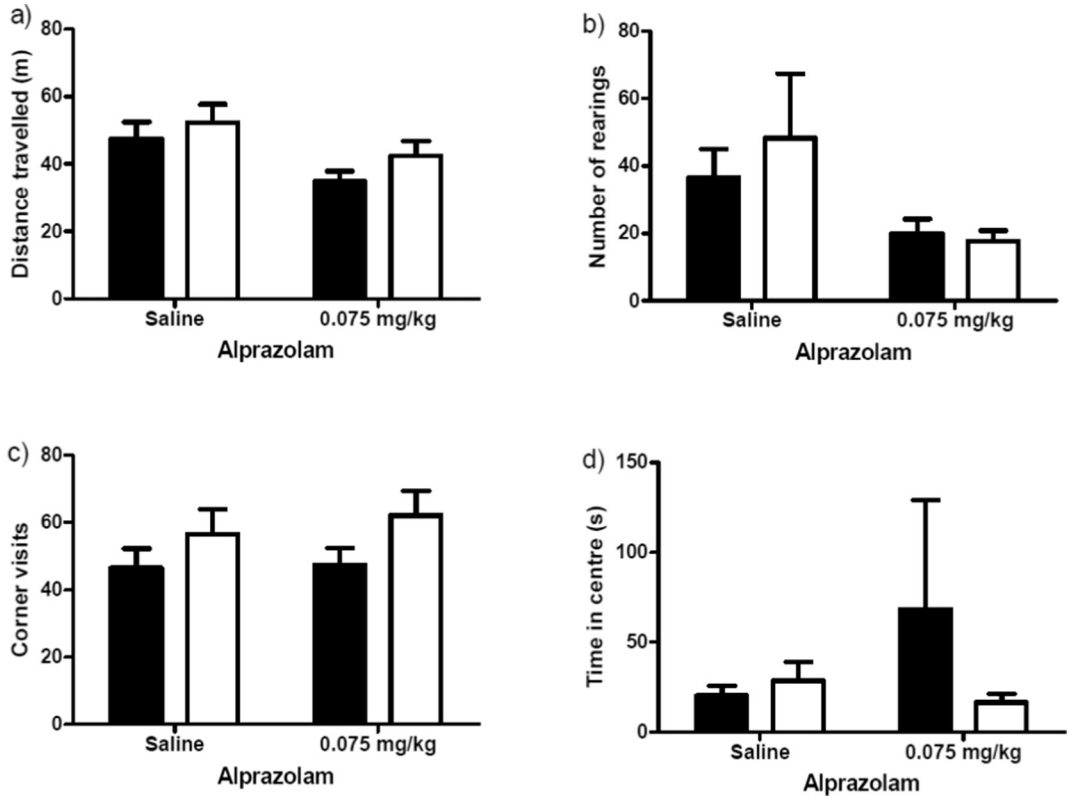

Fig. 8. The locomotor activity test with 0.075 mg/kg of alprazolam. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. *N* = 9–10 in all genotype x treatment groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

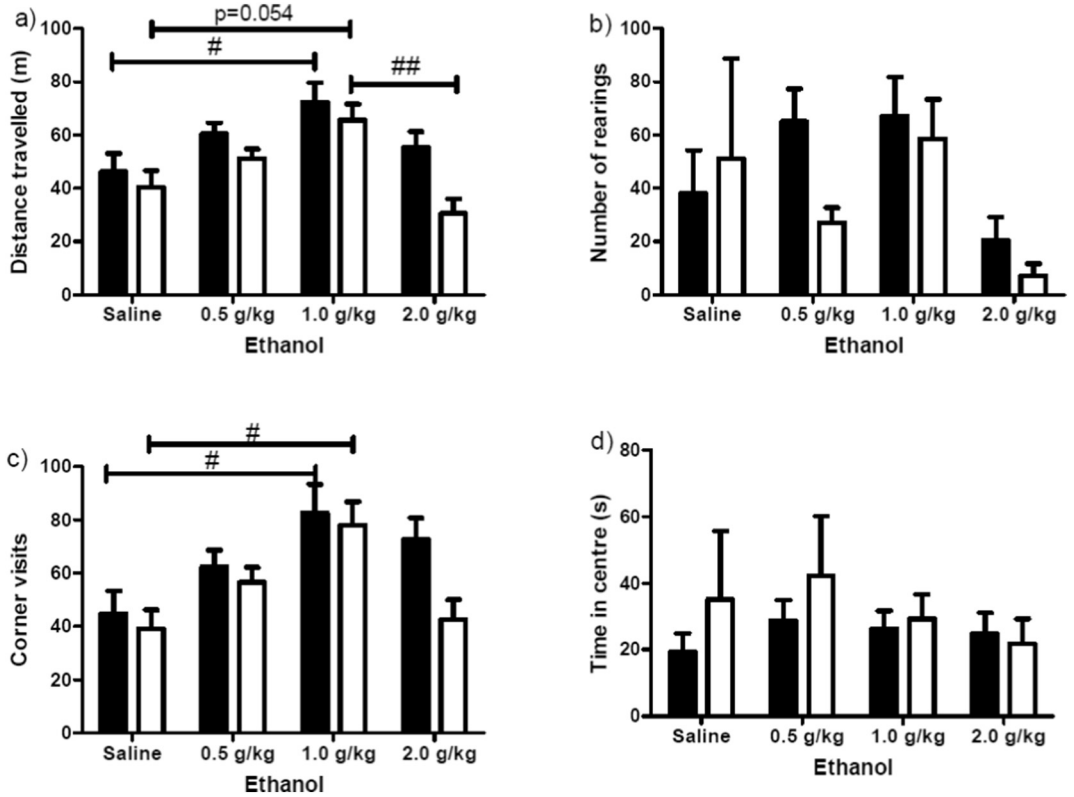

Fig. 9. The locomotor activity test with 0.5, 1.0 and 2.0 g/kg of ethanol. Four parameters – distance travelled (a), the number of rearings (b), the number of corner visits (c), and time spent in the central square (d) – were calculated. Black columns: male wild-type, white columns: male Lsamp−/−. *N* = 13–14 in all genotype x treatment groups. $^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$ vs respective saline group. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

x treatment: F(2, 61) = 0.31, p = 0.73. No post-hoc differences. Time in centre: genotype: F(1, 61) = 4.67, p = 0.035; treatment: F(2, 61) = 0.21, p = 0.81; genotype x treatment: F(2, 61) = 0.55, p = 0.58. No post-hoc differences (Fig. 7a–d).

3.2.5. Alprazolam

Distance travelled: genotype: F(1, 56) = 1.87, p = 0.18; treatment: F(1, 56) = 6.12, p = 0.016; genotype x treatment: F(2, 56) = 0.1, p = 0.75. No post-hoc differences. Number of rearings: genotype: F(1, 56) = 0.21, p = 0.65; treatment: F(1, 56) = 5.32, p = 0.025; genotype x treatment: F(1, 56) = 0.47, p = 0.49. No post-hoc differences. Corner visits: genotype: F(1, 56) = 3.58, p = 0.064; treatment: F(1, 56) = 0.27, p = 0.61; genotype x treatment: F(1, 56) = 0.14, p = 0.71. Time in centre: genotype: F(1, 56) = 0.43, p = 0.52; treatment: F(1, 56) = 0.29, p = 0.59; genotype x treatment: F(1, 56) = 0.81, p = 0.37 (Fig. 8a–d).

3.2.6. Ethanol

Distance travelled: genotype: F(1, 85) = 7.04, p = 0.0095; treatment: F(3, 85) = 9.13, p = 0.00003; genotype x 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). Number of rearings: genotype: F(1, 85) = 1.28, p = 0.26; treatment: F(3, 85) = 4.95, p = 0.0033; genotype x treatment: F(3, 85) = 0.94, p = 0.43. No post-hoc differences. Corner visits: genotype: F(1, 85) = 3.43, p = 0.068; treatment: F(3, 85) = 6.14, p = 0.00079; genotype x 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). Time in centre: genotype: F(1, 85) = 0.92, p = 0.34; treatment: F(3, 85) = 0.54, p = 0.66; genotype x treatment: F(3, 85) = 0.35, p = 0.79 (Fig. 9a–d).

3.3. Loss and regain of righting reflex test

3.3.1. Diazepam

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. 10a–b).

3.3.2. Alprazolam

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. 10a–b).

3.3.3. Ethanol

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. 10a–b).

3.3.4. Pentobarbital

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. 10a–b).

3.3.5. Ketamine

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 wild-type mice (Fig. 10a–b).

3.4. Stress-induced hyperthermia (SIH) test

3.4.1. Diazepam

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 x 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 x dose interaction F(3, 40) = 0.6, p = 0.62 (Fig. 11a–b).

3.4.2. TP003

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 x 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,

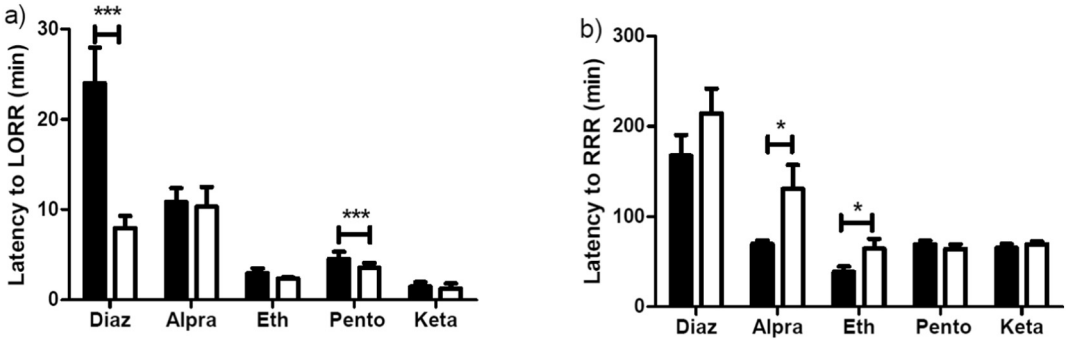

Fig. 10. Loss (LORR, a) and regain (RRR, b) of righting reflex test with 15 mg/kg of diazepam, 3 mg/kg of alprazolam, 3.5 g/kg of ethanol, 45 mg/kg of pentobarbital and 150 mg/kg of ketamine. * $p<0.05$; *** $p<0.001$ vs another genotype. Black columns: male wild-type, white columns: male Lsamp−/−. Diazepam (Diaz): N = 14–15 in both genotype groups. Alprazolam (Alpra): N = 7–8 in both genotype groups. Ethanol (Eth): N = 14–15 in both genotype groups. Pentobarbital (Pento): N = 16 in both genotype groups. Ketamine (Keta): N = 16 in both genotype groups. Data for male and female mice and both sexes combined can be found in Supplementary file 1.

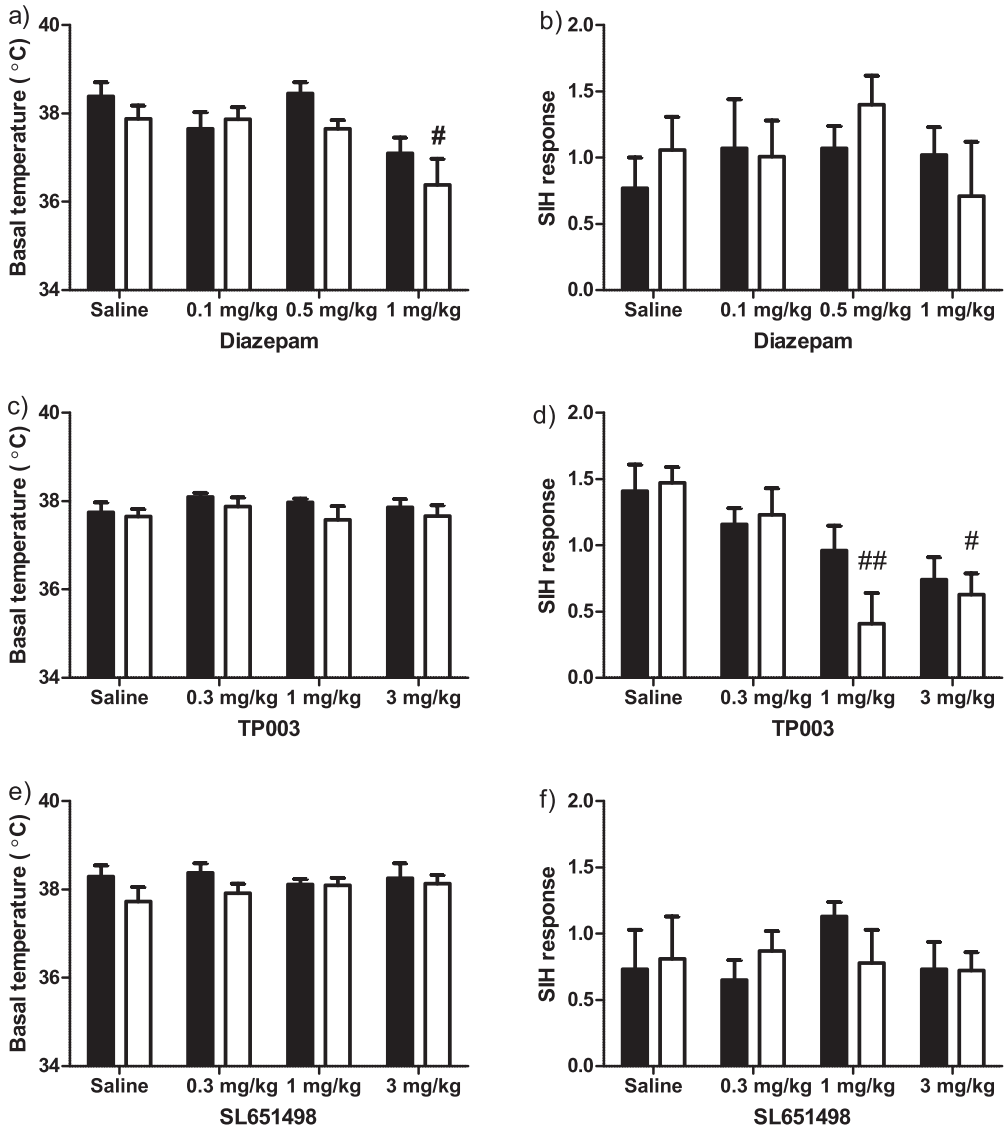

Fig. 11. Stress-induced hyperthermia test with 0.1, 0.5 and 1.0 mg/kg of diazepam, 0.3, 1.0 and 3.0 mg/kg of TP003 and 0.3, 1.0 and 3.0 mg/kg of SL651498. Basal temperature (a, c, e) and stress-induced hyperthermia (SIH) response (b, d, f) were measured for each drug. $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs respective saline group. Black columns: male wild-type, white columns: male Lsamp−/−. N = 9–11 in every genotype x treatment group.

$p = 0.00002$, but not genotype [$F(1, 71) = 1.14$, $p = 0.29$] or genotype x 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. 11c–d).

3.4.3. SL651498

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 x 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 x treatment interaction $F(3, 53) = 0.72$, $p = 0.54$ (Fig. 11e–f).

4. Discussion

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 of the locomotor behaviour of Lsamp−/− mice. Furthermore, the findings that Lsamp−/− mice were hypersensitive to alprazolam (our unpublished pilot study), displayed decreased anxiety and 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

Table 1
An overview of the sensitivity of male Lsamp−/− mice to the effects of psychostimulants, benzodiazepines and other drugs tested in this study. ↑↑ strongly increased sensitivity; ↑ moderately increased sensitivity; = no change in sensitivity (compared to wild-type littermates).

	Male Lsamp−/−	
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	↑↑

has 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 1 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.

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 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 to 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 having 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 wt 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 anesthetic levels acts also on calcium channels and directly opens GABAA receptor-associated chloride channels (Löscher and Rogawski, 2012). Furthermore, Bethmann et al. (2008) have 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, 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 acitivity- 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 pattern of GABAA receptor subunits.

Several experiments with GABA modulators were conducted in both male and female mice to test for possible sex-related differences. However, for consistency and because of space limitations, only the results with male mice are presented in the main text. The results for males, females, and both sexes combined alongside with a discussion on certain sex-specific effects can be found in Supplementary file 1.

In conclusion, the deletion of the Lsamp gene induces extensive dysbalance in several major transmitter systems, most notably serotonergic (Innos et al., 2013) and, as here presented, 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 deficit in GABA signaling 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, on the contrary, drastically reduced in Lsamp−/− mice. In our further studies with 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2019.05.010.

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