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Research report

Deficit in emotional learning in neurotrimin knockout mice

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HIGHLIGHTS

- Initial description of the phenotype of neurotrimin deficient mice is provided.
- Neurotrimin (Ntm) gene knockout mice have a deficit in emotional learning.
- There is no overlap in the behavioural phenotypes of Ntm−/− and Lsamp−/− mice.
- Despite interaction, the roles of IgLONs Ntm and Lsamp seem to be complementary.

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ABSTRACT

Neurotrimin (Ntm) belongs to the IgLON family of cell adhesion molecules with Lsamp, Obcam and kilon that regulate the outgrowth of neurites mostly by forming heterodimers. IgLONs have been associated with psychiatric disorders, intelligence, body weight, heart disease and tumours. This study provides an initial behavioural and pharmacological characterization of the phenotype of Ntm-deficient mice. We expected to see at least some overlap with the phenotype of Lsamp-deficient mice as Ntm and Lsamp are the main interaction partners in the IgLON family and are colocalized in some brain regions. However, Ntm-deficient mice displayed none of the deviations in behaviour that we have previously shown in Lsamp-deficient mice, but differently from Lsamp-deficient mice, 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. Thus, despite being interaction partners, on the behavioural level Lsamp seems to play a much more central role than Ntm and the roles of these two proteins seem to be complementary rather than overlapping.

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1. Introduction

Neurotrimin (Ntm), in humans also called human neurotrimin (Hnt), is a glycophosphatidylinositol (GPI)-anchored cell-adhesion molecule belonging to the IgLON protein family with Lsamp (also Lamp), Obcam (also Opcml) and kilon (also Negr1) [31]. It has been shown that Ntm, and other members of the IgLON family, regulate the development of neuronal projections via cell type specific attractive and repulsive mechanisms that are mediated by both homophilic and heterophilic interactions [6,7,17]. Reed et al. [29] have suggested that only Ntm might be both a homo- and a heterophilic cell adhesion molecule, whereas Lsamp and Obcam act only as heterophilic cell adhesion molecules. Lodge et al. [18] have shown that GPI-anchored Ntm has an alternatively-spliced isoform, possibly modulating the activity of all the IgLONs, that is secreted and co-expressed with the anchored version of the protein in the retina, cerebellum, and DRG neurons. In DRG neurons, Ntm promotes neurite outgrowth via Ntm forming noncovalent homodimers in the plane of the membrane. Conversely, Ntm inhibits neurite outgrowth in sympathetic neurons via heterophilic interactions because sympathetic neurons do not express Ntm [30,34]. McNamee et al. [21] have proposed that IgLONs may not have a primary role in axon guidance, but may be even more important for cell-cell adhesion and recognition.

According to the Eurexpress transcriptome atlas created by Diez-Roux et al. [5], there is a strong widespread expression of Ntm in mouse E14.5 embryonic brain, spinal cord, peripheral nervous system, ganglia, eye, skeleton and limbs. Gil et al. [7] have shown that in adult rat brain, Ntm protein is largely expressed in

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a complementary pattern to that of Lsamp in the nervous system, with co-expression at a few sites. Ntm is expressed at high levels in sensory-motor cortex and, of particular note, is transiently expressed in neurons of cortical barrel fields and corresponding thalamic "barreloids". Ntm is expressed also in the spinal cord [8] and is mediating estrogen-induced sympathetic pruning in some peripheral targets like myometrium; estrogen increases Ntm synthesis and secretion in myometrial smooth muscle cells, so Ntm seems to contribute to sympathetic myometrial neurodegeneration elicited by estrogen [13]. The spatial-temporal expression pattern of Ntm suggests that this adhesion molecule plays a role in axonal fasciculation of specific cerebellar systems and may also be involved in the formation of excitatory synapses and their stabilization into adulthood as it accumulates coincident with synaptogenesis [3], however, in hippocampal neurons *in vitro*, over-expression of Lsamp or Obcam increased synaptic number, while the over-expression of kilon reduced synaptic number and Ntm had no effects [9]. Ntm also mediates the inhibition of Schwann cell proliferation and migration following nerve injury repair through rapid regulation of a certain microRNA (miR-182) which targets FGF9 and Ntm [38].

According to Liu et al. [15], human neurotrimin (Hnt) shows high sequence similarity to the rat Ntm (97%) and has three different transcripts; it has a wider expression pattern than that of rat Ntm; furthermore, the expression of Hnt in fetal brain is higher than that in mature brain and is stronger in nervous tumors than that in normal brain tissues. Ye et al. [37] showed an expression of Hnt in the human fetal heart and a robust and selective expression in the right atrium with much weaker expression in the left atrium and ventricles in the human adult heart.

In 2015 two breakthrough articles were published on IgLONs that shed new light on the function and mechanism of action of these proteins and underline their importance in the nervous system. Sanz et al. [32] showed that metalloproteinase-dependent shedding of IgLON family members regulates neurite outgrowth from mature cortical neurons. The authors suggest that such proteolytic cleavage of IgLON family members could have critical roles in specific targeting and synaptogenesis of cortical neurons similar to roles of other major synaptic cell adhesion molecules like NCAM or N-cadherin. Sharma et al. [33] demonstrated that most IgLON family members were enriched in neurons and oligodendrocytes, Lsamp being the second-most enriched adhesion molecule in neurons and oligodendrocytes. The only exception was Ntm, which was only enriched in neurons.

During the last decade, IgLONs have been linked to several tumours. Ntm has been associated with tumour in two studies. Ntougkos et al. [23] found reduced Obcam, Lsamp and kilon expression and elevated Ntm protein level in human epithelial ovarian cancer relative to normal samples. Ulmer et al. [35] suggested that SNPs in Ntm may increase primary open-angle glaucoma susceptibility in a subset of cases.

Several studies have related Ntm to cognitive functions. Four SNPs in intron 1 of the Ntm gene have been shown to be associated with cognitive function and late-onset Alzheimer's disease [16] and in a family-based association study, Pan et al. [24] showed a link between Ntm polymorphisms and intelligence. The familial case study by Minhas et al. [22] suggested a role for Ntm and Obcam in developmental delay, autistic symptoms and cancer susceptibility, however, Maruani et al. [20] failed to establish Ntm as an autism susceptibility gene in a study including 1256 patients with an autism spectrum disorder.

Recently, intriguing associations between Ntm and heart/circulation have been found. Luukkonen et al. [19] revealed, by using genome-wide paired-end DNA sequencing, an association between a translocation in the Ntm gene and intracranial and thoracic aortic aneurysma, and Cao et al. [1] established Ntm as a novel biomarker for heart failure; in patients with heart failure that responded to long-term treatment with angiotensin-converting enzyme inhibitors and β blockers the expression of Ntm protein was very strongly upregulated compared to non-responders. However, deletion of the Ntm gene failed to induce congenital heart defects in mice [37]. Of relevant note, Li et al. [14] have revealed a link between Ntm and blood lipid levels.

To bring further light to the potential functions of Ntm, here we provide an initial behavioural and pharmacological screening of Ntm−/− mice. We have previously shown [10–12] that mice lacking Lsamp, the main interaction partner of Ntm [29], have decreased anxiety, deficiencies in social behaviour (impaired barbering behaviour, reduced aggressiveness), slight hyperactivity/disinhibition in novel environments, lower exploratory activity and slower swimming speed. Furthermore, Lsamp−/− mice are much less sensitive to the locomotor stimulating effect of amphetamine and much more sensitive to the anxiolytic and sedative effects of ethanol and benzodiazepines. In learning and memory tasks Lsamp−/− mice perform normally, however, in another Lsamp−/− model a spatial memory deficit in the Morris water maze was evident [27]. In this study with Ntm−/− mice, we used a similar behavioural test battery as in our previous studies with Lsamp−/− animals, and also carried out pharmacological screening with amphetamine and ethanol, as Lsamp−/− mice had displayed altered sensitivity to these substances. Besides pointing to possible roles of Ntm in the nervous system, the characterization of the Ntm knockout mouse model helps to bring light on the functional value of Lsamp-Ntm heterodimer forming interaction (which is very close *in vitro*) on the organism level. If the interaction is crucial, deletion of either Ntm or Lsamp should result in a similar or at least partially overlapping phenotype as in both cases no heterodimers are formed.

2. Methods

2.1. Animals

Ntm gene heterozygous mutants were procured from the Mutant Mouse Regional Resource Center at UC Davis. In the Ntm gene, consisting of 8 exons, coding exon 1b was targeted by homologous recombination. Complete deletion of functional Ntm transcripts encoded by both 1a and 1b promoters was achieved as the genomic structure and the assembly of the alternative transcripts of the Ntm gene is analogous to the Lsamp gene described in detail in Philips et al. [26]. Lexicon ES cell line derived from 129S5/SvEvBrd was used for making the construct. Mice were further bred at the animal facility of the University of Tartu. All studies were performed in male F2 hybrids [(129S5/SvEvBrd × C57BL/6) × (129S5/SvEvBrd × C57BL/6)]. Ntm deficient −/− and +/− animals and their wild-type (+/+) littermates were used in the study.

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 ± 1 °C under a 12:12 h light/dark cycle (lights off at 19:00 h). 2 cm layer of aspen bedding (Tapvei, Estonia) and 0.5 l of aspen nesting material (Tapvei, Estonia) was used in each cage and changed every week. No other enrichment was used besides nesting material. Tap water and food pellets (R70, Lactamin AB, Sweden) were available ad libitum. Unless noted otherwise, all experiments were performed with male mice aged 2–4 months.

2.2. Behavioural testing

Testing was carried out between 10:00 and 17:00 of the light phase. Before each experiment, mice were let to habituate to the

experimental room and the lighting conditions therein for 1 h. To reduce the number of animals, the mice were used in several experiments with at least a four-day interval between the experiments. Mice were first tested in tests sensitive to previous experimental experience [36]; stressful tests involving injections or electric shocks were performed last. The first batch of mice was used in the following tests (in the following order): elevated plus maze, motility box, motility box with amphetamine; the second batch: light-dark box, hyponeophagia, active avoidance; the third batch: reflex tests, Morris water maze, active avoidance; the fourth batch: reflex tests, marble burying, fear conditioning; the fifth batch: nesting behaviour, loss/regain of righting reflex with ethanol; the sixth batch: social interaction, loss/regain of righting reflex with ethanol; seventh batch: hot plate. The first, third and sixth batch consisted of 15–17 mice in each genotype group and the second, fourth, fifth and seventh batch consisted of 8 mice in each genotype group. For some of the experiments, only a part of the batch was used. We always tried to keep group sizes as even as possible; slight fluctuations are caused by excluding a couple of mice for technical reasons (*e.g.* for avoiding electric shocks by climbing). No mice were excluded on the basis of being "outliers".

2.3. Reflex tests

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 [10]. 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 it's forepaws for a "soft landing" was observed. In the ear twitch test, ear twitching reflex in response to a pen click was observed. Buried food finding test, measuring olfactory abilities, was carried out as described by Radyushkin et al. [28]. Starting two days prior to testing, mice received each day 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 at the one end of the cage. The mouse was positioned in the right corner at the opposite end of the cage, and the food-finding time, *i.e.* the time from the moment the mouse was placed into the cage to the time it located the cookie and initiated burrowing, was recorded. A clean cage and new bedding was used for each trial. Hot plate test was carried out for the assessment of pain sensitivity. The mouse was confined to plate was heated to 53 °C and the mouse was confined there by a plexiglass cylinder (diameter 15 cm, height 20 cm). Latency (s) to show hind paw response (licking or shaking) was measured.

2.4. Locomotor activity test

Locomotor activity 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) made of transparent Plexiglas and connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). The floor of the boxes was cleaned with 5% of ethanol and dried thoroughly after each mouse. Computer registered the distance travelled, the number of rearings and time spent in the central part of the box.

2.5. Elevated plus maze test

The elevated plus maze test was carried out as described earlier [10]. In short, the apparatus consisted of two opposite open (17.5 × 5 cm) arms without sidewalls and two closed 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 (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. Standard 5 min test duration was employed and the sessions were videotaped. 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: (1) latency to enter an open arm; (2) number of entries on open arms; (3) number of entries on closed arms; (4) time spent on close arms; (5) total number of head-dippings and (6) number of unprotected head-dippings defined as head-dippings made on open arms.

2.6. Light-dark box test

The light-dark box (TSE, Technical & Scientific Equipment GmbH, Germany; same as in 2.8 and 2.10) 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. An 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 number of transitions were measured.

2.7. Hyponeophagia test

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.

2.8. Active avoidance test

The active avoidance test was carried out in a rectangular two-way automated shuttle-box (TSE, Technical and Scientific Equipment GmbH, Germany; same as in 2.6 and 2.10), 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 (10 lx) illuminated 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 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. Inter-trial interval was 10 s. The floor of the testing apparatus was cleaned with 5% ethanol and dried thoroughly after each mouse.

2.9. Morris water maze test

The water maze consisted of a circular pool (diameter 150 cm), escape platform (diameter 16 in diameter) video camera and computer with software (TSE, Technical & Scientific Equipment GmbH, Germany). The pool (depth 50 cm) was filled with tap water (22 °C, to a depth of 40 cm) that was made opaque by adding a small amount of non-toxic white putty. The escape platform was positioned in the centre of the Southwest quadrant (Q2), 20 cm from the wall. The water level was 1 cm above the platform, making it invisible. Each trial, the animals were put into the water, facing the wall, at pseudo-randomly assigned starting positions (East, North, South, or West). The acquisition phase of the experiment consisted of a series of 16 training trials (four trials per day for four consecutive days, inter-trial interval ca 1 h). Mice were allowed to search for the platform for a maximum of 60 s at which time the mice were gently guided to the platform by means of a metal sieve. 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 diffuse white light (20 lx). Distance travelled during the trial, latency to find the submerged platform and swim velocity were registered. We used average values per day, which was obtained by collapsing data of 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 indicator of spatial memory.

2.10. Fear conditioning test

In this classical 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; same as in 2.6 and 2.8) according to the method described by Paylor et al. [25] with some modifications. The apparatus was located in a quiet, dimly (10 lx) illuminated room. 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.

2.11. Marble burying test

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.

2.12. Nesting behaviour test

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 [4].

2.13. Social interaction test

Social interaction test was carried out as described previously [10] with some modifications. Two male mice (one Ntm+/+ 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. Illumination level of the testing arena was 25 lx. Mice were videotaped for 10 min. The videotapes were later scored by a trained observer. The following measures were registered for each mouse: (1) episodes of aggressive behaviour (attacks, biting, chasing, rattling the tail), (2) anogenital sniffing of the other mouse, (3) sniffing the body of the other mouse, (4) digging episodes and digging time, (5) grooming episodes and grooming time, and (6) rearings and rearing time.

2.14. Locomotor activity test with 5 mg/kg of amphetamine

Ntm+/+, +/− and −/− mice were randomly assigned to groups that received an i.p. injection of either saline or 5 mg/kg of amphetamine. After the 30 min post-injection waiting period in small individual cages measuring 22 (L) × 16 (W) × 14 (H) cm, mice 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; same as in 2.4). The floor of the testing apparatus was cleaned with 5% of ethanol and dried thoroughly after each mouse. Computer registered the distance travelled, the number of rearings and time spent in the central part of the box.

2.15. Loss and regain of righting reflex test with 4.0 g/kg of ethanol

The mice were injected intraperitoneally with 4.0 g/kg of ethanol. At the onset of ethanol-induced sedation (the loss of righting reflex), each mouse was placed on its back in a V-shaped trough made of cardboard. Time between the injection and the loss of righting reflex and time between the loss of righting reflex and the regain of righting reflex defined as the ability to right itself on all four paws three times within a 30 s interval were taken.

2.16. Statistical analysis

Results are expressed as mean values ± SEM. Statistica for Windows 12.0 software was used for statistical analysis. One-way ANOVA was used for the following measurements and tests: body

Table 1
Overview of statistically not significant differences between the genotypes, not reported in figures. Results are presented as means ± standard error of mean. Abbreviations: N = number of animals in each experimental group; g = grams; % = percentage of animals that displayed the reflex/ability; s = seconds; m = metres; 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	100	100	
Ear twitch test (%)	8, 8, 8	100	100	100	
Buried food finding (%)	8, 8, 8	100	87.5	100	
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 ± 31.2	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	$F_{(2,61)} = 1.55$, $p = 0.22$

weight, reflex tests, locomotor activity test, elevated plus maze, light-dark box, hyponeophagia, probe trial in the Morris water maze, contextual part of the fear conditioning test, marble burying test, nesting behaviour test, social interaction, and loss/regain of righting reflex test. Two-way independent-groups ANOVA was used for the locomotor activity test with amphetamine (genotype x treatment). Repeated measures ANOVA was used for calculating the learning curve in the Morris water maze and active avoidance tests and for the cued part of the fear conditioning test. Comparisons between individual groups were performed by means of Newman-Keuls *post hoc* test. P value below 0.05 was considered to be significant.

2.17. Ethics

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.

3. Results

3.1. Body weight, basic reflexes and viability

At 2.5 months of age, there were no differences in body weight between Ntm+/+, Ntm+/− and Ntm−/− animals (Table 1). 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 1). Knockouts were viable both *in utero* and postnatally as the analysis of 150 litters revealed an expected Mendelian ratio of +/+, +/− and −/− genotypes.

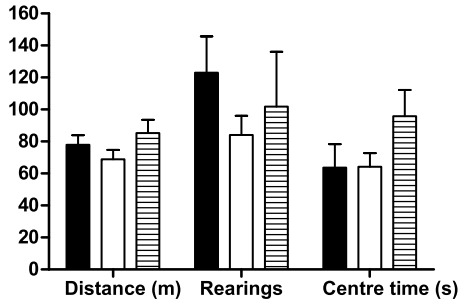

Fig. 1. Distance travelled (in metres), the number of rearings performed, and time spent (in seconds) in the central part of the box in the locomotor activity test. Black colums = Ntm+/+ (N = 15); white colums = Ntm+/− (N = 16); striped columns = Ntm−/− (N = 13). m = metres; s = seconds.

3.2. Locomotor activity test

There were no statistically significant differences between the genotype groups (+/+ N = 15; +/− N = 16; −/− N = 13) in the distance travelled (+/+ 77.9 ± 6.0; +/− 68.8 ± 5.9; −/− 85.3 ± 8.2; $F_{(2,41)} = 1.55$, $p = 0.22$), in the number of rearings (+/+ 123 ± 22.7; +/− 84.1 ± 11.9; −/− 101.8 ± 34.3; $F_{(2,41)} = 0.74$, $p = 0.49$) and in time spent in the central part of the box (+/+ 63.7 ± 14.6; +/− 64.2 ± 8.6; −/− 95.8 ± 16.4; $F_{(2,41)} = 1.82$, $p = 0.17$) (Fig. 1).

3.3. Elevated plus maze test

There were no statistically significant differences between the genotype groups in any of the parameters measured (Table 1).

3.4. Light-dark box test

No statistically significant differences between the genotype groups were detected in this test (Table 1).

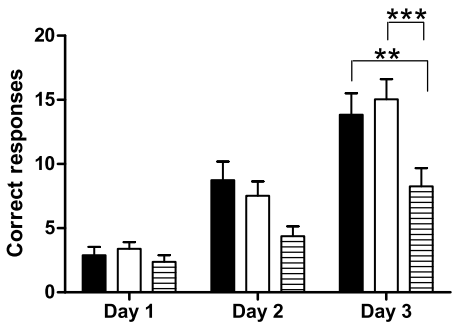

Fig. 2. The learning curve (Days 1–3) in the active avoidance test showing the number of corrected responses on each day. Black colums = Ntm+/+ (N = 19); white colums = Ntm+/− (N = 23); striped columns = Ntm−/− (N = 19). **p < 0.01, ***p < 0.001, compared to Ntm−/− mice.

3.5. Hyponeophagia test

The latency to start eating was similar in all the groups (Table 1).

3.6. Active avoidance test

All the genotypes showed progress in Days 1–3, but +/+ mice (N = 19) and +/− mice (N = 23) had a much steeper learning curve than −/− mice (N = 19) (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 +/+ ($p = 0.002$) and +/− ($p = 0.0002$) mice had significantly more correct responses compared to −/− mice (Fig. 2).

3.7. Morris water maze test

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 1), no thigmotaxis was observed and floating was minimal. In 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).

3.8. Fear conditioning test

Fig. 4 gives an overview of the fear conditioning. No freezing behaviour was evident in the pre-conditioning phase in any of the genotypes (N = 8 in all groups). In the contextual part, there was no difference between the groups in freezing time (+/+ 37.6 ± 16.5 s; +/− 42.1 ± 9 s; −/− 22.2 ± 5.4 s; $F_{(2,21)} = 1.2$, $p = 0.32$). In the cued part, freezing time (no stimulus: +/+ 5.7 ± 5.7 s; +/− 23.4 ± 12.1 s; −/− 6.3 ± 4.6 s; sound stimulus: +/+ 39.8 ± 19.9 s; +/− 60.6 ± 24.9 s; −/− 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: +/+ 13.3 ± 3.5; +/− 8.9 ± 2.7; −/− 12.7 ± 5; sound stimulus: +/+ 11.4 ± 4.5; +/− 13.1 ± 10.5; −/− 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 ($F_{(2,21)} = 0.27$, $p = 0.76$).

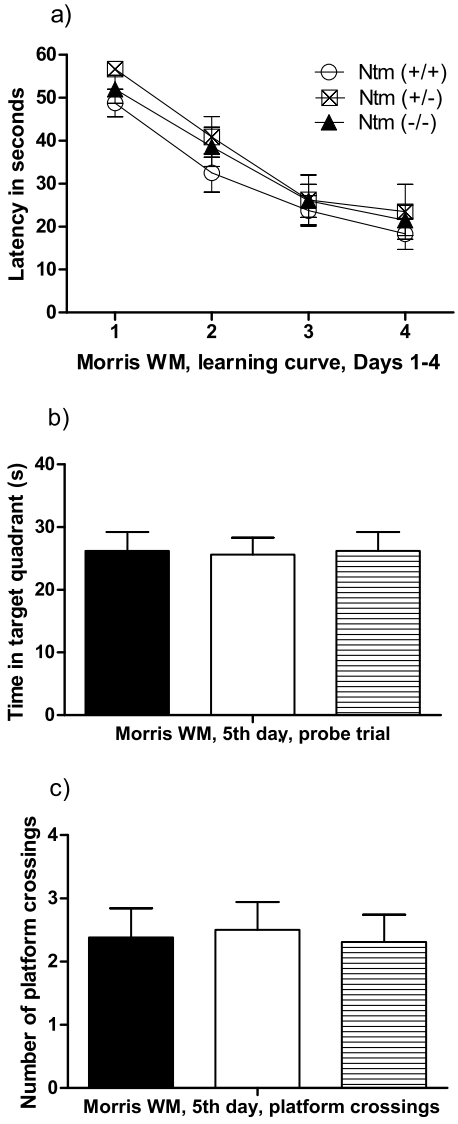

Fig. 3. The learning curve (a) in the Morris water maze showing the time (in seconds) to reach the submerged platform in Days 1–4. Average values per day, obtained by collapsing data of four trials for each animal, are presented. In (b) and (c), time spent in the target quadrant and the number of platform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

3.9. Marble burying test

There was no difference between the genotypes in the number of marbles buried (Table 1).

3.10. Nesting behaviour test

There were no differences between the nest scores (Table 1).

3.11. Social interaction test

Of the 15 Ntm+/+ and 16 Ntm−/− mice tested, only two +/+ mice and one −/− 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 1).

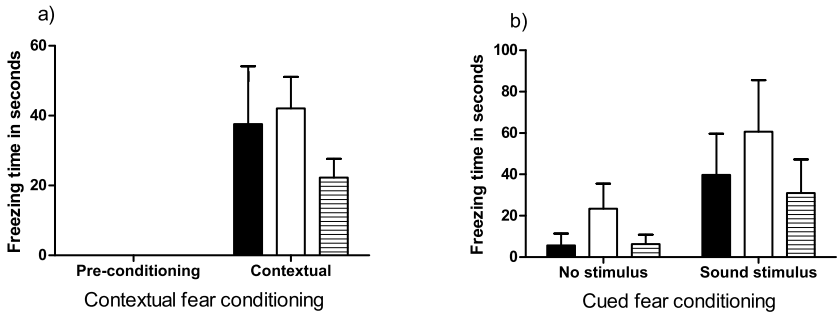

Fig. 4. Results of the contextual (a) and cued (b) fear conditioning. N = 8 mice in every group. Black colums = Ntm+/+; white colums = Ntm+/−; striped columns = Ntm−/−.

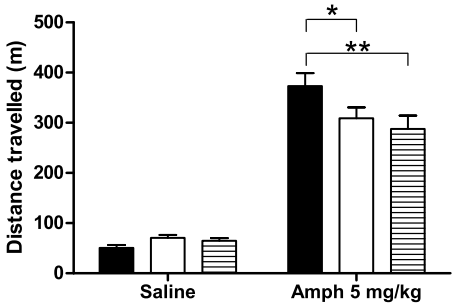

Fig. 5. The locomotor stimulating effect of 5 mg/kg of amphetamine. Black colums = Ntm+/+; white colums = Ntm+/−; striped columns = Ntm−/−. *p < 0.05; **p < 0.01: compared to Ntm+/+ mice. N = 7–8 in all groups. m = metres; Amph = amphetamine.

3.12. Locomotor activity test with 5 mg/kg of amphetamine

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 +/+ mice compared to +/− ($p = 0.02$) and −/− mice ($p = 0.009$) (Fig. 5).

3.13. Loss and regain of righting reflex test with 4 g/kg of ethanol

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 1).

4. Discussion

This study gives an initial characterization of the phenotype of Ntm deficient mice. We found these mice to have normal body weight, no gross vision, hearing or olfaction deficiencies and no alterations in pain sensitivity. Ntm and Lsamp are colocalized 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 colocalization 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. [10–12]. 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). Learning deficiency revealed in active avoidance is indirectly in line with studies associating Ntm with cognitive function and intelligence [16,24]. Table 2 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 2
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)	=/↓*	=
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 gene-dose dependent manner.

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