DISENTANGLING THE ROLE OF TRPM4 IN HIPPOCAMPUS-DEPENDENT PLASTICITY AND LEARNING: AN ELECTROPHYSIOLOGICAL, BEHAVIORAL AND fMRI APPROACH.

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Abstract

Hippocampal long-term potentiation (LTP) has been extensively studied as a cellular model of learning and memory. Recently, we described a central function of the Transient Receptor Potential M4 (TRPM4) channel in hippocampal LTP in mice *in vitro*. Here, we used *Trpm4* knock-out (*Trpm4*^{-/-}) rats to scrutinize TRPM4's role in the intact brain *in vivo*. After having confirmed the previous *in vitro* findings in mice, we studied hippocampal synaptic plasticity by chronic recordings in freely-moving rats, hippocampus-dependent learning by a behavioral battery and hippocampal-cortical connectivity by fMRI. The electrophysiological investigation supports an involvement of TRPM4 in LTP depending on the induction protocol. Moreover, an exhaustive analysis of the LTP kinetics point to mechanistic changes in LTP by *trpm4* deletion. General behavior as measured by open field test, light-dark box and elevated plus maze was inconspicuous in *Trpm4*^{-/-} rats. However, they showed a distinct deficit in spatial working and reference memory associated to the Barnes maze and T-maze test, respectively. In contrast, performance of the *Trpm4*^{-/-} in the Morris water maze was unaltered. Finally, fMRI investigation of the effects of a strong LTP induction manifested BOLD responses in the ipsilateral and contralateral hippocampus and the prefrontal cortex of both groups. Yet, the initial BOLD response in the stimulated

hippocampal area of *Trpm4*^{-/-} was significantly enhanced compared to WT rats. Our findings at the cellular, behavioral and system level point to a relevant role for TRPM4 in specific types of hippocampal synaptic plasticity and learning but not in hippocampal-prefrontal interaction.

Introduction

The hippocampus (HP) and its multiple forms of synaptic plasticity have been extensively studied in recent decades (Citri & Malenka, 2008). The most relevant forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) have been proven to model many of the molecular, cellular and morphological underpinnings of learning and memory (Kandel, Dudai, & Mayford, 2014; Takeuchi, Duzsikiewicz, & Morris, 2013). Numerous studies have explored the phenomenon at the perforant path to dentate gyrus synapse (PP-DG) (Bliss & Lomo, 1973; Nicoll, 2017), but also at the afferent fibre connection between CA3 and CA1 pyramidal neurons, known as the Schaffer collateral-commissural pathway (SCCP). Induction of LTP in the SCCP typically involves postsynaptic calcium entry via N-methyl-D-aspartate receptors (NMDAR), subsequent activation of protein kinases (Lüscher & Malenka, 2012; Park et al., 2014; Volianskis et al., 2015) and the release of calcium from intracellular calcium stores (Lisman, 1989; Malenka, Kauer, Zucker, & Nicoll, 1988). However, LTP can also be expressed in the presence of NMDAR-antagonists, in a process that involves L-type voltage-gated calcium channels (VGCC) (Freir & Herron, 2003; Morgan & Teyler, 1999) depending on the type and strength of the particular induction protocol.

A novel player in synaptic plasticity that has only recently been involved in brain processes is the Transient Receptor Potential channel M4 (TRPM4). TRPM4 is a calcium-activated but calcium-impermeable non-selective cation (CAN) channel that regulates Ca²⁺-dependent changes in membrane potential in excitable and non-excitable cell types (Launay et al., 2002). TRPM4 belongs to the melastatin subfamily of the Transient Receptor Potential (TRP) channels, which form an evolutionary conserved protein channel superfamily and are widely recognized for their role in sensory signal transduction and a variety of monogenic and complex human disorders (for a review on Trp channels, Nilius & Owsianik, 2011). We have recently shown that TRPM4 has an important function in LTP induction *in vitro* as a possible co-activator of NMDAR, since mice lacking the TRPM4 protein showed an impaired LTP in the hippocampal CA1-region, which was rescued by promoting NMDAR activation or post-synaptic membrane depolarization (Menigoz et al., 2015).

However, experimentally induced regional synaptic modifications also alter the activity of global, widespread networks that cannot be observed *in vitro*. Therefore, electrophysiological recordings in the intact brain in combination with functional imaging of the whole brain network are emerging as outstanding complementary methods to study *in vivo* physiological functions at the system level. This synergistic approach allows new insights into the complex physiological changes across different brain areas during the induction of LTP. For instance, recent studies have shown that induction of hippocampal PP-DG LTP result in significant BOLD responses not only in the dentate gyrus but also in the entire hippocampal formation (including the contralateral side) and in various target regions of the neocortex, such as the prefrontal lobe (Álvarez-salvado, Pallarés, Moreno, & Canals, 2013; Angenstein, Krautwald, Wetzel, & Scheich, 2013; Canals, Beyerlein, Merkle, & Logothetis, 2009). Activation of the bidirectional pathway from the hippocampus (HP) to the prefrontal cortex (PFC) by hippocampal LTP induction is functionally very interesting since the monosynaptic connection from ventral HP (vHP) to medial PFC (mPFC) has been associated with episodic and working memory, and other indirect HP-mPFC projections have been related to contextual memory retrieval and emotion (Eichenbaum, 2017; Jin & Maren, 2015).

Here, we pursued a characterization of the role of TRPM4 in the intact brain. Since *in vitro* data already indicated a role of TRPM4 in synaptic plasticity in mice, we aimed in this study to validate these findings in the rat knock-out model (*Trpm4*^{-/-}) *in vivo*. To go further beyond, we investigated the role of TRPM4 in different types of learning and in hippocampal-cortical connectivity using a combination of electrophysiological stimulation, fMRI recording and behavioral analysis.

Experimental Procedures

Animals

Trpm4 knock-out rats (*Trpm4*^{-/-}) and wild-type (WT) rats about 7-10 weeks old (300-450g) male littermates were used for the experiments. The animals were housed and maintained in standard cages under conventional laboratory conditions with *ad libitum* access to food and water and 12h light/12h dark cycle. The experiments were conducted during the light phase of the animals' activity cycle. The animal care and experimental protocols were performed following the regulations of the European Community Council Directive (2010/63/EU) and approved by the local ethics committee under the number P079/2013 and P123/2012, KU Leuven.

Genetic alteration of *Trpm4*^{-/-} knockout rats

Trpm4^{-/-} knockout rats were generated by Sigma Advanced Genetic Engineering (SAGE®) Labs. *Trpm4* gene specific Zinc finger constructs directed against exons 18-19, which contain the coding sequence for TM3-5 and the pore region of the TRPM4 protein, were injected in zygotes from Sprague-Dawley rats. Numerous founder animals were born and genotyped. An animal with a 514bp deletion in the TRPM4 gene was selected as the founder for further breeding. This deletion completely removes exon 18 and a piece of exon 19 from the *Trpm4* gene, plus the intron 18-19. In the mutant protein TM3, TM4 and a piece of TM5 are completely removed, and a frame shift of the reading frame is introduced. The deletion was confirmed via genomic sequencing and western blotting.

Electrophysiology

In vitro recording from hippocampal slices

Slice preparation

Rats were sacrificed by cervical dislocation and brains transferred to ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF; in mM): NaCl 124; KCl 3; NaHCO₃ 26; NaH₂PO₄ 1.25; MgSO₄ 1; CaCl₂ 2; glucose 10. Hippocampi were horizontally sliced (250 µm thick) in a vibratome filled with ice-cold oxygenated aCSF. Hippocampal slices were allowed to recover in an interface chamber filled with oxygenated solution at room temperature for at least 2 hours. Then they were placed in a MEA (Multi Electrode Array) recording chamber and perfused continuously at a rate of 2 ml/min with ACSF at 32°C.

Extracellular recordings from a MEA-chip

Extracellular stimulations and recordings were performed with a MEA set-up (Multi Channels Systems, Reutlingen, Germany) consisting of a MEA1060-BC pre-amplifier, a filter amplifier (gain 1100X) and a two-channel stimulus generator (STG2002). MEA biochips with 8 X 8 titanium nitrite electrodes (30 µm diameter and 200 µm spacing) were used. The bath was grounded with an internal reference electrode. Slice position and contact with the electrodes were secured with a nylon mesh (ALA Scientific Instruments, USA). Stimulation electrodes were positioned at the Schaffer collateral pathway and evoked field excitatory postsynaptic potentials (fEPSP) were monitored at proximal stratum radiatum. Data acquisition was performed and controlled by MC_Rack 3.2.1.0 and MC_Stimulus II software (Multichannel Systems, Reutlingen, Germany). Input/output curves were obtained 60 minutes after placing the slice on the biochip. Test stimuli were delivered as biphasic pulses (100 µs pulse width) at increasing voltages. For each stimulation, data from three consecutive fEPSPs were collected. Baseline stimulation strength for the rest of the experiments was set to produce 40% of maximal response. Paired-pulse facilitation (PPF) was investigated by applying two pulses in rapid succession (inter-pulse intervals of 20, 50, 100, 150, 200 and 250 ms, respectively). Data from three consecutive fEPSPs were collected for each inter-pulse interval. PPF was calculated as the ratio of the slope from the second to first response for each pair of stimulations. LTP was induced after 40 min of stable baseline by one of the following protocols: One, three or eight trains of theta-burst stimulation (TBS; 5 bursts of 4 pulses at 100 Hz repeated at 200 ms interval, inter-train interval 5 min), or high-frequency stimulation (HFS: 1s at 100 Hz).

In vivo recording from the intact hippocampus

Chronic implantation of electrodes in the hippocampal CA1-region

The animals were anesthetized using an isoflurane/oxygen pump system (Iso-vet Surgivet at 3% for induction and 1-2% maintenance; O₂ 1mL/min). A drop in body temperature was prevented by a ventral

heat pad during the whole surgery. Additional subcutaneous anesthetics were applied on the shaved head (Xylocaine 2%, 1:200000 adrenaline, AstraZeneca). Then, the animals were chronically implanted with custom-made stimulation and recording electrodes as described earlier (Seidenbecher, Balschun, & Reymann, 1995). They consisted of Teflon-coated tungsten wire (inner core diameter 0.05 μm and 0.075 μm , respectively; Advent Research Materials), and were connected to a miniature socket. The stereotaxic coordinates were determined by a dual standard digital stereotaxic instrument (Stoelting Co) and referenced to bregma for anteroposterior (AP) and mediolateral (ML) distance, and to the brain surface for dorsoventral (DV) distance. The bipolar stimulation electrode was implanted in the *Schaffer collaterals* of the right hippocampus (AP -3.5, ML 3, DV. 2-3) to evoke a field excitatory post-synaptic potential (fEPSP) that was recorded by a monopolar recording electrode implanted in the apical dendritic layer of *stratum radiatum* of the hippocampal CA1 area (AP -3.4, ML 2, DV 2-3). The depth of the electrodes was adjusted such that the fEPSP amplitude and slope were maximal, by using biphasic test-pulses of 200 μA (0.1 ms pulse width). Additionally, two stainless steel screws were fixed on the contralateral side of the skull, serving as reference and ground electrodes. Once the fEPSP was stable, the whole assembly was fixed on the skull with UV dried dental cement (Tetric EvoFlow, Ivoclar Vivadent). Pain relief was ensured for 24 h with 0.06 mg/kg Vetergesic (Ecu phar). The rats were group-housed until the surgery, after which they were placed individually to promote recovery and protect the head post. The correct position of the electrodes was confirmed a posteriori via post-mortem histological analysis (e.g. in [Fig. 2a](#)).

Electrophysiological recordings in freely-moving animals

After at least 7 days of recovery, the animals were placed into the experimental box (40 x 40 x 40 cm) with *ad libitum* access to water and food and the electrodes connected by means of a flexible cable, allowing the animal to move freely. Single biphasic square waves of constant current (0.1 ms duration per half-wave), were generated by an isolated pulse stimulator (A-M Systems, 2100) at low frequency (0.025 Hz) and the evoked signals were amplified by a differential AC amplifier (AM systems 3100), filtered by a low-pass filter at 5 kHz and by a high-pass filter at 1 Hz, digitized through a data acquisition unit (1401, 625 kHz, 16 bit ADC, CED) and stored on-line by a PC. Additionally, 50 Hz noise removal was performed by a HumBug noise eliminator (Quest Scientific). Test stimuli were delivered to the Schaffer-collateral fibers every 5 min at the stimulation strength that evoked field EPSPs of 35% of the maximum determined according to an input-output (I-O) curve, ranging from 50 – 800 μA and established for each individual animal 24 h before the experiment. LTP was induced by a high-frequency stimulation (HFS) protocol consisting of 1, 4 or 6 trains of 20 pulses at 200 Hz (0.1 ms stimulus duration, inter-train interval 2 s), repeated three times in a time interval of 10 min. For a more detailed explanation of the protocols, see [Fig. 2b](#). Animals that showed electrophysiological and behavioral signs of seizures were discarded.

Behavior

We examined the general behavior of the *Trpm4*^{-/-} rats by means of the open field test (OFT), light-dark box (LDB) and the elevated plus maze (EPM), and characterized hippocampus-dependent learning with an aversive motivated protocol the Barnes maze (BM), the Morris water maze (MWM) test and the elevated T-maze test. A general description of the common features is summarized here, while an extensive explanation of the tests can be found in the **online supplementary Information**. Behavioral testing was carried out on males, 6 - 7 weeks old (at the start of the habituation period), socially housed (4 animals per polycarbonate cage) with wood chip bedding, nesting material and wood chew sticks as enrichment. All tests were performed between 9 a.m. and 5 p.m. Wild type and *Trpm4*^{-/-} animals were randomly tested. In order to accomplish a general characterization of the *Trpm4*^{-/-}, all animals were submitted to the behavioral test battery, and independent groups to the hippocampus-dependent tasks. The common procedures for all behavioral tests included habituation to the experimenter, corresponding training sessions (depending on the task), and a probe test. After every session the animals were returned to their home cage.

Electrical stimulation and functional imaging fMRI

All combined electrophysiology/fMRI measurements were performed in the Leibniz Institute for Neurobiology (LIN), Magdeburg, Germany as previously described (Angenstein et al., 2007; Riemann,

Helbing, & Angenstein, 2016) using a 4.7 T Bruker Biospec 47/20 animal scanner (free bore of 20 cm) equipped with a BGA09 (400 mT/m) gradient system (Bruker BioSpin GmbH, Ettlingen, Germany) and slightly adapted to our area of interest and specific conditions. A 50 mm Litzcage small animal imaging system (DotyScientific Inc., Columbus, SC, USA) was used for the RF signal reception. The animals were chronically implanted as described above for the electrophysiological experiments but under pentobarbital anesthesia (40 mg/kg, i.p.). The coordinates needed to be slightly adapted so the distance between the electrodes was bigger (still staying in the same layers of CA1), silver wire was used for reference and grounding, and plastic screws were used to ensure the implant. Then everything was fixed with air-dried dental cement. Moreover, females were also included in this study, thus each group consisted on 5 males and 4 females. Combined electrophysiological/fMRI recordings were performed one week after the surgery. All animals were initially anesthetized with isoflurane (2%; in 50:50 N₂:O₂; v:v), which allowed to fix the head of the animals in a custom-made animal cradle and to connect the recording and stimulation electrodes. Then, the isoflurane concentration was reduced to 1.5% and medetomidine (50 µg/kg) was subcutaneously applied. After 10 min, isoflurane was further reduced to 0.4% and a continuous s.c. medetomidine injection (100 µg/kg/h) started. Five minutes later isoflurane administration was terminated. All necessary MRI and electrophysiological adjustments for the simultaneous fMRI experiment were set in parallel before the measurements began. Breathing, heart rate, and oxygen saturation were monitored throughout the experiment by an MRI-compatible pulse oximeter (MouseOXTM; Starr Life Sciences Corp., Pittsburgh, PA, USA) and heating was provided from the ventral site. To keep the intensity of the stimulation constant through all fMRI experiments 150 µA was chosen as the stimulation intensity. The CA1 region was stimulated with the strong LTP protocol described for *Electrophysiology* above (6 trains of 20 pulses at 200 Hz, 0.2 ms stimulus duration, inter-train interval 2 s, repeated three times in a time interval of 10 min). The first stimulus train was presented 2 min after starting the fMRI session. The total scanning time for the combined electrophysiology/fMRI experiment was 25 min. The combined fMRI and electrophysiology session started 30 min after placement of the rat in the scanner, thus the total time for pre-adjustments and data acquisition was less than 1 h. The electrophysiological responses during stimulation were recorded using the same equipment as mentioned for *Electrophysiological recordings in freely-moving animals* and sampled at 5 MHz. For anatomical images, 10 horizontal T₂-weighted spin-echo images were obtained with rapid acquisition relaxation enhanced sequence (RARE sequence, Hennig, Nauerth, & Friedburg, 1986) with the following parameters: TR 4000 ms, TE 15 ms, slice thickness 0.8 mm, FOV 37 x 37 mm, matrix 256 x 256, RARE factor 8, and number of averages four. The total scanning time was 8 min 32 s. fMRI was performed with an echo-gradient EPI (echo planar imaging) sequence with the following parameters: TR 2000 ms, TE 24 ms, slice thickness 0.8 mm, FOV 37 x 37 mm, matrix 92 x 92, and total scanning time per frame 2 s. The slice geometry, i.e., the position and size of all 10 horizontal slices, was identical to the previously obtained anatomical T₂-weighted spin-echo-images.

Data processing and analysis

Electrophysiology and behavior

To analyze the electrophysiology data between groups, a repeated measures analysis of variance (RM-ANOVA, SPSS, IBM Corp.19; Armonk, NY) was applied. The fEPSP slopes from the period after the electrical stimulation (min 30) to the end of the experiment (min 340) were compared between groups in order to assess the statistical difference between genotypes. For the analyses of the different phases of LTP, a RM-ANOVA was performed for initial potentiation (I-LTP, from min 30 to min 60; i.e. 10 to 40 min post tetanus), early-LTP (E-LTP, min 65 to min 180) and late-LTP (L-LTP, min 85 to min 340). Therefore, the post-tetanic potentiation (PTP, first minutes after HFS) was excluded. All intervals in between the 3 HFS were excluded too. Column analyses for comparison between two behavioral groups were performed by Student t-tests. When repeated measures were required, then RM-ANOVA analyses were applied. Additionally, for comparing quadrants preference, a one-way ANOVA was performed.

Dynamic data-based modelling

Discrete-time transfer function (TF) models were used to quantify the dynamics of the obtained LTP responses for the different experimental conditions (see Tambuyzer et al., 2013; Young, 2011 for more details on this modelling approach). The models describe the relation between the LTP induction protocol (model input; i.e. HFS) and the long-term potentiation responses (model output; i.e. measured

fEPSP slopes as a percentage of the baseline). For each data set, the obtained LTP responses were averaged and the resulting mean response curves were used to estimate the best first and second order TF models, which can be interpreted as a representation of the complexity of the LTP response dynamics (Tambuyzer et al., 2013). Three criteria were used to select the best models: R^2 (Young, 1984), the Akaike information criterion (AIC; Akaike, 1974) and visual inspection based on a priori biological knowledge (Ljung, 1987). Finally, time constants (TC) and steady-state gain (SSG) values were calculated for each model. In practical terms, assuming zero initial conditions, the TC is the time taken for the output (i.e. fEPSP slope) to reach 63% of its steady state value in response to a step input (i.e. HFS protocol). The SSG can be defined as the steady state change of output per unit change of input. One-paired Wilcoxon rank sum tests were used to compare the TC and SSG values obtained for the different LTP responses. All calculations were performed in Matlab using the Statistics Toolbox for the statistical comparisons and the Captain Toolbox for the TF model analyses (Taylor, Pedregal, Young, & Tych, 2007).

Functional imaging

The functional data were loaded and converted into BrainVoyager data format. A standard sequence of pre-processing steps implemented in the BrainVoyager QX 2.6.1 software (Brain Innovation, Maastricht, the Netherlands), such as slice scan time correction, 3D motion correction (trilinear interpolation and reduced data using the first volume as a reference), and temporal filtering (high pass GLM-Fourier: three sines/cosines and Gaussian filter; FWHM 3 data points) were applied to each data set. Because the reconstruction of the fMRI images resulted in a 128 x 128 matrix (instead of the 92 x 92 imaging matrix), a spatial smoothing (Gaussian filter of 1.4 voxel) was added.

To visualize the effect of high-frequency stimulation on variations in local BOLD signals two forms of analysis were performed: (i) a multi-subject general linear model (GLM) analysis and (ii) a volume of interest (VOI) analysis.

GLM analysis: each individual functional data set was used for a multiple-subject GLM analysis implemented in BrainVoyager QX 2.6.1 software. Functional activation was analyzed by using the correlation of the observed BOLD signal intensity changes in each voxel with a predictor (hemodynamic response function), generated from the given stimulus protocol (see above). Based on this, the appropriate 2D activation map could be generated. To calculate the predictor, the square wave representing stimulus on and off conditions was convolved with a double gamma hemodynamic response function (onset 0 s, time to response peak 5 s, time to undershoot peak 15 s). To exclude false-positive voxels, we only considered those with a significance level (p) above the threshold set by calculating the false discovery rate (FDR) with a q -value of 0.01 (which corresponds to a t value greater than 3.17 or $p < 0.0016$). Each individual BOLD time series was normalized using the averaged BOLD signal intensity of 100%. All normalized BOLD time series were then averaged and depicted as mean BOLD time series $\pm$ SEM. Based on the calculated BOLD time series, event-related BOLD responses were calculated by measuring the signal intensities starting 10 frames before stimulus onset (-20 s until 0 s), during stimulus presentation (between 0 s and 12 s, which corresponds to 6 frames) and the following 30 frames (12 s to 72 s) after the end of the stimulus. To avoid the confounding effect of putative variations in baseline BOLD signal intensities on the calculated BOLD response (i.e., $\text{BOLD signal}_{\text{stimulus}} / \text{BOLD signal}_{\text{baseline}} * 100\%$), each BOLD response was related to BOLD signal intensities of the stimulus over the preceding 20 s. To compare average maximal BOLD responses between conditions, a two-tailed unpaired Student's t -test was performed. Differences were considered significant at a calculated p -value less than 0.05.

VOI analysis: each individual functional imaging data set was aligned to a 3D standard rat brain using the 3D volume tool implemented in BrainVoyager QX 2.6.1 software. Individual VOIs, i.e., right/left hippocampus and prefrontal cortex region, were marked in the 3D standard rat brain. The averaged BOLD time series of all voxels located in one VOI was then calculated for each individual animal using the volume-of-interest-analysis tool implemented in the BrainVoyager QX 2.6.1 software.

Results

Genetic deletion of TRPM4 in rats results in a severe impairment of LTP in vitro

First, we tested the electrophysiological properties of synaptic communication in slices, via MEA recordings of field potentials (Menigoz et al., 2015; Vennekens et al., 2007). As shown in [Fig. 1a](#), basal

synaptic transmission and paired pulse facilitation were unaffected in *Trpm4*^{-/-} rats. Next, we examined long-term plasticity and induced LTP with TBS-protocols of increasing strength. When LTP was induced with the weakest TBS-protocol, consisting of only one TBS-train, potentiation in WT reached a stable level of $130 \pm 5.65\%$ 30 min after induction. In contrast, in *Trpm4*^{-/-} only a short-term potentiation (STP) of low magnitude ($103.50 \pm 3.56\%$) was induced, which returned to baseline level within 30 min (**Fig. 1b**) ($F_{(1,15)} = 20.93$, $p = 0.0004$; WT $n = 9$, *Trpm4*^{-/-} $n = 8$; 2-way RM-ANOVA). The induction of LTP by 3-TBS caused a higher LTP of $164.52 \pm 7.24\%$ at 30 min in WT but again only STP in *Trpm4*^{-/-} although with a slightly higher initial magnitude ($136.0 \pm 10.0\%$ at 5 min). At 30 min the values were back to baseline ($107.32 \pm 7.01\%$; **Fig. 1d**). Overall, this resulted in an even higher difference between WT and *Trpm4*^{-/-} ($F_{(1,15)} = 116.3$, $p < 0.0001$; WT $n = 8$, *Trpm4*^{-/-} $n = 9$; 2-way RM-ANOVA). Increasing the strength of the induction protocol further to 8 TBS, caused in WT a very stable LTP of $219.94 \pm 18.93\%$. Interestingly, this protocol was strong enough to generate LTP even in *Trpm4*^{-/-} ($149.81 \pm 5.96\%$ at 30 min, **Fig. 1e**). However, the difference between both genotypes in the level of potentiation was pronounced ($F_{(1,10)} = 12.7$, $p = 0.0051$; WT $n = 6$, *Trpm4*^{-/-} $n = 6$; 2-way RM-ANOVA). Finally, we tested the potency of high-frequency stimulation (HFS), an induction protocol that is often used for LTP-induction. As depicted in **Fig. 1f**, HFS caused a robust LTP of $190.95 \pm 10.35\%$ at 30 min, compared to the $136.33 \pm 4.35\%$ in *Trpm4*^{-/-} ($F_{(1,12)} = 45.97$, $p < 0.0001$; WT $n = 7$, *Trpm4*^{-/-} $n = 7$; 2-way RM-ANOVA). Taken together, *Trpm4*^{-/-} rats show a severe impairment in LTP depending on the strength of the induction protocol, whilst basal synaptic transmission and short-term plasticity are apparently unchanged.

Basal synaptic transmission is unaltered in freely-moving Trpm4^{-/-} animals

After having confirmed that *Trpm4*^{-/-} rats express a similar ‘plasticity’ phenotype as previously found in *Trpm4*^{-/-} mice, we moved on to examine the function of TRPM4 in synaptic plasticity in the intact hippocampus *in vivo*. The day prior to the experiment, all animals were exposed to the recording cage for habituation and testing the electrophysiological responses. Stimulation of the Schaffer collaterals evoked fEPSPs in the CA1 region of the right hippocampus (e.g. in **Fig. 2a**). 24h later, input-output (I-O) curves were determined, which provided a measurement for basal synaptic transmission and for the saturation level of the field excitatory post-synaptic potential (fEPSP) for each animal. When stimulated at progressively increasing stimulation strengths, both groups followed a similar tendency, although the *Trpm4*^{-/-} seemed to reach saturation at a higher level as compared to the WT. However, the differences were not statistically significant ($p > 0.05$, t-student test) (**Fig. 3a**). The stimulation strength that evoked the 35% of the maximum slope for each animal was used as the standard stimulation strength ($\sim 150 \mu A$) for the experiment 24h later. Additionally, paired-pulse facilitation (PPF), a presynaptically mediated form of short-term plasticity, was tested in all animals by applying two stimulation pulses at a 30 ms interval. As shown in **Fig. 3b**, PPF did not differ between the two groups.

Trpm4 has a differential role in in vivo CA1-LTP depending on the strength of the induction protocol

Next we explored the effect of *Trpm4* deletion on the induction of LTP in the intact brain *in vivo*. Previous investigations in our laboratories had shown that the genetic deletion of this channel in mice results in a severe deficit of CA1-LTP *in vitro* (Menigoz et al., 2015). To determine whether the functional importance of TRPM4 for LTP depends on the strength of its induction, rats lacking the TRPM4 protein and their controls were tested with weak, intermediate or strong induction protocols that varied in strength due to the number of trains applied, consisting of 1, 4, or 6 trains of 20 bursts at 200 Hz (repeated three times, 3 HFS, at 10 min interval), respectively. For consistency of the analyses, we followed the LTP classification proposed by Sweatt (Sweatt, 2010) which distinguishes three different coexisting phases of LTP: initial potentiation (I-LTP, 30-60 min, starting 10 min after the last HFS), early-LTP (E-LTP, 65-180 min) and late-LTP (L-LTP, 185-340 min).

Strong HFS (S, 3 x 6 bursts of 20 pulses at 200 Hz) applied to wild type (WT) freely-moving rats resulted in a robust LTP. In contrast, animals lacking *Trpm4* expressed LTP which declined faster, resulting in a statistically significant difference between WT and *Trpm4*^{-/-} ($F_{(1,12)} = 24.92$, $p = 0.0003$, 2-way RM ANOVA). Looking more closely at the different phases of LTP, there are no differences in LTP induction, but in early-LTP and late-LTP (E-LTP $F_{(1,12)} = 11.97$, $p < 0.005$; L-LTP $F_{(1,12)} = 50.82$, $p < 0.0001$; 2-way RM ANOVA) (**Fig. 4a**). As depicted in **Fig. 4b**, WT rats expressed a LTP of $182 \pm 6.59\%$, which stayed stable for about 3h ($179 \pm 5.56\%$) and slightly decreased to $158 \pm 7.15\%$ after 6h. In contrast, LTP in

Trpm4^{-/-} animals, although surpassing the induction level of the WT ($210 \pm 7.89\%$), decreased faster to around $139 \pm 6.99\%$ (3h) and kept progressively decreasing (6h, $125 \pm 5.91\%$ LTP). Intermediate HFS (I, 3 x 4 bursts of 20 pulses at 200 Hz) produced a LTP of striking similarity in controls and *Trpm4*^{-/-} (Fig. 5a). Both groups reached a similar LTP induction level ($210 \pm 13.32\%$ in WT vs. $196 \pm 7.29\%$ in *Trpm4*^{-/-}) that immediately started decreasing to progressively converge to similar levels by three hours ($140 \pm 21.58\%$ WT, $140 \pm 12.59\%$ *Trpm4*^{-/-}) and stabilized thereafter at $120 \pm 18.47\%$ in WT and $138 \pm 14.96\%$ in *Trpm4*^{-/-} (Fig. 5b). The weakest HFS (W, 3 x 1 burst of 20 pulses at 200 Hz) still induced LTP in both groups, but the induction period and first hours of LTP were significantly different in *Trpm4*^{-/-} compared to WT. Whilst the WT exhibited a LTP that slowly decreased for 3 h until reaching a stable level, the LTP of the *Trpm4*^{-/-} animals started decreasing right after the induction to achieve stability around 80 min post-induction (I-LTP $F_{(1,14)} = 5.6$, $p < 0.05$; E-LTP, $F_{(1,14)} = 5.35$, $p < 0.05$; 2-way RM ANOVA) (Fig. 6a). Moreover, the WT reached an induction of $175 \pm 9.88\%$ LTP that progressively decreased to reach $132 \pm 4.61\%$ by 3 h, while the *Trpm4*^{-/-} expressed a LTP induction of $147\% \pm 8.05$ that rapidly decayed to $125 \pm 9.45\%$ by 3 h. By the end of the experiment the recordings of both groups were almost at baseline levels with a $108 \pm 7.06\%$ in WT and $108 \pm 11.17\%$ in *Trpm4*^{-/-} (Fig. 6b). Together, the different time-course of LTP in WT and *Trpm4*^{-/-} rats in response to certain induction protocols indicated that deletion of *Trpm4* results in mechanistic changes in LTP.

This was confirmed by an exhaustive analysis of the effect of the specific induction protocols on LTP kinetics by dynamic data-based modelling, which pointed to diverse characteristics for LTP in WT and *Trpm4*^{-/-} (Fig. 7a). In general, all models could describe the LTP responses in an accurate way (R^2 close to 1 in all cases, Table 1). Moreover, all model parameter values were reliable (see confidence interval of model parameters in Table 1). In support of our supposition, the first order models were selected as best models for the LTP curves of WT rats (Model selection criteria: R^2 , AIC and visual inspection; see Table 1), whereas second order models were more suitable for the LTP curves of *Trpm4*^{-/-} rats (Model selection criteria: R^2 , AIC and visual inspection; see Table 1). Modelling the LTP decay data (Table 2) resulted in lower time constant (TC) values for WT, denoting slow decay after the strong LTP induction (lower values of TC, Table 2). Conversely, *Trpm4*^{-/-} evidenced a LTP induction that decayed promptly (fast, high TC values, Table 2) over the 6 h of the experiment. Interestingly, in WT the strong HFS caused a stable LTP that worked as an integrator [i.e. a step change in the input (HFS) causes the system output (LTP) to change nearly linearly, as indicated by an autoregressive parameter (a-par) that is reflecting the response transient dynamics reaching a value of almost -1]. Thus, the effect of the HFS will remain for a long time after the induction protocol (see Tambuyzer et al., 2013 for further explanation). In WT rats the strong and intermediate protocols evoked similar LTP induction levels ("First value" in Table 2) whilst the intermediate and weak protocols caused similar features of LTP maintenance, resulting in different end levels (See also "SSG" and "Last value" in Table 2). In contrast, the *Trpm4*^{-/-} displayed similar tendencies after the application of all protocols, just following different intensities that decreased succeeding the HFS strength but converged to similar end levels (Fig. 6; SSG and "Last value", Table 2).

***Trpm4* absence does not affect general behavior but affects an aversive hippocampus-dependent task**

The significant effects of *Trpm4* disruption on some forms of LTP prompted us to check whether these deficits in hippocampal synaptic plasticity might be accompanied by changes in behavior. Fig. 8 summarizes the results of the inspection of general behavioral traits in the open field test (OFT, Fig. 7a), the light-dark box (LDB, Fig. 8b) and the elevated plus maze (EPM, Fig. 8c), which did not reveal any differences. The data suggest normal level of activity and exploratory response (as denoted by the zone entries, % center vs. periphery, and latency to center in the OFT; % of time in darkness, latency to darkness, light-dark transitions in the DLB; latency to center, number of transitions, % time in closed arms in EPM). Moreover, the anxiety levels (measured as % of time in the center vs. periphery in OFT and % of time in open arms vs. center in EPM) were not significantly different between WT and *Trpm4*^{-/-}. Thus, *Trpm4*^{-/-} rats were inconspicuous in these general behavioral characteristics.

In order to assess hippocampus-dependent learning behavior, the animals were trained in an aversive version of the Barnes maze (BM). The animals were trained to find a fixed escape tunnel position from different starting points. As depicted in Fig. 9a, *Trpm4*^{-/-} rats seemed to need more time during the training phase to learn the task, especially in day 1 and day 4 (latency, upper panel; not significant, n. s., $F_{(1, 22)} = 3.582$, $p = 0.0716$; 2-way RM ANOVA; WT d1-d4 $p < 0.01$, *Trpm4*^{-/-} $p < 0.001$),

and made significantly more mistakes (lower panel; significant, $F_{(1,22)} = 6.328$, $p = 0.0197$; 2-way RM ANOVA; WT d1-d4 $p < 0.05$, *Trpm4*^{-/-} n. s.). The good performance of the two genotypes in the probe trial (**Fig. 9b**) evidenced that they had learnt the task, reaching similar levels of % of right pokes (upper graph) and % of time spent in the wrong quadrants (lower graph). Additionally, we measured the number of working memory mistakes per training day, i.e. how many times the animals visited an already visited hole. The upper panel in **Fig. 9c** shows that the *Trpm4*^{-/-} rats made a significantly higher percentage of mistakes per day during training ($t = 2.311$, $p = 0.031$; Welch's t-test). Finally, 48h after the probe trial, the animals were tested in a serial working memory (WM) task where they had to remember a new escape tunnel position learnt in a previous session in the same day (ITI 30 min), during consecutive days. In this test, the *Trpm4*^{-/-} performed significantly worse than the WT in all variables measured: latency ($t = 3.029$, $p = 0.009$; Welch's t-test) and number of mistakes ($t = 4.5$, $p = 0.0005$; Welch's t-test) (**Fig. 9c**, middle and lower panel). We also analyzed the search strategy that animals used to localize the target hole. No significant differences between WT and *Trpm4*^{-/-} were found. As depicted in **Fig 9d**, both groups started searching based on a random strategy, to switch then to a serial strategy, and eventually changed to a spatial strategy after 4 days of training.

To further examine spatial learning capacity in the *Trpm4*^{-/-} rats, they were trained in a Morris water maze (MWM) with hidden platform (Morris, 1984). During acquisition, both groups learned the position of the hidden platform in a similar way. However, *Trpm4*^{-/-} animals displayed during the first day of training a significantly better performance as evidenced by a significantly smaller path-length ($t = 3.247$, $p = 0.0039$; Welch-test) (**Fig. 10a**). This difference was not due to a different swimming speed because the swim velocity of both genotypes was almost identical from day 1 (WT 23.215 ± 0.862 cm/s; *Trpm4*^{-/-} 20.555 ± 1.103 cm/s). On the next day, however, WT animals achieved a similar performance level (path-length, 352.6 ± 57.1 cm) as *Trpm4*^{-/-} (path-length, 263.8 ± 40.1 cm) and the acquisition curves of both genotypes were then almost identical for the remainder of the first phase of acquisition training. When tested for spatial reference memory during the 1st probe trial, *Trpm4*^{-/-} and WT animals showed a significant preference for the target quadrant (**Fig. 10c**) (WT $p = 0.0187$; *Trpm4*^{-/-} $p < 0.0001$; One-way ANOVA with post-hoc Dunnet-test) and this preference was further increased by the second week of training (trial block 6-10) as revealed in probe trial 2 (**Fig. 10d**) (WT $45.6 \pm 4.0\%$; *Trpm4*^{-/-} $47.6 \pm 3.7\%$; $p < 0.0001$ for both groups; One-way ANOVA with post-hoc Dunnet-test). During the reversal training for the new platform position, there was again the tendency of a better performance of *Trpm4*^{-/-} rats on the first day of reversal but both groups attained already on the next day similar values as before platform reversal and the performance reached an asymptotic high level, i.e. low path-length (WT 94.8 ± 3.0 cm; *Trpm4*^{-/-} 98.0 ± 3.9 cm) (**Fig. 10b**). In accordance with the very similar performance of both groups during the reversal acquisition, the probe trials did not reveal any significant difference in spatial reference memory since WT and *Trpm4*^{-/-} rats displayed a significant target preference after 5 days of acquisition training (**Fig. 10e**) (WT $p < 0.001$; *Trpm4*^{-/-} $p = 0.044$; One-way ANOVA with post-hoc Dunnet-test) which was further increased after another three days of training (**Fig. 10f**) (WT and *Trpm4*^{-/-} $p < 0.0001$; One-way ANOVA with post-hoc Dunnet-test). In conclusion, the MWM task does not provide any evidence for changed/impaired spatial learning or memory or a different cognitive flexibility in *Trpm4*^{-/-} rats.

Finally, taking advantage of the natural tendency of rats and mice to explore novel environments (Dember, 1989), we trained the animals in an alternated protocol of the elevated T-maze (**Fig. 11**). Control animals turned towards the novel arm at higher frequency than expected by chance whilst *Trpm4*^{-/-} rats did not (WT $t = 10.11$, $p = 0.0021$; *Trpm4*^{-/-} $t = 0.3310$, $p = 0.7624$; One-sample t-test to hypothetical value 0.5, chance level). Moreover, the ratio time spent in the new arm compared to the old one was significantly higher in WT compared to *Trpm4*^{-/-} rats (**Fig. 11a**, $F_{(1,6)} = 39.76$, $p = 0.0007$; 2 way-ANOVA). This was also reflected by the amount of total time the animals spent exploring the new arm over the four days of training, with the control animals spending significantly more time in the new arm compared to the *Trpm4*^{-/-} rats (**Fig. 11b**, $t = 5.794$, $p = 0.002$; Welch-test). The analysis of the total distance walked in the maze was not significantly different between the two groups, indicating no exploration bias (**Fig. 11c**).

Absence of *Trpm4* affects the initial BOLD response only in the right hippocampus but not in other brain regions

Since the LTP recordings unveiled differences in the activation of the hippocampus in *Trpm4*^{-/-} and WT rats in response to electrical stimulation, we speculated whether such stimulation could also evoke

genotype-specific activation patterns in brain regions known to be strongly anatomically and functionally connected to the hippocampus. First, we confirmed that the chronically implanted tungsten electrodes caused only minor artifacts in anatomic magnetic resonance (MR) images and in the corresponding functional magnetic resonance images (fMRI) as shown in [Fig. 12a](#). Of note, anatomical MR images did not indicate any gross anatomical differences between WT and *Trpm4*^{-/-}.

Next we selected the strong LTP induction protocol (3 x 6 bursts of 20 pulses at 200 Hz) as the standard protocol for fMRI measurement, because pilot experiments have shown that it evokes bold responses of sufficient strength. As compiled in [Fig. 12b](#), stimulation of the hippocampal Schaffer collaterals to CA1 inputs with the strong HFS protocol resulted in significant blood oxygen level-dependent (BOLD) responses in the stimulated ipsilateral (right) dorsal hippocampus (HP), in the contralateral (left) dorsal HP and scattered significant BOLD responses in parts of the frontal cortex. Whereas high-frequency stimulation (HFS) of the Schaffer collaterals induced positive BOLD responses in the right and left dorsal hippocampus, the concurrently induced BOLD responses in frontal cortex areas were negative ([Fig. 12b](#) left). Similar results were obtained when a VOI analysis of voxels in each corresponding region were considered ([Fig. 12c](#)).

Further analysis revealed that there was no significant difference in the distribution of significantly activated voxels in *Trpm4*^{-/-} and WT rats as indicated in the 3D images on [Fig. 12b](#) right, which represent all significantly activated voxels (threshold: $\text{fdr } q = 0.01$, $t_{\min}$ value > 3.52) converted into volumes of interest (VOI), from which surface clusters were created. Thus the general activation pattern, reflected by BOLD signal changes, was similar in the two genotypes.

A detailed examination of the BOLD responses to each of the three consecutive strong HFS revealed similar BOLD signals in the dorsal hippocampus except for the right dorsal hippocampus of the *Trpm4*^{-/-} rats, where the first HFS caused a significantly sustained increase in BOLD signals after the stimulation had ceased ([Fig. 12b](#), middle). To test whether the first strong HFS also caused different responses in target regions of the right hippocampus, a VOI (volume of interest) analysis was performed. Comparing BOLD time series in additional 13 predefined VOIs revealed no significant differences in BOLD responses in WT and *Trpm4*^{-/-} rats to the first HFS period, thus the sustained increase in BOLD signal intensities in the right dorsal hippocampus of *Trpm4* knock-out rats was not accompanied with significant changes in BOLD signal intensities in other brain regions.

Taken together, the results at the system level show that the stimulation of Schaffer collaterals with bursts of 200 Hz pulses induced positive BOLD responses in the ipsilateral and contralateral hippocampus and negative BOLD responses in the prefrontal cortex. The initial BOLD response in the right hippocampus was significantly prolonged in *Trpm4*^{-/-} when compared to wild type rats whilst all subsequent BOLD responses were similar.

Discussion

Here we examined the functional contribution of TRPM4 to hippocampal synaptic plasticity in the intact brain *in vivo* and its putative involvement in certain types of behavior. Electrophysiological measurements at the synaptic level, fMRI-generated BOLD responses at the network level and a series of behavioral tests were used as functional read-outs in *Trpm4*^{-/-} and wild type (WT) rats.

First, we compared at the synaptic level the phenotype *in vitro* with previous result in mice and found essentially the same phenotype. Therefore, the mechanisms underlying TRPM4's function in LTP are conserved across rodent species. Basal synaptic transmission and short-term plasticity were intact in *Trpm4* knock-outs rats, but LTP was profoundly affected by TRPM4's absence. Although the two weakest TBS protocols (1 and 3 TBS) caused a severe depletion of LTP, stronger protocols (8 TBS and HFS) were sufficiently strong to surpass the threshold and induce LTP in the *Trpm4*^{-/-}, although of lower magnitude than in the WT. These data point to a role for TRPM4 in setting a stimulus threshold for LTP induction. In the absence of functional TRPM4 channels, stronger stimuli are necessary to induce LTP.

The *in vivo* experiments in freely-moving rats also show that the diverse HFS protocols (strong, intermediate and weak) caused different degrees of LTP in both genotypes and, interestingly, entailed specific effects in *Trpm4*^{-/-} compared to WT. In general, WT expressed a higher and more stable LTP, and the strong and intermediate protocols evoked similar LTP induction levels whilst the intermediate and weak protocols shared features of LTP maintenance, although resulting in different end levels. In contrast, LTP in *Trpm4*^{-/-} displayed similar features after the application of all protocols, characterized by

a fast decay of potentiation that depended on the particular HFS strength, and by convergence to similar end levels. The putatively mechanistic differences in LTP induction and maintenance between *Trpm4*^{-/-} and WT animals that are already apparent by visual inspection were corroborated by dynamic data-based modelling using discrete-time transfer function models. They confirmed a robust, slowly (nearly linearly) decaying form of LTP induced by the strong HFS in WT but not in *Trpm4*^{-/-} rats. Thus, strong LTP works as an integrator in WT rats, whilst deletion of *Trpm4* causes a change that apparently increases the dynamics of the LTP response, resulting in a faster decaying form of LTP as indicated by the larger α -parameters and smaller time constants. This robust LTP in WT animals was from 1 hour after LTP induction significantly different from the lower LTP in *Trpm4*^{-/-} that decayed for the first three hours. In contrast, the weak HFS induced a LTP that decayed slowly for three hours in WT whilst in *Trpm4*^{-/-} the magnitude of the induction was evidently reduced and stabilized precociously. The current hypothesis of TRPM4 as a key component in NMDA-mediated types of LTP can explain the results obtained by both methods. Ca^{2+} influx through NMDA receptors and membrane depolarization would trigger the activation of TRPM4, which will further depolarize the membrane via a TRPM4-mediated Ca^{2+} -dependent cation current in order to fully unblock NMDA receptors (full mechanism in Menigoz et al., 2015). Thereby, upon deletion of *Trpm4*, the activation of NMDA receptors would be insufficient to properly induce LTP and the downstream signaling mechanisms. This would lead to an increase in the threshold for LTP induction, which could explain the different effects on LTP induced by weak and strong protocols. Thus, TRPM4 is involved in hippocampal synaptic processes depending on the particular recording conditions and the physiological context.

We suggest that the strong and weak HFS protocols used here *in vivo*, trigger different forms of LTP that rely on different mechanisms, as has been reported before by us and others (Ballesteros, Buschler, Köhr, & Manahan-Vaughan, 2016; Straube & Frey, 2003; Wilsch et al., 1998). There are few critical factors on determining the magnitude and persistence of LTP that might explain this variability. For instance, not only the strength but also the pattern of tetanic stimulation affects the amount of excitatory neurotransmission. Stronger stimulation protocols provoke larger and longer-lasting post-synaptic depolarizations that activate more afferent fibers and synapse populations (Grover & Teyler, 1990; Martin, Shires, & Spooner, 2013) by virtue of phenomena termed cooperativity and associativity (Bliss & Collingridge, 1993; McNaughton, Douglas, & Goddard, 1978). They also engage other calcium sources besides the well reported NMDARs, such as voltage-gated Ca^{2+} (VGCC) channels (Freir & Herron, 2003), and intracellular Ca^{2+} stores (Raymond & Redman, 2002; Wilsch et al., 1998). Notably, the intermediate HFS induced similar LTP magnitude and tendency in both groups, indicating that this LTP might be independent of TRPM4 or in between thresholds for activating different LTP mechanisms than the induced by the other protocols. Taken together, these results are indicative of marked differences in LTP kinetics between the WT and animals lacking *Trpm4*. Since the *Trpm4*^{-/-} evidenced altered LTP mechanisms after the application of both strong and weak protocols, the involved underlying pathways might be different. This was also confirmed by the fact that first order models fit the LTP decay curves of WT whilst second order models of *Trpm4*^{-/-}, suggesting more complex dynamics of the LTP response curves in *Trpm4* absence.

Although the involvement of TRPM4 in synaptic plasticity had already been reported *in vitro*, the role of TRPM4 in the intact brain and the possible effect of its absence in behavior were unknown. Our *in vivo* data show that TRPM4 has a relevant role in LTP induced by a strong HFS, whilst the weak HFS highlights a putatively different role of TRPM4 in a tender form of LTP. The effect of the diverse *in vivo* and *in vitro* protocols cannot be exactly translated (Saeidnia, Manayi, & Abdollahi, 2016) since there are many different factors involved, such as the strength of the protocols themselves (discussed above), the disconnection of the HP with the rest of the brain *in vitro*, or the presence of more feed-forward inhibition *in vivo* (Ashwood, Lancaster, & Wheal, 1984; Buzsaki, 1984; Dingledine & Gjerstad, 1980). Regardless the differences, the phenotype *in vivo* recapitulates the differences observed *in vitro* in rats and mice but at a more moderate level.

The deletion of TRPM4 proteins did not affect general activity, exploration and anxiety levels analyzed by the open field test, dark-light box and elevated plus maze, but impaired the performance of the *Trpm4*^{-/-} animals in the slightly aversive Barnes maze (BM) test. Although both groups eventually learnt to locate the escape tunnel based on the spatial cues provided, when they were trained for 4 consecutive days, the *Trpm4*^{-/-} evidenced difficulties as they made more mistakes while learning the task. The animals lacking *Trpm4* never reached the performance levels of the WT, which could be an indication of not remembering the position of the escape hole (spatial reference memory deficit) or which holes had already been visited (spatial working memory impairment). Indeed, when looking at the

number of times that the animals re-visited holes, the levels of the knock-outs were significantly higher, pointing to a working memory deficit. Interestingly, when the animals were exposed 48h later to a serial working memory task in the aversive BM, the *Trpm4*^{-/-} animals failed to remember a new escape tunnel position which was presented 30 minutes before the test, again indicating impaired spatial reference and working memory in *Trpm4*^{-/-} rats. In contrast to the Barnes maze results, no impairments were found in the *Trpm4*^{-/-} when the animals were trained in a hidden-platform version of the Morris water maze (MWM). Both groups learnt the task and performed well during the probe trial, although the *Trpm4*^{-/-} showed a slightly better performance during training day 1. Since a better motor performance can be excluded because of a lacking difference in swimming velocity, most likely both genotypes differed in the adaptation to the test environment during the first day of training. The difference in spatial reference memory performance of the *Trpm4*^{-/-} in the MWM compared to the BM may be due to procedural differences (type of training over the days), together with the degree of aversion that is used to motivate the animals to perform the task, since swimming in the water is more stressful than running around the brightly lit surface of the Barnes maze (Harrison, Hosseini, & McDonald, 2009). Finally, the animals were exposed to an elevated T-maze, after previous habituation. This test is often used to investigate hippocampus-dependent spatial reference memory and working memory, since the response during each trial varies according to what they have just done (Olton, 1979). When following alternated-arm training, the WT rats spent significantly more time in the new arm per day compared to the old one, as expected. This effect was not observed in the knock-out rats, which showed no preference for the novel arm versus the familiar one. Thus, *Trpm4*^{-/-} rats apparently forgot which arm had been previously visited, corroborating the spatial reference and/or working memory deficits of *Trpm4*^{-/-} rats found in Barnes maze. Taken together, the behavioral data expose a very specific *Trpm4*^{-/-} genotype, characterized by the absence of a role for TRPM4 in general behavior but a role in distinct hippocampus-dependent tasks, especially when working and reference memory are required in the context of the Barnes Maze and T-maze tests, but not in the Morris Water Maze test. These data constitute the first description of a specific spatial memory deficit due to the lack of *Trpm4*.

Given these effects of *Trpm4* deletion on specific types of spatial memory, the hippocampal synaptic plasticity deficit and the close anatomical and functional connection between HP and PFC (Liu, Bai, Xia, & Tian, 2017), it was tempting to check by fMRI whether differences in hippocampal activation in *Trpm4*^{-/-} rats might be conveyed to hippocampal subareas or even other brain areas. Indeed, tetanic stimulation of Schaffer collaterals caused a BOLD response of both ipsilateral and contralateral hippocampus and prefrontal cortex, indicating that the activation might not be confined to the stimulated area but instead spread to other hippocampal subareas and neocortical areas with which the hippocampus connects. Of note, this is the first report of region-specific BOLD activation after strong electrical stimulation of the hippocampal Schaffer commissural-collateral pathway to CA1 region. A previous study found a BOLD activation not only in the hippocampal formation but also in the prefrontal lobe after the stimulation of the perforant pathway to the dentate gyrus connection (Angenstein et al., 2013). The broad activation of the whole hippocampal formation verifies the presence of associational fibers connecting intrinsically its parts (the so-called trisynaptic circuit). Moreover, the HP is extrinsically connected with other brain areas. Previously described positive BOLD responses in the mPFC during perforant pathway stimulation (reference above) might be mediated by direct projections from CA1 or subiculum neurons (Cenquizca & Swanson, 2007; Verwer, Meijer, van Uum, & Witter, 1997) or via entorhinal cortex projections (Insausti et al., 1997; Valenti and Grace, 2009). On the other hand, current observations that Schaffer collateral stimulation causes negative BOLD responses in prefrontal cortex corroborate previous findings that CA3 stimulation alleviates the otherwise induced positive BOLD responses in the prefrontal cortex (Scherf & Angenstein, 2017). Together these results indicate that projections from entorhinal cortex mediate positive BOLD responses in the prefrontal cortex whereas projections from hippocampal neurons cause negative BOLD responses.

Interestingly, the stimulation in *Trpm4*^{-/-} rats caused a sustained increase of BOLD signals after the first HFS train in the ipsilateral (significant) and contralateral HP (not significant), which lasted for around 4 min, whereas in WT rats BOLD signals quickly returned to the initial level. A sustained increase in BOLD signals was previously also observed when high-frequency pulse stimulation of the perforant pathway caused heavy neuronal afterdischarges of granule cells in the dentate gyrus (Helbing et al., 2013). In the current experiments we did not observe long-lasting afterdischarges of pyramidal cells in the CA1 region of *Trpm4*^{-/-} rats, excluding sustained increased pyramidal cells spiking activity as cause for the observed sustained BOLD signal increase. It is known that depolarization of the post-synaptic cells by strong HFS activates various molecular cascades that control vasoconstriction (i.e., via VDCCs) or

vasodilation of arterioles, which then mediate an increase (i.e., functional hyperemia) or a decrease in local cerebral blood flow (Longden, Hill-Eubanks, & Nelson, 2016). Moreover, TRPM4 seems to be vitally important for the myogenic response. Pressure-induced constriction of ex-vivo cerebral arteries requires functional TRPM4 channels (Earley, Waldron, & Brayden, 2004), and blocking TRPM4 causes almost maximal vasodilation of pressurized arteries (Gonzales, Garcia, Amberg, & Earley, 2010). Thus, the lack of *Trpm4* could temporally extend the stimulus-induced vasodilation, which in turn would have led to the sustained increase of BOLD signals after the first tetanic stimulation in *Trpm4*^{-/-} rats. Since the delayed increase in BOLD signals only occurred after the first stimulation period but not after the following two stimulation periods, a general effect on neurovascular coupling mechanisms seems unlikely. More likely, high-frequency stimulation of Schaffer collaterals in *Trpm4*^{-/-} rats triggered a different neuronal activation pattern than in WT rats, in particular an altered and probably more sustained increase in the activity of interneurons. All in all, the results at the system level show that the stimulation of Schaffer collaterals with bursts of 200 Hz pulses induced positive BOLD responses in the ipsilateral and contralateral hippocampus and negative BOLD responses in the prefrontal cortex. Interestingly, the initial BOLD response in the right hippocampus was significantly prolonged in *Trpm4*^{-/-} when compared to wild type rats whilst all subsequent BOLD responses were similar.

The main findings at the different levels of analyses indicate a relevant role of TRPM4 in hippocampal synaptic plasticity (LTP) and some involvement in specific types of hippocampal-dependent behavior. At the electrophysiological level, the distinct involvement of TRPM4 depending on the strength of the LTP protocol points to specific mechanisms underlying its function. Together with the behavioral results pointing to a role of TRPM4 in specific types of spatial working and reference memory processes, this indicates a novel relevant role for TRPM4 that has not been studied yet. The fact that absence of TRPM4 only altered BOLD responses in the ipsilateral hippocampus after the first electrical stimulation period implies that it rather controls the quality of signal processing in local neuronal circuits (Angenstein et al., 2009) rather than general neurovascular coupling mechanisms. The novel physiological functions of TRPM4 in the intact brain revealed here at the cellular, behavioral and system level point to a specific role in neuronal plasticity and cognition depending on the particular activation level of the neuronal circuit.

Legends to Figures and Tables

Fig. 1 Electrophysiological results *in vitro*. **a** Both genotypes showed similar input/output properties when the slices were stimulated at progressively increasing intensities (V), supporting an intact basal transmission in *Trpm4*^{-/-} rats. **b** Short-term plasticity as measured by a paired-pulse responses did not differ between genotypes. **c** One theta-burst stimulation (1xTBS) induced LTP in WT rats but only short-term potentiation (STP) in *Trpm4*^{-/-} ($F_{(1,15)} = 20.93$, $p = 0.0004$, WT $n = 9$; *Trpm4*^{-/-} $n = 8$, 2-way RM-ANOVA). **d** Likewise, 3xTBS generated LTP in WT animals whilst only STP in *Trpm4*^{-/-} ($F_{(1,15)} = 116.3$, $p < 0.0001$; WT $n = 8$; *Trpm4*^{-/-} $n = 9$, 2-way RM-ANOVA). **e** 8xTBS caused LTP in both genotypes, which was significantly higher in WT ($F_{(1,10)} = 12.77$, $p = 0.0051$; WT $n = 6$, *Trpm4*^{-/-} $n = 6$, 2-way RM-ANOVA). **f** High-frequency stimulation (HFS) caused a robust potentiation in WT and *Trpm4*^{-/-}, but of much lower level in the latter ($F_{(1,12)} = 45.97$, $p < 0.0001$; WT $n = 7$, *Trpm4*^{-/-} $n = 7$, 2-way RM-ANOVA).

Fig. 2 Schematic representation of the electrode placement and the stimulation protocol (weak). **a** Histological image of the hippocampus with the location of a stimulation electrode in the *Schaffer collateral commissural pathway* (SCCP) and a recording electrode in the *stratum radiatum* layer of CA1 (indicated by the arrows). Inset on the right depicts a representative fEPSP example, quantified as the first negative deflection of the slope of the evoked potential. **b** Representative example of the tetanization protocol. For each time point measured, 3 consecutive fEPSP responses are collected every 10 s, and then averaged for each set of stimuli that is imposed every 5 minutes. After one hour of baseline recording, LTP is induced by a high-frequency stimulation (HFS) protocol consisting of 1, 4 or 6 trains of 20 pulses at 200 Hz (0.1 ms pulse duration, inter-train interval 2 s), repeated three times in a time interval of 10 min. LTP is then recorded for 6 h (360 min).

Fig. 3 *In vivo* basal synaptic transmission and short-term plasticity is similar in both genotypes. **a** Basal synaptic transmission is not altered in *Trpm4*^{-/-} rats as established by input-output (I-O) curves. The curves of both genotypes increase to a similar degree, and although the wild type (WT) reaches the saturation level seemingly faster, this difference is not significant. **b** Paired-pulse responses (left) induced a facilitation at 30 ms interval that does not differ between WT and *Trpm4*^{-/-}. The right panel shows a representative analogue recording in response to paired pulse stimulation at 30 ms inter-pulse interval.

Fig. 4 Electrophysiological results for Strong HFS (S-HFS) *in vivo*. **a** Strong HFS (3 x 6 bursts of 20 pulses at 200 Hz) applied to *Schaffer collateral-CA1 stratum radiatum* synapses in freely-moving rats induce a robust LTP in wild types (WT) in contrast to the detrimental LTP in rats lacking *Trpm4* (*Trpm4*^{-/-}). This results in a significantly lower level of potentiation in *Trpm4*^{-/-} rats (2-way ANOVA: $F_{(1,12)} = 24.92$, $p = 0.0003$). A close examination of the three phases of LTP, shows statistically significant differences between WT and *Trpm4*^{-/-} in E-LTP and L-LTP (E-LTP $F_{(1,12)} = 11.97$, $p < 0.005$; L-LTP $F_{(1,12)} = 50.82$, $p < 0.001$). Inset on the right shows representative analogue recordings for WT (top) and *Trpm4*^{-/-} (bottom) illustrating the robust LTP in WT rats (min 30) that stayed stable until the end (min 340) compared to potentiation in *Trpm4*^{-/-} which decreases progressively. **b** The percentages of WT LTP remain stable ($182 \pm 6.59\%$) since the first 30 min post-induction until 3 h of experiment ($179 \pm 5.56\%$), to end with a potentiation of $158\% \pm 7.15$ by min 340. In contrast, *Trpm4*^{-/-} LTP shows a high induction ($210 \pm 7.89\%$) that quickly converts into exponential decrease for 3 h until $139 \pm 6.99\%$, to reach a $125 \pm 5.91\%$ LTP by the end of the experiment.

Fig. 5 Electrophysiological results for Intermediate HFS (I-HFS) *in vivo*. **a** *In vivo* LTP after the application of an intermediate HFS protocol (3 x 4 bursts of 20 pulses at 200 Hz) is very similar between WT and *Trpm4*^{-/-} rats. An initial decrement (60-180 min) follows a later stabilization after 3 h LTP in both groups until the end of the experiment (2-way ANOVA, $F_{(1,12)} = 0.36$, $p = 0.56$). The right panel shows the representative analogue recordings for WT (top) and *Trpm4*^{-/-} (bottom) show a higher potentiation in WT (min 30) compared to the *Trpm4*^{-/-} but later reach the same level (min 340). **b** Although the percentage for LTP induction is slightly higher in WT ($210 \pm 13.32\%$ vs. $196 \pm 7.29\%$), LTP in both groups decays similarly to reach after 3 h a $140 \pm 21.48\%$ and $140 \pm 12.59\%$ in WT and *Trpm4*^{-/-} rats, respectively, and remain similar until the end of experiment at 6 h ($120 \pm 18.47\%$ in WT vs. $138 \pm 14.96\%$ in *Trpm4*^{-/-}).

Fig. 6 Electrophysiological results for Weak HFS (W-HFS) *in vivo*. **a** Significant differences in the initial potentiation (I-LTP, 21-60 min) and Early-LTP (65-180 min) induced by a weak protocol (3 x 1 burst of 20 pulses at 200 Hz) (2-way ANOVA I-LTP $F_{(1,14)} = 5.6$, $p < 0.05$ and E-LTP, $F_{(1,14)} = 5.35$, $p < 0.05$). The potentiation in wild type (WT) is higher and slowly reaches the level of the *Trpm4*^{-/-} after 3 h, whilst the potentiation of the *Trpm4*^{-/-} is weaker and vastly decays to reach a stable LTP at around 80 min for the rest of the experiment. The right shows the representative for WT (top) and *Trpm4*^{-/-} (bottom), in which WT animals attain a higher potentiation after induction (min 30) compared to the *Trpm4*^{-/-} but reach about the same level as the *Trpm4*^{-/-} at the end of the experiment (min 340). **b** The much higher LTP magnitude of WT rats at 30 min ($175 \pm 9.38\%$) contrasts with the lower induction ($147 \pm 8.05\%$) in *Trpm4*^{-/-}. This difference become smaller but is still evident during the first 3 h of experiment (min 180; WT $132 \pm 4.61\%$ vs. *Trpm4*^{-/-} $125 \pm 9.45\%$), after which the values of both groups merge to reach almost baseline levels by the end of recording (min 340); WT $108 \pm 7.06\%$, *Trpm4*^{-/-} $108 \pm 11.17\%$).

Fig. 7 Evaluation of the differential LTP kinetics by dynamic data-based modelling. **a** LTP in wild type (WT) decays slowly (TC, Table 2) after strong HFS induction (SSG, Table 2) fitting First Order Models (visual inspection + R^2 + AIC, Table 1). Strong HFS (red) evokes a very stable and close-to-saturation LTP in WT (works as an integrator, a-par close to 1, Table 2), whilst the weaker protocols induces a decaying form of LTP (SSG and TC values, Table 2). **b** HFS of all strengths induce in *Trpm4*^{-/-} a LTP of lower values of induction (SSG, Table 2) and faster decay (TC, Table 2) which fits Second Order Models (visual inspection + R^2 + AIC, Table 1). The LTP induction level varies with the HFS strength and potentiation rapidly decays alongside until the end of the experiment.

Fig. 8 General behavior is unaltered in animals lacking *Trpm4*. The general behavioral characterization battery shows normal behavior of the *Trpm4*^{-/-} compared to the wild types (WT) in the Open field test (OFT) (**a**), the light-dark box (LDB) (**b**) and the Elevated plus maze (EPM) (**c**) in all parameters measured (Unpaired t-test, no significant differences). Normal levels of activity and exploratory responses are found (as denoted by the zone entries, % center vs. periphery, and latency to center in the OFT; % of time in darkness, latency to darkness, light-dark transitions in the DLB; latency to center, number of transitions, % time in closed arms in EPM). Likewise, anxiety levels measured as % of time in the center vs. periphery in OFT and % of time in open arms vs. center in EPM do not differ significantly between genotypes.

Fig. 9 Hippocampus-dependent behavioral characterization by the Barnes maze (BM). **a** *Trpm4*^{-/-} rats needed more time (latency, upper panel: tendency not significant, n. s., 2-way ANOVA, $F_{(1,22)} = 3.582$, $p = 0.0716$; WT d1-d4 $p < 0.01$, *Trpm4*^{-/-} $p < 0.001$) and made more mistakes (lower panel: 2-way ANOVA, $F_{(1,22)} = 6.328$, $p = 0.0197$; WT d1-d4 $p < 0.05$, *Trpm4*^{-/-} n. s.) during training for the BM than the wild types (WT). **b** The probe test on day 5 shows similar performance for both groups in the BM based on % of right pokes (upper graph) and % of time spent in wrong quadrants (lower graph). **c** The *Trpm4*^{-/-} animals made significantly more working memory mistakes per training day (upper panel, Welch-test = 2.311, $p = 0.031$). Moreover, the *Trpm4*^{-/-} rats performed significantly worse than the WT in the working memory task, needing more time (latency, middle panel, Welch-test = 3.029, $p = 0.009$) and making more mistakes (lower panel, Welch-test = 4.5, $p = 0.0005$) when learning a new escape tunnel position. **d** The analysis of the search strategies used by the animals to localize the target hole shows no significant differences between WT and *Trpm4*^{-/-}. Both groups started searching based on a random strategy, switched then to serial and eventually, after 4 days of training, to spatial.

Fig. 10 Spatial learning by the hidden platform version of the Morris water maze (MWM). Both groups acquired the task properly over training. **a** *Trpm4*^{-/-} animals show a better performance during the first day of training (smaller path-length; $t = 3.247$, $p = 0.0039$; Welch-test) but achieve a similar performance level from day 2 (path-length; WT 352.6 ± 57.1 cm; *Trpm4*^{-/-} 263.8 ± 40.1 cm). **b** During the reversal training, both groups reach an asymptotic high performance level (low path-length). **c** Both genotypes express a significant preference for the target quadrant in the 1st probe trial (WT $p = 0.0187$, *Trpm4*^{-/-} $p < 0.0001$; One-way ANOVA with post-hoc Dunnet-test). **d** Performance is further improved by the probe trial 2 (WT $45.6 \pm 4.0\%$, *Trpm4*^{-/-} $47.6 \pm 3.7\%$; $p < 0.0001$ for both groups; One-way ANOVA with post-hoc Dunnet-test). **e** The reversal probe trials do not reveal any significant difference in spatial reference memory between WT and *Trpm4*^{-/-} rats in probe 1 (preference for new target quadrant: WT $p < 0.001$, *Trpm4*^{-/-} $p = 0.044$; One-way ANOVA with post-hoc Dunnet-test) **f** In reversal probe 2, preference for the

new target quadrant is further improved (WT and *Trpm4*^{-/-} $p < 0.0001$; One-way ANOVA with post-hoc Dunnett-test).

Fig. 11 Hippocampus-dependent performance in the elevated T-maze. **a** WT rats spend significantly more time than by chance level in the new arm compared to the old one, whilst *Trpm4*^{-/-} rats do not (WT $t = 10.11$, $p = 0.0021$; *Trpm4*^{-/-} $t = 0.3310$, $p = 0.7624$; student t-test). Additionally, WT animals spend significantly more ratio time in the new arm compared to the old one than the *Trpm4*^{-/-} in every test day ($F_{(1,6)} = 39.76$, $p = 0.0007$; 2 way-ANOVA). **b** The average of ratio time spent on new arms over the four days of testing highlights the difference between the *Trpm4*^{-/-} and WT ($p = 0.002$; Welch-test). **c** The analysis of the total distance walked in the maze shows no differences between genotypes, discarding exploration bias.

Fig. 12 BOLD response pattern generated by strong HFS (3 x 6 bursts of 20 pulses at 200 Hz) in the right Schaffer collaterals. **a** Anatomical MR images only show minor artifacts of the stimulation and recording electrode (indicated by green arrows) and no obvious anatomical differences between the wild type (WT) and *Trpm4*^{-/-} brains. **b** Left side: Averaged BOLD time series of all significantly activated voxels in the right dorsal hippocampus (top), left dorsal hippocampus (middle) and in the frontal cortex (bottom) during the entire stimulation period (25 min). Time course of BOLD signal changes during the first high frequency stimulation is magnified and depicted in the middle panel. Significant changes in signal intensities between *Trpm4*^{-/-} and WT rats ($p < 0.05$, indicated by green dots) only occur in the right dorsal hippocampus after high-frequency stimulation ceased. Comparing the spatial distribution of significantly activated voxels in *Trpm4*^{-/-} and WT rats reveals no significant differences (right side). **c** Left side: Averaged BOLD times series of all voxels in the right dorsal hippocampus (top) and in the medial prefrontal cortex (bottom). The analyzed regions are depicted at the right side.

Table 1 Modelling results for the data after imposing First or Second order models on both groups. R^2 (Young, 1984) and the Akaike information criterion (AIC; Akaike, 1974) were analyzed together with visual inspection based on a priori biological knowledge (Ljung, 1987). HFS = High-frequency stimulation; S = strong, I = intermediate, W = weak.

Table 2 Modelling results for the decay of LTP (from min 30). Assuming zero initial conditions, TC is the time taken for the output (i.e. fEPSP slope) to reach 63% of its steady state value in response to a step input (i.e. HFS protocol). The SSG can be defined as the steady state change of output per unit change of input (Young et al., 1984). Int. = Integrator; See table 1 for further explanation; HFS = High-frequency stimulation; S = strong, I = intermediate, W = weak; WT= wild type.

Compliance with Ethical Standards

Ethical approval:

- All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the European Community Council Directive (2010/63/EU) and following the regulations of and approved by the local ethics committee under the number P079/2013 and P123/2012, KU Leuven.
- This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1

Figure 2

a

Stimulation electrode

Recording electrode

b

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Table 1

HFS	Group	Best First Order Model			Best Second Order Model			Best Model Order
		Model Struct	R ²	AIC	Model Struct	R ²	AIC	
S	WT	[1 2 0]	0.95	-19.60	[2 2 0]	0.92	-22.14	1st
I	WT	[1 2 0]	0.93	-17.33	/	/	/	1st
W	WT	[1 2 0]	0.95	-17.38	/	/	/	1st
S	<i>Trpm4</i> ^{-/-}	[1 2 0]	0.96	-17.90	[2 2 0]	0.96	-18.06	2nd
I	<i>Trpm4</i> ^{-/-}	[1 2 0]	0.94	-17.43	[2 2 0]	0.94	-18.24	2nd
W	<i>Trpm4</i> ^{-/-}	[1 2 0]	0.91	-17.23	[2 2 0]	0.92	-19.21	2nd

Table 1

Table 2

HFS	Group	Model Struct	R ²	AIC	a-par (std)	b-par (std)	SSG (SE)	TC (min) (SE)	First& Last Value	Int.
S	WT	[1 1 0]	0.93	-17.18	-0.9950 (0.0028)	0.6723 (0.0449)	134.5 (9.36)	997.5 (560)	194-156	Yes
I	WT	[1 1 0]	0.98	-19.21	-0.9631 (0.0020)	3.3235 (0.1063)	90.1 (3.08)	132.9 (7.35)	203-116	No
W	WT	[1 1 0]	0.99	-20.95	-0.9822 (0.0011)	1.7679 (0.0411)	99.3 (6.08)	278.4 (17.4)	173-102	No
S	<i>Trpm4</i> ^{-/-}	[1 1 0]	0.99	-21.69	-0.9459 (0.0012)	4.9519 (0.0789)	91.5 (1.57)	89.9 (2.05)	214-122	No
I	<i>Trpm4</i> ^{-/-}	[1 1 0]	0.99	-22.00	-0.9383 (0.0012)	4.1622 (0.0603)	67.5 (1.06)	78.5 (1.57)	190-125	No
W	<i>Trpm4</i> ^{-/-}	[1 1 0]	0.94	-16.07	-0.9122 (0.0063)	2.9621 (0.1814)	33.7 (2.30)	54.4 (4.08)	151-107	No

Table 2

Article title: DISENTANGLING THE ROLE OF TRPM4 IN HIPPOCAMPUS-DEPENDENT PLASTICITY AND LEARNING: AN ELECTROPHYSIOLOGICAL, BEHAVIORAL AND fMRI APPROACH.

Brain Structure and Function

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Methodology for behavior (set-ups and protocols)

Open field test (OFT):

The dark PVC square shaped open field arena box (100 x 100 cm, 50 cm walls) was located under bright lights, with a video camera above for test recording and later analysis. The floor of the open field arena was divided into 16 squares with equal dimensions to delimit central and periphery areas and to allow visual scoring.

At the beginning of each trial, the animal was individually placed for 20 seconds in a dark opaque starting chamber, positioned in the center of the arena, which was lifted by the researcher for the beginning of the test. Then the animal could explore the arena for 3min.

Light-dark box (LDB):

The box made of PVC consisted in two dark-light units separated by an opaque divider. Each unit consisted on a dark (25 x 50 x 50 cm) and a light compartment (25 x 50 x 50 cm), separated by an opening that allowed the passage from one zone to the other. The dark zone consisted on dark walls shielded on top and the bright zone on an open-air chamber with transparent walls and illuminated by a 50-Watts floodlight.

The test started by placing the rats (two animals were tested simultaneously in a different unit) in the lit chambers at the opposite end facing the entrance to the dark chamber. They were allowed to explore the maze and recorded for 3 minutes.

Elevated plus maze (EPM):

The elevated plus-shaped maze consisted of two opposite open arms (50 x 10 cm) and two opposite closed arms (50 x 10 x 50 cm walls) standing 50 cm above the floor.

At the start of every EPM task, the rats were placed on the central platform, facing towards a closed arm and allowed to explore the maze freely for 3 minutes.

Barnes maze (BM):

The maze consisted on a white wheeled circular platform (122 cm diameter) elevated from the floor 100 cm and with 20 holes (10 cm diameter each), one of which allows the escape to a tunnel positioned under the maze. The starting chamber is an opaque box where the animals were positioned before each trial to let them turn into a random direction when the box was

lifted. In the main platform, bright light and a high intensity fan blowing on the surface of the maze encouraged the escape.

Around the maze, four A4 size coloured proximal visual cues (Δ ; O; $\square$; $\diamond$) were placed within 50 cm distance, and a camera above the maze for recordings. Prior the beginning of the experiment, the animals were habituated both to the researcher and the behavioural set-up.

Training: The training phase consisted on 3 sessions per day of 3 min, ITI 30min, during 4 days. At the beginning of each trial, the rats were placed for 20 seconds in the starting chamber from one of four start positions, wherein the 2 first positions were equidistant from the escape hole. Rats were assigned to one escape hole from four different escape locations available. In between animals, the location of the escape hole was changed by 45° rotation of the maze, while the tunnel was fixed throughout the experiment in the same maze spatial location. Rats unable to find the goal tunnel after 3 minutes were cautiously placed next to the correct hole and allowed to descend into the tunnel. All animals were granted 1 minute inside the tunnel before the next session.

Probe trial: On day 5 the acquisition probe trial was conducted after 2 training sessions of 4 min with the escape tunnel removed. The rats were placed in the starting chamber on the middle of the maze for 30 seconds, after which the door was raised by the investigator and the animals were tested for the subsequent 3 minutes.

Serial working memory task: 48 h after the probe, the animals were trained on a serial reverse learning task which consisted on 2 sessions of 3 min per day, ITI 30min, during 3 days. In the first session a new random allocation of the tunnel was presented and in the second session they were assessed how well they remembered the previous position. Each day the animals had a new position to find.

The error definitions were: 1. if any part of the animal's head dropped below the level of the maze surface into a hole that did not lead to the escape tunnel, 2. repetitive errors at the same hole were counted only if the animal moved away from the hole and then returned to investigate it.

The search strategies were analyzed by dividing the animal behavior in three categories: i) random: without specific pattern; ii) serial: structured way of checking the holes one by one in a serial order; iii) spatial: based on spatial clues and directly to the target quadrant.

Morris water maze test (MWM):

The water maze consisted of a large circular pool (diameter 150 cm; depth 32.5 cm) filled to a depth of 16 cm with water ($26 \pm 1^\circ\text{C}$) made opaque with non-toxic white paint. The circular platform (diameter 15 cm; height 15 cm) was hidden 1 cm beneath water surface at a fixed position. The pool was at the centre of a brightly lit room with various fixed cues (e.g. posters, computers, tables) and the experimenter always sat in the same place. The entire learning protocol consisted of 4 phases. During the initial acquisition phase, rats were trained for ten days to find the hidden escape platform. Each rat did a total of 10 training days, 4 trials per day with 15-min inter-trial interval. The platform remained at the same position during all acquisition trials. Rats were placed into the pool facing the wall of the pool at one of four randomly chosen starting locations. When rats failed to find the platform within 2 min, they were guided to the platform and were to stay on it for 15 s, before being returned to their cages. Path length and velocity were recorded with *Ethovision* video tracking equipment and software (Noldus Bv, Wageningen, The Netherlands). After five training days, there were 2 days of rest, and a probe trial on the 8th day and on the 15th day. During probe trials, the platform was removed, and the swimming path recorded during 100 s. Time spent in each quadrant as well as path length, velocity, latency of first entrance and frequency of entries in the target quadrant were measured.

Subsequently to the 2nd probe trial on day 15, the platform was placed into the opposite quadrant and rats underwent a reversal training to examine the cognitive flexibility of both genotypes. After 5 days of training and 2 days of rest, there was another probe trial (Reversal probe 1) and training was continued for another 3 days followed by a probe trial on the day thereafter (Reversal probe 2).

Elevated T- maze test (T-maze):

The elevated T-maze consisted of a starting arm (47 x 10 cm) and two identical goal arms (35 x 10 cm), surrounded by a 50 cm high wall. The maze was placed in a dedicated room where four simple colored-paper shapes (squares, triangles, circles) were mounted around as visual cues, in addition to the asymmetry of the room itself. The rats were habituated to the maze for seven days in order to avoid anxiety, and then the spatial non-matching-to-place test started. The animals were tested over a 4-day period with a single trial per day. Each trial consisted of two phases: a sample run and a choice run, separated by an inter-trial-interval of 2 min (ITIs of 2 min allow assessing the persistence of short-term working memory). On the sample run, the animals were allowed to explore one of the arms for 5 min, according to a pseudorandom sequence (with equal numbers of left and right turns per session, and no more than two consecutive turns in the same direction). In the choice run, the block was removed, and after placing the rat at the end of the starting arm, it was allowed to freely explore either of the two arms for 5 min. The location of the maze in the testing room was changed between days to provide new extra-maze cues and to promote environmental novelty. The ratio of time spent in the new arm versus the old one was calculated per day and also averaged over the 4 days.