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Contrasting functions of mitogen- and stress-activated protein kinases 1 and 2 in recognition memory and *in vivo* hippocampal synaptic transmission

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Abstract

The mitogen-activated protein kinases (MAPK) are major signaling components of intracellular pathways required for memory consolidation. Mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and MSK2) mediate signal transduction downstream of MAPK. MSKs are activated by Extracellular-signal Regulated Kinase 1/2 (ERK1/2) and p38 MAPK. In turn, they can activate cyclic AMP-response-element-binding protein (CREB), thereby modulating the expression of immediate early genes crucial for the formation of long-term memories. While MSK1 has been previously implicated in certain forms of learning and memory, little is known concerning MSK2. Our goal was to explore the respective contribution of MSK1 and MSK2 in hippocampal synaptic transmission and plasticity and hippocampal-dependent recognition memory. In *Msk1*- and *Msk2*-knockout mice, we evaluated object and object-place recognition memory, basal synaptic transmission, paired-pulse facilitation and inhibition, and the capacity to induce and sustain long-term potentiation (LTP) *in vivo*. We also assessed the level of two proteins downstream in the MAPK/ERK1/2 pathway crucial for long-term memory, CREB and the immediate early gene EGR1. Loss of *Msk1*, but not of *Msk2*, affected excitatory synaptic transmission at perforant path-to-dentate granule cell synapses, altered short-term presynaptic plasticity, impaired selectively long-term spatial recognition memory, and decreased basal levels of CREB and its activated form. LTP *in vivo* and LTP-induced CREB phosphorylation and EGR1 expression were unchanged after *Msk1* or *Msk2* deletion. Our findings demonstrate a dissimilar contribution of MSKs proteins in cognitive processes and suggest that *Msk1* loss-of-function only has a deleterious impact on neuronal activity and hippocampal-dependent memory consolidation.

Highlights:

- MSK1 but not MSK2 plays a prominent role in the consolidation of hippocampal-dependent spatial recognition memory.
- *Msk1* but not *Msk2* deletion reduces excitatory glutamatergic transmission and short-term presynaptic plasticity in the DG.
- *Msk2* deletion, as opposed to *Msk1* deletion, is associated with an increase in CREB, pCREB and EGR1 basal levels in the DG.

- MSK1 and MSK2 are not essential for LTP induction and stabilization in the DG, and LTP-induced pCREB and EGR1 expression.
- In contrast to MSK2, MSK1 represents a key regulator of neuronal activity thereby optimizing information storage.

Keywords: Short- and long-term synaptic plasticity, Long-term potentiation, Transcription factors, Dentate gyrus, Knockout mice

Abbreviations:

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor;

CREB, cyclic AMP-response element binding protein;

DG, Dentate gyrus

EGR1, Early growth response 1

ERK, Extracellular signal-regulated kinase;

ERK1/2, Extracellular signal-regulated kinase 1/2;

fEPSP, Field excitatory postsynaptic potential;

KO, Knockout

IEG, Immediate early gene;

ISI, Inter-stimulus interval;

LTP, Long-term potentiation;

MPP, Medial perforant path;

MSK1/2, Mitogen stress-activated kinase 1 and 2;

NMDA, N-methyl-D-aspartate

Nr4a1/2, Nuclear Receptor Subfamily 4 Group A Member 1 and 2

PKA, Protein kinase A

PPF, Paired-pulse facilitation;

PPI, Paired-pulse inhibition;

PTP, Post-tetanic potentiation;

RSK, 90-kDa ribosomal S6 kinase;

SEM, Standard error of the mean;

STP, Short-term potentiation;

WT, Wild-type.

INTRODUCTION

The elucidation of the mechanisms underlying learning and memory is one of the major challenges in neuroscience research. The role in memory formation and storage of synaptic integrity and activity-dependent changes in synaptic strength, as exemplified by the phenomenon of long-term potentiation (LTP), has been well established (Mayford et al., 2012). A wealth of experimental data has also highlighted the key role of intracellular signaling cascades via kinases pathways leading to the recruitment of specific gene programs in these processes. The contribution of specific components of these molecular cascades underlying synaptic plasticity and memory formation, however, are still not fully understood.

The Mitogen-Activated Protein Kinase (MAPK) cascade is one such major signaling pathway transducing extracellular signals into cellular responses critical for memory. Numerous studies have shown that phosphorylation of extracellular signal-regulated kinases (ERKs) and of the transcription factor cyclic AMP-response-element-binding protein (CREB), and the subsequent regulation of immediate early genes (IEG), such as Early Growth Response 1 (EGR1), are necessary for the maintenance of LTP (English and Sweatt, 1997; Davis et al., 2000; French et al., 2001) and the consolidation of several forms of memory (Bourtchuladze et al., 1994; Jones et al., 2001; Bozon et al., 2003; Kelly et al., 2003; Carlezon et al., 2005; Alberini, 2009).

Mitogen- and stress-activated protein kinases (MSKs) act at the downstream end of the MAPK signaling pathway. MSKs can be activated by Extracellular-signal Regulated Kinase 1/2 (ERK1/2) and p38 MAPK pathways, and regulate the transcription of IEGs and chromatin remodeling via phosphorylation of CREB and histone H3 (Deak et al., 1998; Arthur et al., 2004; Chwang et al., 2007; Anjum and Blenis, 2008; Vermeulen et al., 2009; Cargnello and Roux, 2011; Choi et al., 2012; Karelina et al., 2012). Two *MSK* genes (*MSK1* and *MSK2*) have been identified in mammals, which exhibit 75% homology in amino acid sequence and harbor two distinct kinase domains (Deak et al., 1998). Analysis of *MSK1* and *MSK2* expression in different human tissues revealed that they are ubiquitously expressed, with predominant expression of *MSK1* and *MSK2* mRNAs in the brain (Deak et al., 1998). In the mouse, *MSKs* mRNA are expressed in adult CNS, with *MSK1* mRNA expressed at higher levels than *MSK2* mRNA, in particular in cortex and hippocampus (Arthur et al., 2004). Although *Msk* single or double knockout mice

showed no major alteration of gross brain morphology (Brami-Cherrier et al., 2005; Chwang et al., 2007; Chandramohan et al., 2008; Karelina et al., 2012), subtle anomalies of neuron morphology were found in *Msk1* mutant mice hippocampus. Specifically, *Msk1* mutant mice exhibit reduced dendritic branching complexity of dentate gyrus (DG) granule cells and CA1/CA3 pyramidal cells, reduced spinogenesis in CA1 and CA3 areas, and decreased proliferation of newborn neurons in the DG (Choi et al., 2012; Karelina et al., 2012). Along with changes in hippocampal neuroanatomy, *Msk1* mutant mice show cognitive alterations in certain hippocampal-dependent tasks, including delayed spatial learning in the Barnes maze (Karelina et al., 2012), deficient spatial search strategy in the Morris watermaze and impaired contextual fear memory (Chwang et al., 2007), suggesting an important role of MSK1 in learning and memory formation. In addition, MSK1 was shown to regulate in the hippocampus the expression of an extensive number of genes that underlie neuronal-specific cellular signaling (Choi et al., 2017; Privitera et al., 2020).

While the importance in memory formation and plasticity of kinases modulating MSKs, such as ERK1/2, and of MSKs downstream targets, such as CREB and histone H3, has been demonstrated, whether both MSK1 and MSK2 are necessary and have similar or different functions in these processes still remains unknown. Moreover, little is known about MSK2 function in the brain and no study has specifically investigated the role of MSK2 in cognition or synaptic plasticity. To gain a more comprehensive understanding of the specific physiological roles of these closely related kinases, we simultaneously investigated memory performance and synaptic transmission and plasticity in the DG, a major cortical input to the hippocampus, and examined basal and LTP-regulated CREB phosphorylation and EGR1 expression of mice lacking either *Msk1* (*Msk1*-KO) or *Msk2* (*Msk2*-KO). Collectively, our study supports the idea that MSK1, in contrast to MSK2, represents a key regulator of basal synaptic transmission and a molecular homeostat regulating dentate gyrus synaptic function and hippocampal memory consolidation.

EXPERIMENTAL PROCEDURES

Animals and statements of ethics

The generation of *Msk1*-KO and *Msk2*-KO mice has been described previously (Arthur and Cohen, 2000; Wiggin et al., 2002). *Msk* heterozygous mice were backcrossed onto a C57BL/6 line. Experimental groups were generated by

heterozygote mating at University Louis Pasteur by Dr. André Hanauer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). The genotype of the mice was determined using primer sets and cycling conditions described by Wiggin et al. (Wiggin et al., 2002). Mouse siblings (20-30 weeks old) were housed in a temperature and light-controlled colony room under a 12-hr light/dark cycle (light on: 7.00 a.m.) in groups of 4/5 with food and water *ad libitum*. All experiments were conducted during the light phase and performed on independent groups of naïve adult animals. All efforts were made to minimize the number of animals and their discomfort throughout the experiments. The total number of mice used in our study is 96 and 107 for *Msk1* and *Msk2* lines respectively (WT controls and mutants). The research protocol of the present study was approved by the Animal Review Board of University Paris-Sud. Experiments were performed in accordance with the guidelines of the European Communities Council Directive (2010/63/EU Council Directive Decree) and the French National Committee (87/848). All experiments were conducted blind to genotype.

Behavioral analyses

Memory performance of *Msk1*-, *Msk2*-KO mice and control littermates were evaluated in object and object-place recognition memory tasks using standardized procedures as previously described (Jones et al., 2001; Bozon et al., 2002). These memory tasks are based on the spontaneous preference of rodents for novelty and their ability to remember previously encountered objects and their location. The apparatus consisted of a circular arena (65 cm in diameter, 70 cm height) with wood shavings on the floor, placed in a dimly illuminated room. A cue card was placed at a fixed location on the top of the wall of the open field and prominent distal extra-maze cues were arranged in the room around the area to facilitate spatial mapping. The behavior of the animals was video-tracked and automatically recorded using the ANY-maze™ software (Stoelting Co., USA).

Male and female mice were handled once a day for 5 min during two consecutive days and then familiarized to the empty arena and test environment during two 20-min sessions on two consecutive days. Following habituation, mice were submitted to a single training session of three 5-min exploration trials separated by a 5-min interval in their home cages. On each trial, mice were placed at the center of the arena. In the object-place recognition version of the task, mice were left to

explore three differently shaped and colored objects. These objects were constructed from assembling interlocking block pieces and placed in fixed positions. After the last training trial, the group was divided into two subgroups to assess short- and long-term memory; half of the animals were tested for retention at 10 min, and the other half at 48h. For the retention session (lasting 10 min), the spatial position of one of the objects was changed to a new spatial location in the arena. In the object recognition task, mice were left to explore two identical objects (A1 and A2, made out of interlocking block pieces) placed in fixed positions (at the same distance from the wall of the arena) during the acquisition session (three 5-min trials separated by 5 min intervals). They were submitted to a retention session 48h after the last training trial, during which one of the objects was replaced by a novel object (B) of a different shape and color, while the other object was replaced by a novel object (A3) identical to the objects A1 and A2 used in the sampling session. Of note, spatial recognition memory (in the object-place recognition task) is more strongly dependent on the hippocampus than object recognition memory (in the object recognition task) which engages predominantly the perirhinal cortex when the two objects explored during the acquisition phase are identical copies of each other (Winters et al., 2004; Barker and Warburton, 2011). Due to possible spontaneous preference for a given object or its position in the arena, the nature and the spatial position of the objects were counterbalanced between mice within each experimental group. During the retention test, in order to counterbalance for individual side preferences among mice, the replaced object and its relative position in the context were also counterbalanced between mice within each experimental group as was the displaced object and its relative relocation in the area. The time spent exploring each object was scored during both acquisition and retention sessions. The criteria for exploration were based strictly on active behavioral exploration, where the mouse had its forelimbs close to an object and its head oriented toward the object or touching it with its nose. To examine retention memory in the object-place recognition task, a discrimination ratio was calculated for each mouse by dividing the time spent exploring the displaced object by the time spent exploring the displaced object plus the mean time spent exploring the non-displaced objects (for example, if the three objects are C1, C2, C3 and the displaced object is C3, the discrimination ratio would be: $C3/(C3+((C1+C2)/2))$). For the object recognition task, the discrimination ratio was calculated by dividing the time spent exploring the novel object by the total time of

objects' exploration.

***In vivo* electrophysiology**

Male mice were anaesthetized with a mixture of oxygen (Air Liquid Santé, Bonneuil sur Marne, France) and isoflurane (1-2%, CSP Translab, Cournon, France) during surgery and throughout the electrophysiological recordings using an anesthesia gas workstation (Tem Segal, Lormont, France). Animals were held in a stereotactic frame (Narishige, Japan, Micromécanique SAS, Evry, France) and maintained at a constant body temperature of 37.0 ± 0.5 °C (Harvard Apparatus, Les Ulis, France). A concentric bipolar stimulating electrode, made from 150- μ m diameter stainless steel wire inserted into a 300- μ m diameter microtube, was positioned in the medial perforant path (MPP, coordinates: 3 mm lateral to lambda, depth ~1.5 mm from brain surface), and a glass micropipette recording electrode containing a silver wire immersed in saline was lowered into the dendritic layer of the ipsilateral dentate gyrus or in the hilus (2 mm posterior to bregma, 1.6 mm lateral, ~1.5 mm from brain surface). Electrodes depth was adjusted to maximize the amplitude of evoked field potential (Fig. 1).

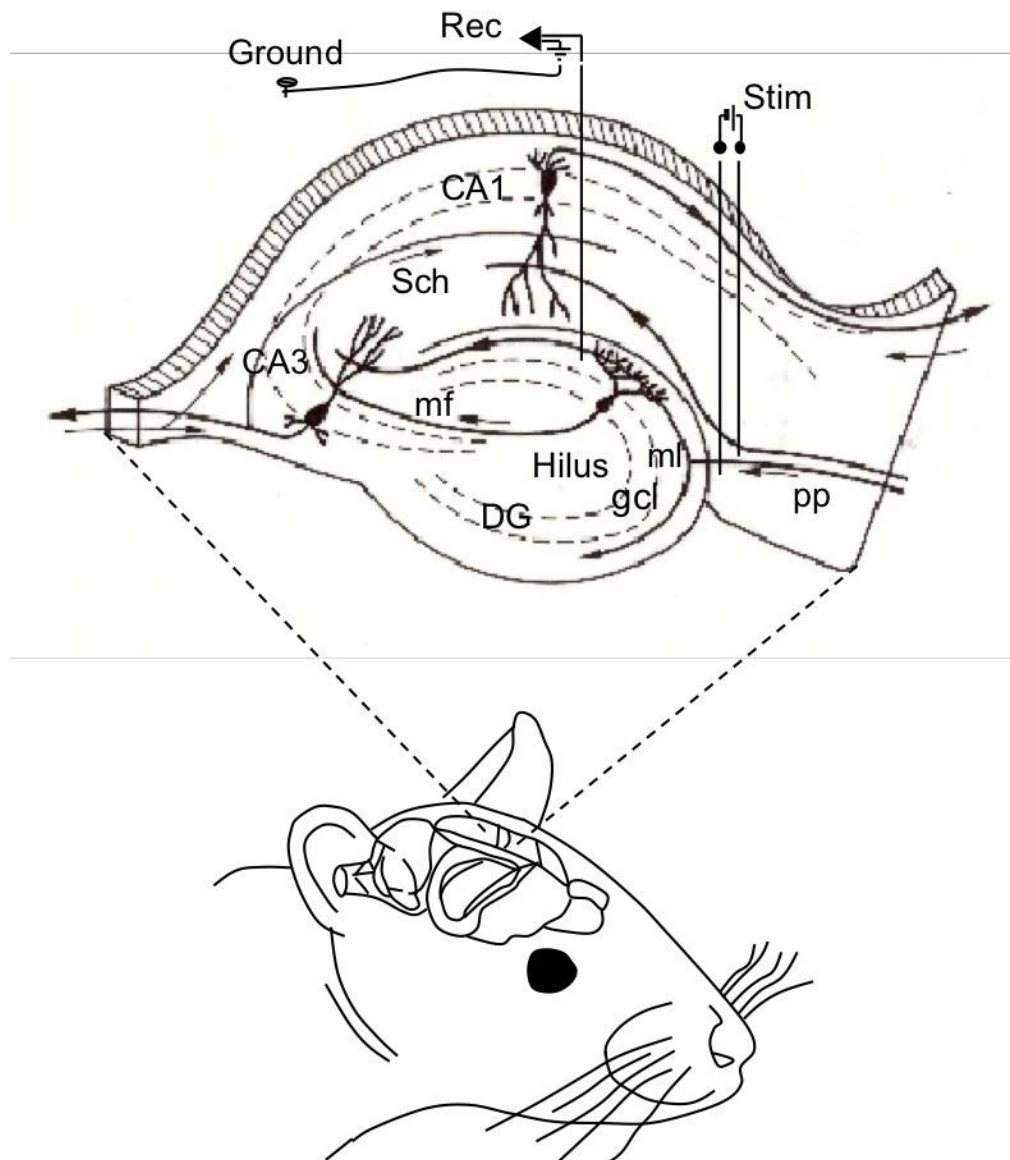


Fig. 1 Experimental design for *in vivo* electrophysiology recordings.

Animals were implanted with a stimulating (Stim) electrode at the medial perforant path (pp) and with a recording (Rec) electrode in the molecular layer (ml) of the dentate gyrus (DG). gcl, granular cell layer; mf, mossy fibers; Sch, Schaffer collaterals

After surgery, low frequency baseline stimuli (monophasic pulse, 60- μ s pulse-width, 0.033 Hz) were delivered via a photically-isolated constant current unit to evoke field excitatory postsynaptic potentials (fEPSP). Responses were stored for off-line analyses of the maximum slope of the rising phase of the fEPSP and amplitude of the population spike as described previously (Errington et al., 1987; Morice et al., 2013). After a stable response was established, input-output (I/O) curves were

generated using a range of stimulus intensities from 0 to 600 μ A. Three responses recorded from the hilus of the DG were collected at each intensity and averaged. Another group of animals was used to examine paired-pulse facilitation (PPF) and inhibition (PPI) at varying inter-stimulus intervals (ISI 10-1000 ms) and at two intensities, one to evoke a pure fEPSP (1 mV amplitude) and the other above threshold for evoking a 1 mV population spike. Three responses were collected at each interval and intensity and averaged. In LTP experiments, negative-going fEPSPs were recorded from the dendritic layer of the DG. MPP test stimuli (60 μ s) were first delivered at 0.033 Hz for 30 min at an intensity to evoke a fEPSP slope at 50% of maximum to establish baseline. A tetanus consisting of six series of six trains of six stimuli at 400 Hz, 200 ms between trains, 20 s between series was then delivered to the MPP. During the tetanus, pulse-width was doubled. This protocol has been shown to produce robust LTP in mice in *in vivo* experiments (Morice et al., 2013). After the tetanus, low-frequency stimulation was resumed for 15 min, and continued for 120 min in a sub-group of mice. Data are expressed as percent change in fEPSP slope relative to the pre-tetanus baseline. Animals were killed either 15 min or 3h after induction of LTP for biochemical analyses. Additional groups of controls, naïve mice, held in the stereotactic frame under anaesthesia and maintained at a constant body temperature of 37.0 ± 0.5 °C during 3 hours but without surgery, were used for biochemical experiments.

Western blot analyses

Levels of CREB, phosphorylated CREB and of the transcription factor EGR1 (Early Growth Response 1) in left dentate gyri of WT and KO mice were evaluated by western blot analyses. Briefly, the dentate gyri were dissected out from the brain on ice as previously described (Kelly et al., 2003; Morice et al., 2013; Penke et al., 2014). Tissue was snap frozen in liquid nitrogen and stored at -80° for western blot analyses. Twenty micrograms of total proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8%) and then transferred onto 0.45 μ m nitrocellulose membranes (Schleicher & Schuell). Membranes were pre-blocked with TBST+skimmed milk (5%) and incubated overnight at 4°C with primary antibody. The next day membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, GE Healthcare, France) for 1 h at room temperature. Membranes were washed with TBST buffer and proteins were

visualized using enhanced chemiluminescence (ECL) (Amersham, GE Healthcare, France). After development and fixation, the films were scanned and staining intensity of the protein bands determined using TotalLab software (Nonlinear dynamics, UK). Membranes were then subsequently stripped of antibodies and re-probed with other antibodies using the same procedure. Antibodies against β -actin (1:10000) were from Sigma-Aldrich (France) and antibodies against CREB (1:1000), pCREB (1:1000), and EGR1 (1:500) from Cell Signaling Technology (The Netherlands). Each experiment was replicated two to four times on independent blots. Signal density values were normalized to the reference protein (actin) and the CREB phosphorylated form to total CREB.

Statistical analysis

Statistical analyses were carried out using R (Comprehensive R Archive Network, CRAN), StatView (Abacus) and Prism (GraphPad) softwares. Normality and homogeneity of variance were confirmed using Shapiro-Wilk and Bartlett test respectively. For two-way ANOVA with repeated measures, normality of residuals and homogeneity of variance of residuals were confirmed graphically using the diagnostic plots method. Thus, if these two criteria were satisfied, parametric tests were used, while nonparametric tests were performed when these assumptions were not met. For behavioral analyses, statistics were conducted with the unpaired *t*-test or Mann-Whitney test to compare the total exploration time between genotypes. Two-way analyses of variance (ANOVA) were performed to assess the interaction between genotypes and retention time. One-sample *t*-test was used to compare the time spent exploring the new or the displaced object to the chance level set at 50%. For *in vivo* electrophysiology, data were analysed using a mixed effects model for repeated measures. Two-way ANOVA were performed with genotypes (between factor), and intensity, ISI, and time (within factors). For western blotting analyses, statistics were conducted with the unpaired *t*-test or with two-way ANOVA to assess the interaction between genotypes and groups. One-sample *t* test was used to compare pCREB/CREB ratio to the theoretical mean value of the normalized WT level set at 1. To control the family wise-error rate, the adjustment Sidak's method was used. Thus, significant main effects were analyzed further by *post hoc* comparisons of means using Sidak's multiple comparisons test. Statistical significance was set at $p < 0.05$. Data are given as mean \pm SEM.

RESULTS

SHORT- AND LONG-TERM MEMORY IN *MSKS* MUTANT MICE

Loss of *Msk1* but not *Msk2* impairs long-term place recognition memory

To investigate the consequences of MSKs loss on cognitive function, we characterized the performance of *Msk1*- and *Msk2*-KO mice for short- and long-term memory in two distinct recognition memory paradigms that differentially engage the hippocampus. Both recognition memory paradigms were conducted in a context showing exactly the same characteristics (same experimental room, open field, experimenter, kind of objects).

In the object-place recognition task (Fig. 2A), no significant difference in total exploration time was observed in either *Msk1*- or *Msk2*-KO mice and their respective WT littermates during the training session (for *Msk1* mutants and littermate controls: WT: n = 15; *Msk1*-KO: n = 13: $t_{26} = 0.5951$, $p = 0.5569$; for *Msk2* mutants and littermate controls: WT: n = 15; *Msk2*-KO: n = 15: $t_{28} = 1.878$, $p = 0,0709$) or the test session (for *Msk1* mutants and littermate controls: $U = 75.5$, $p = 0.3216$; for *Msk2* mutants and littermate controls: $U = 76$, $p = 0,1370$). Thus, deletion of *Msk1* or *Msk2* did not impair spontaneous exploratory behavior during training. This is in accordance with previous studies showing that *Msk* null mouse lines do not exhibit abnormal anxiety, spontaneous locomotor activity, habituation to a new environment, or sensory or nociceptive phenotypes (Brami-Cherrier et al., 2005; Chwang et al., 2007; Chandramohan et al., 2008). This indicates that subsequent deficits in the object recognition tasks may not be attributed to changes in exploratory behavior.

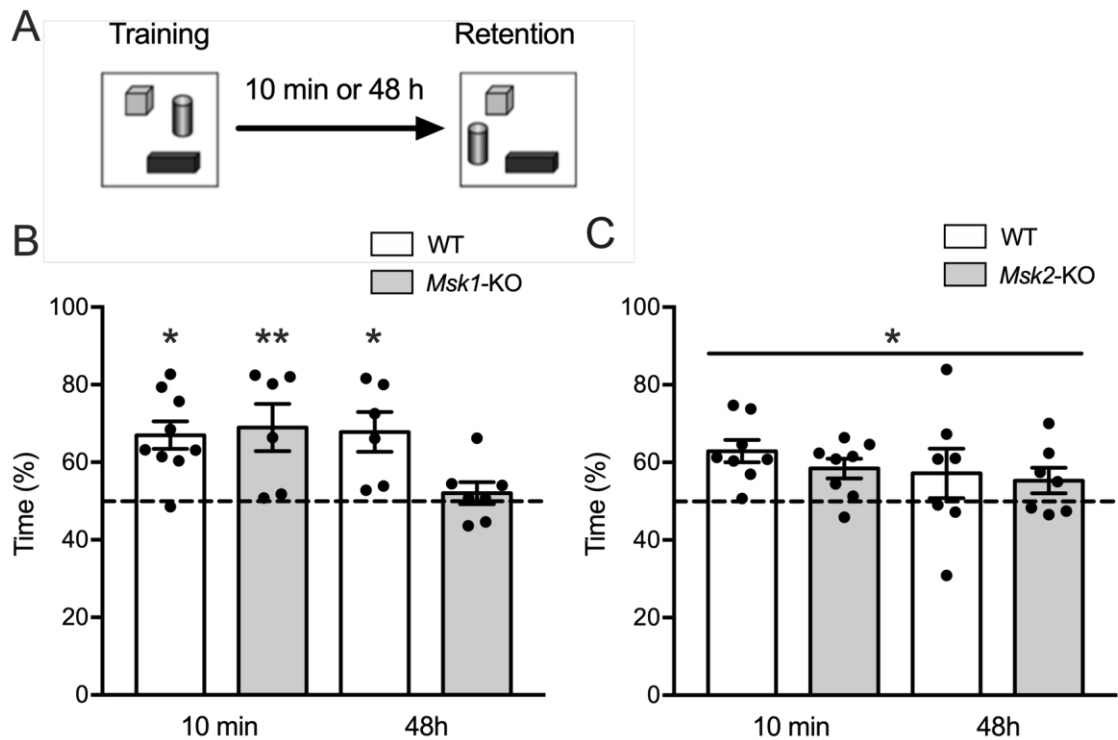


Fig. 2. Object-place recognition memory in *Msk1*- and *Msk2*-KO mice.

Schematic representation of the object-place recognition memory paradigm (A) and retention performance of *Msk1*- (B) and *Msk2*-KO (C) mice 10 min and 48h after exposure to the objects. Results are expressed as the percent time spent exploring the displaced object. (B) *Msk1*-KO mice show preserved object-place recognition memory at a 10-min delay (WT, n = 9; *Msk1*-KO, n = 6), but impaired long-term object-place recognition memory at a 48-h delay (WT, n = 6; *Msk1*-KO n = 7). (C) *Msk2*-KO mice show normal short-term (10-min: WT, n = 8; *Msk2*-KO, n = 8) and long-term (48h: WT, n = 7; *Msk2*-KO, n = 7) object-place recognition memory. White and gray bars correspond to the percent time spent exploring the displaced object in WT and KO mice respectively. The horizontal line represents equal exploration of the unmoved and displaced objects. Values are means \pm SEM. *p < 0.05 and **p < 0.01 compared with chance level.

A significant genotype x retention time interaction was observed in *Msk1* mutants and their littermate controls (Fig. 2B, genotype effect: $F_{1,24} = 2.464$, p = 0.1296, ns; retention time effect: $F_{1,24} = 3.343$, p = 0.0799, ns; genotype x retention time interaction: $F_{1,24} = 4.066$, p = 0.0551) while no significant difference was observed between *Msk2* mutants and littermate controls (Fig. 2C, genotype effect: $F_{1,26} =$

0.6561, $p = 0.4253$, ns; retention time effect: $F_{1,26} = 1.266$, $p = 0.2708$, ns; genotype x retention time interaction: $F_{1,26} = 0.1119$, $p = 0.7407$, ns). During the retention session, 10 min after the last training trial, *Msk1* mutants and their littermate controls spent significantly more time exploring the displaced object than the unmoved objects (compared to chance level set at 50%: littermate controls: $t_8 = 4.497$, $p < 0.01$; *Msk1* mutants: $t_5 = 3.112$, $p < 0.05$) indicating normal short-term spatial recognition memory in *Msk1*-KO. However, 48h after the training session, *Msk1*-KO mice failed to show significantly greater exploration of the displaced object when compared with the control group (littermate controls vs *Msk1* mutants: $p < 0.05$; compared to chance: littermate controls: $t_5 = 3.491$, $p < 0.05$; *Msk1* mutants: $t_6 = 0.7236$, $p = 0.4965$, ns), indicating that long-term spatial recognition memory was impaired in *Msk1* mutant mice. By contrast, *Msk2*-KO mice and their WT littermates spent significantly more time exploring the displaced object at both delays (compared to chance: *Msk2* mutants and littermate controls: $t_{29} = 4.477$, $p < 0.0001$), indicating that short- and long-term spatial recognition memories were preserved in *Msk2*-KO mice.

In the object recognition task (Fig. 3A), no significant difference in total exploration time was observed in either *Msk1*- or *Msk2*-KO mice and their respective WT littermates during the training session (*Msk1* mutants and littermate controls: $t_{12} = 0.1022$, $p = 0.9203$; *Msk2* mutants and littermate controls: $t_{14} = 1.576$, $p = 0.1373$), as in the object-place recognition task. During test session after a 48-h delay, no significant difference in total exploration time was observed in *Msk1*-KO mice and their WT littermates ($t_{12} = 0.351$, $p = 0.7317$), while *Msk2* mutants spent slightly less time exploring the objects compared to their WT littermates ($U = 8$, $p = 0.0104$).

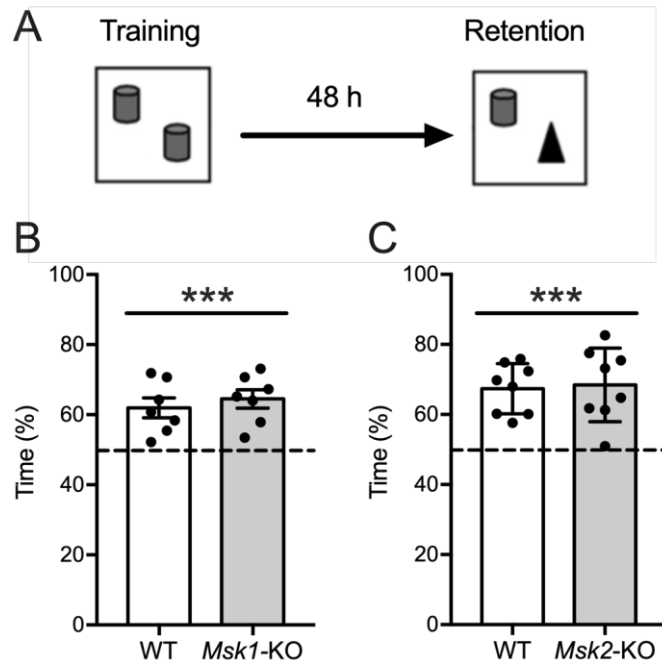


Fig. 3. Object recognition memory in *Msk1*- and *Msk2*-KO mice.

Schematic representation of the object recognition memory paradigm (A) and retention performance of *Msk1*- (B) and *Msk2*-KO (C) mice 48h after training. Results are expressed as the percent time spent exploring the novel object. Both *Msk1*-KO (B) (WT, n = 7; *Msk1*-KO, n = 7) and *Msk2*-KO (C) (WT, n = 8; *Msk2*-KO, n = 8) mice show normal long-term object recognition memory at a 48-h delay. White and gray bars correspond to the percent time spent exploring the novel object in WT and KO mice respectively. The horizontal line represents equal exploration of the familiar and novel objects. Values are means \pm SEM. ***p < 0.0001 compared with chance level.

During the retention session, 48h after the last training trial, no significant difference in the percent time spent exploring the novel object was observed between control and mutant mice. All mice spent significantly more time exploring the novel object than the familiar object (compared with chance level set at 50%: *Msk1* mutants and littermate controls: Fig. 3B, $t_{13} = 7.037$, $p < 0.0001$; *Msk2* mutants and littermate controls: Fig. 3C, $t_{15} = 8.244$, $p < 0.0001$), indicating that both *Msk1*- and *Msk2*-KO mice remembered the objects they have previously experienced and could form long-term object recognition memory.

Altogether these results indicate that neither MSK1 nor MSK2 is essential for short-term spatial recognition memory and long-term object recognition memory,

while MSK1 is required for long-term spatial recognition memory. Since spatial recognition memory is more strongly dependent on the hippocampus than object recognition memory, which predominantly engages the perirhinal cortex (Barker and Warburton, 2011), these results suggest that MSK1 is implicated in hippocampal-dependent recognition memory.

SYNAPTIC TRANSMISSION AND PLASTICITY IN *MSKS* MUTANT MICE

***Msk1* but not *Msk2* deletion reduces basal synaptic transmission in the DG**

Synaptic transmission and plasticity are essential forms of neuron-to-neuron communication for signal processing during the encoding of memories. Here, we investigated the consequences of *Msk1* or *Msk2* genetic deletion on synaptic transmission and plasticity at MPP to granule cell synapses in the DG, a major cortical input to the hippocampus and a sub-hippocampal area strongly implicated in recognition memory. For example, granule cells of the DG were shown to be strongly activated during the exploration of objects in an object-place memory task (Soule et al., 2008) and the DG is important for the encoding of objects, places, and contextual information (Spanswick and Sutherland, 2010). Numerous studies have also illustrated the critical role of the DG in spatial pattern separation, e.g., the ability to discriminate subtle environmental changes when distinct spatial locations have a high degree of similarity or spatial overlap (McHugh et al., 2007; Kesner, 2013). Using different object recognition paradigms, it has been shown that lateral and medial perforant path inputs into the dorsal DG are involved in spatial information processing (Hunsaker et al., 2007; Hunsaker et al., 2008; Albasser et al., 2010) and that the dorsal DG supports specifically object-place feature information while its lesion does not disrupt simple object feature configuration (Lee et al., 2005; Dees and Kesner, 2013; Kesner et al., 2015). Interestingly, object-place recognition triggers rapid upregulation in the DG of plasticity-associated IEG and of postsynaptic density proteins with key roles in long-term synaptic plasticity and long-term memory (Soule et al., 2008). We therefore investigated basal synaptic transmission *in vivo*, forms of short-term plasticity such as paired-pulse facilitation (PPF) and inhibition (PPI), and LTP induced by high-frequency stimulation at perforant path inputs to the DG.

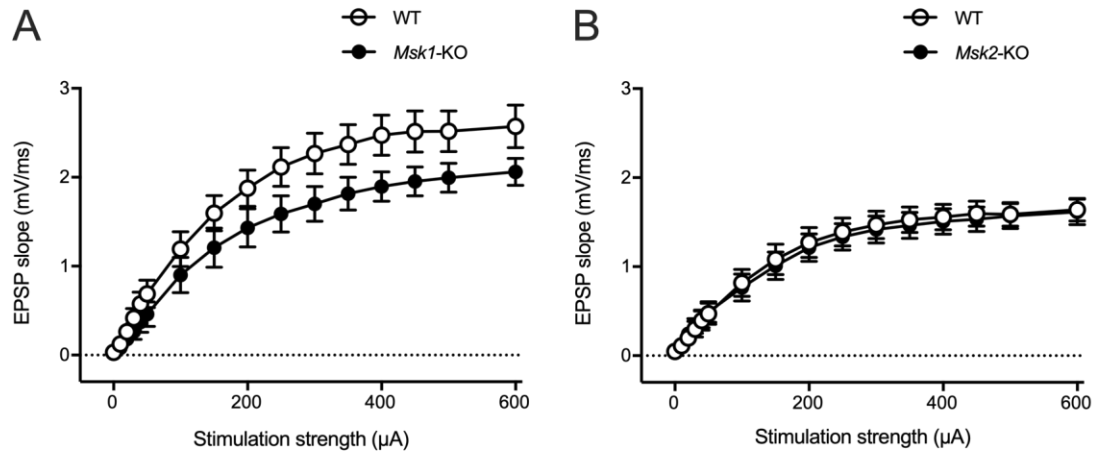


Fig. 4. Synaptic transmission in the DG of *Msk1*- and *Msk2*-KO mice.

Synaptic transmission at MPP granule cell synapses in *Msk1*- (A) and *Msk2*-KO (B) mice. The graphs represent stimulus-response relationships using a range of stimulation intensities (0 to 600 μ A). For each animal, three responses were collected at each intensity and averaged. For each intensity, data points are averages of fEPSP slope values (mV/ms) per group of mice. (A) Significant changes in the I/O relationship were observed between WT (open circles, $n = 13$) and *Msk1*-KO mice (filled circles, $n = 13$), while (B) no significant difference in fEPSP slopes at the different intensities was observed between WT (open circles, $n = 13$) and *Msk2*-KO mice (filled circles, $n = 13$). Values represent means \pm SEM.

For basal synaptic transmission, analysis of I/O curves showed a typical increase in the fEPSP with increasing stimulus strength both in *Msk1*- and *Msk2*-KO mice and their respective littermates (for *Msk1* mutants and littermate controls: Fig. 4A, intensity effect: $F_{15,360} = 143.246$, $p < 0.0001$; for *Msk2* mutants and littermate controls: Fig. 4B, intensity effect: $F_{15,360} = 185.710$, $p < 0.0001$). However, there was an overall reduction of the fEPSP slope at all intensities in *Msk1*-KO mice compared to their WT littermates (genotype effect: $F_{1,24} = 3.221$, $p = 0.0853$; genotype \times intensity interaction: $F_{15,360} = 2.039$, $p < 0.05$), while no significant difference in fEPSP slopes at the different stimulus intensities were observed in *Msk2*-KO mice. At the saturation level, a 20% reduction of the fEPSP slope was observed in *Msk1*-KO mice compared to their WT littermates, suggesting decreased basal synaptic transmission in these mutants.

We next tested PPF of the fEPSP at intensities sub-threshold for evoking a population spike, a form of short-term plasticity attributable to presynaptic mechanisms (Katz and Miledi, 1968). Stimulus intensity required to evoke fEPSPs with comparable amplitudes was independent of genotype (for *Msk1* mutants and littermate controls: WT: $59 \pm 13 \mu\text{A}$, *Msk1*-KO: $70 \pm 10 \mu\text{A}$; for *Msk2* mutants and littermate controls: WT: $69 \pm 11 \mu\text{A}$, *Msk2*-KO: $80 \pm 11 \mu\text{A}$).

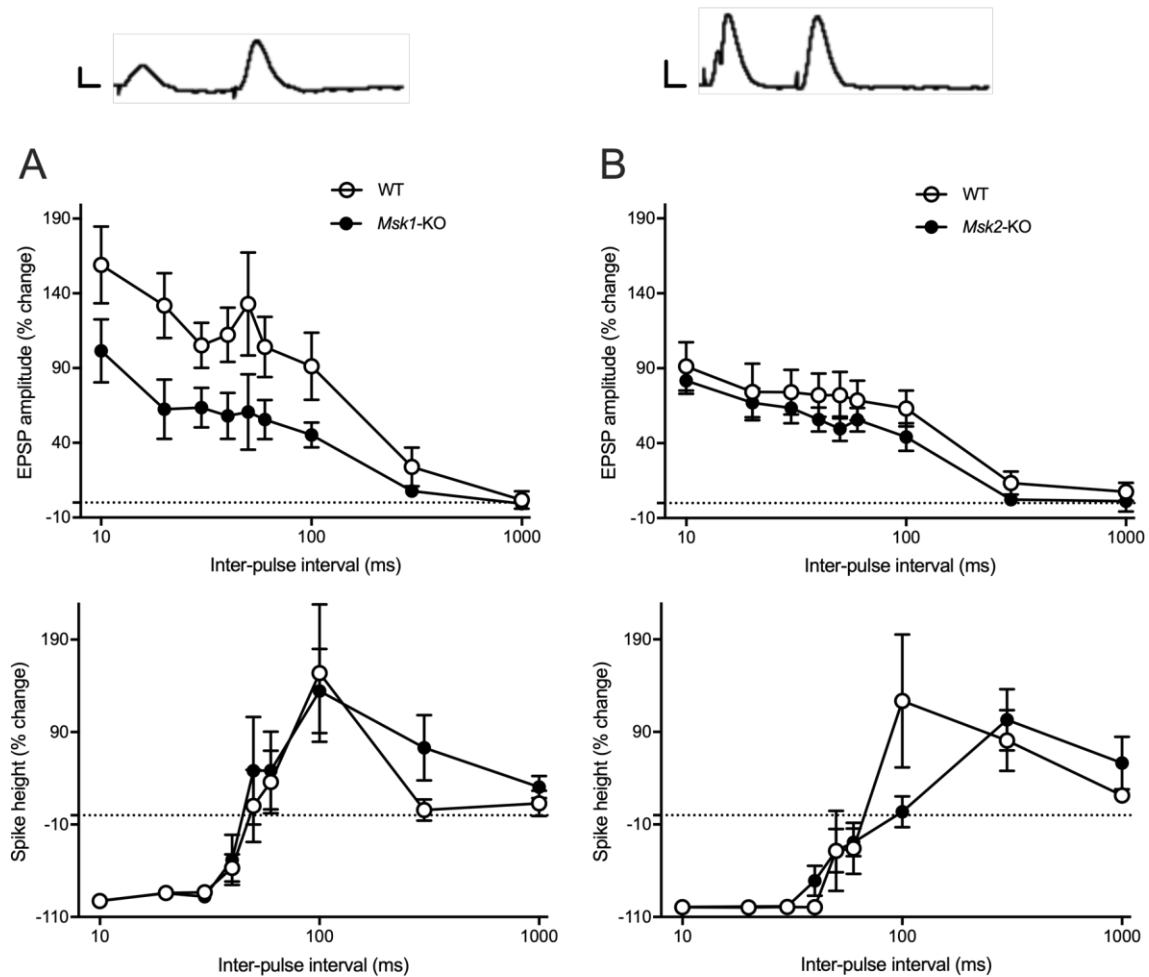


Fig. 5. Short-term synaptic plasticity in the DG of *Msk1*- and *Msk2*-KO mice.

Representative examples of paired-pulse facilitation of the fEPSP (top left) and depression of the population spike (top right) at an inter-pulse interval of 50 and 40 ms, respectively, in a control mouse. Calibration bars: 1 mV, 10 ms. (top) Paired-pulse facilitation at low stimulus intensity set to evoke a pure fEPSP of 1 mV amplitude in *Msk1*- (A) and *Msk2*-KO (B) mice. The graph represents the percent change in fEPSP amplitude of the second response relative to the first response, plotted against a range of inter-stimulus intervals (ISI: 10-1000 ms, log scale). For each animal, three responses were collected at each ISI and averaged. For each ISI,

data points represent averaged fEPSP amplitudes (% change) per group of mice. Paired-pulse facilitation of fEPSP amplitude showed the same dependence on ISI in all mice (**A**: WT (open circles, n = 10) and *Msk1*-KO (filled circles, n = 10) mice; **B**: WT (open circles, n = 10) and *Msk2*-KO (filled circles, n = 15) mice). (bottom) Paired-pulse inhibition at stimulus intensities just above threshold for evoking a 1 mV population spike in *Msk1*- (**A**) and *Msk2*-KO (**B**) mice. Paired-pulse depression and facilitation of the population spike also showed the same dependence on ISI in all mice (**A**: WT (open circles, n = 10) and *Msk1*-KO (filled circles, n = 9) mice; **B**: WT (open circles, n = 10) and *Msk2*-KO (filled circles, n = 17) mice). For each animal, three responses were collected at each ISI and averaged. Data points represent averaged population spike amplitudes (% change) from each group plotted against ISI (10-1000 ms, log scale). Values represent means \pm SEM.

Paired-pulse stimulation (inter-stimulus interval, ISI 10-100 ms) at this intensity resulted in facilitation of the fEPSP, maximal at an inter-pulse interval of 10 ms, in both *Msk1*- (Fig. 5A left) and *Msk2*- (Fig. 5B left) KO mice, and their respective WT littermates. PPF showed the same dependency on ISI in *Msk1*-, *Msk2*-KO mice and their respective WT littermates and decreased significantly with increasing ISI for all mice (for *Msk1* mutants and littermate controls: ISI effect: $F_{8,144} = 16.710$, $p < 0.0001$; for *Msk2* mutants and littermate controls: ISI effect: $F_{8,184} = 35.624$, $p < 0.0001$). The amplitude of facilitation was however reduced in *Msk1*-KO mice compared to their WT littermates (genotype: $F_{1,18} = 6.248$, $p < 0.05$), suggesting impaired short-term presynaptic plasticity in the DG of *Msk1*-KO mice.

In the PPI experiment, stimulation at intensities just above threshold for evoking a population spike resulted in complete suppression of the population spike evoked by a second pulse at short ISIs and in spike facilitation at longer intervals in all mice. This profile of spike depression at short ISIs followed by facilitation at longer intervals reflects recurrent inhibition and disinhibition, respectively, and is in part a postsynaptic phenomenon. The stimulus intensity required to evoke a 1 mV population spike did not significantly differ between mutants and their respective controls (for *Msk1* mutants and littermate controls: WT = $144 \pm 22 \mu\text{A}$, *Msk1*-KO = $165 \pm 21 \mu\text{A}$; for *Msk2* mutants and littermate controls: WT = $182 \pm 28 \mu\text{A}$, *Msk2*-KO = $243 \pm 24 \mu\text{A}$). Paired-pulse depression and facilitation of the population spike

showed the same dependence on ISI in WT and *Msk1*-KO mice (Fig. 5A right, ISI effect: $F_{8,136} = 15.577$, $p < 0.0001$). Spike facilitation occurred at 60 ms and the amplitude of spike facilitation peaked at an ISI of 100 ms in both groups. In *Msk2*-KO mice, although the maximal amplitude of spike facilitation reached a similar level in both controls and mutant mice, spike facilitation occurred at longer ISIs in *Msk2*-KO mice (300 ms) compared to WT mice (100 ms) (Fig. 5B right, ISI effect: $F_{8,200} = 21.267$, $p < 0.0001$; genotype x ISI interaction: $F_{8,200} = 1.959$, $p = 0.0533$). Thus, the PPI analysis suggested normal network excitability in the DG of *Msk1*-KO and a slight decrease in excitability in *Msk2*-KO mice.

The loss of *Msk1* or *Msk2* does not alter *in vivo* DG LTP

Post-tetanic (PTP) and short-term (STP) potentiation were examined between 0 and 15 min following the tetanus (for *Msk1* mutants and littermate controls: Fig. 6A left; for *Msk2* mutants and littermate controls: Fig. 6B left). Stimulus intensities required to evoke fEPSPs of comparable slope values were not significantly different between KO mice and their littermates (for *Msk1* mutants and littermate controls: WT = $175 \pm 23 \mu\text{A}$; *Msk1*-KO = $220 \pm 35 \mu\text{A}$; for *Msk2* mutants and littermate controls: WT = $184 \pm 25 \mu\text{A}$, *Msk2*-KO = $188 \pm 24 \mu\text{A}$). For all mice, the amplitude of PTP-STP increased rapidly to a steady level (for *Msk1* mutants and littermate controls: time effect: $F_{14,336} = 15.309$, $p < 0.0001$; for *Msk2* mutants and littermate controls: time effect: $F_{14,336} = 21.934$, $p < 0.0001$) with a small but significant higher magnitude of PTP-STP in *Msk1*-KO mice compared to WT mice (genotype effect: $F_{1,24} = 4.567$, $p = 0.05$), and no significant differences between *Msk2*-KO mice and their WT littermates.

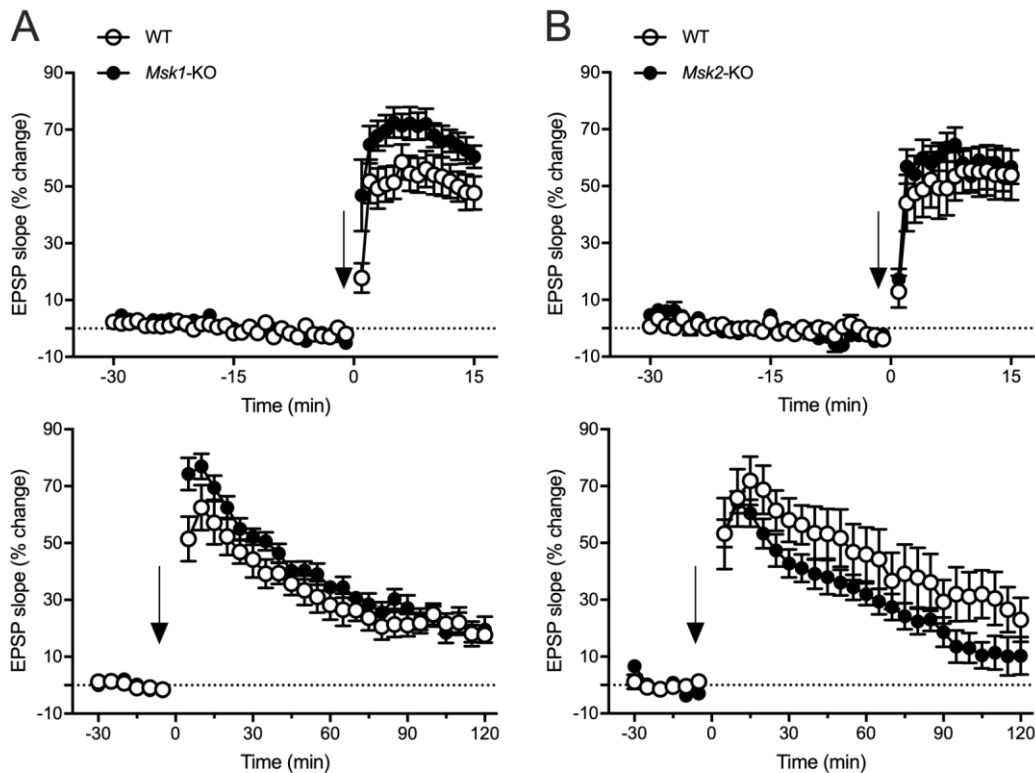


Fig. 6. Synaptic plasticity in the DG of *Msk1*- and *Msk2*-KO mice.

(top) PTP-STP of the fEPSP in *Msk1*- (A) and *Msk2*-KO (B) mice. Test stimuli were applied once every 30 s during 30 min before and 15 min after the tetanus (arrow). For each animal, two successive responses recorded over 1 min were averaged. Data points represent averaged fEPSP slope expressed as percent change from baseline (dashed line) for each group of mice. (A) Magnitude of PTP-STP was significantly higher in *Msk1*-KO mice (filled circles, $n = 13$) compared to WT mice (open circles, $n = 13$). (B) In contrast, a similar level of PTP-STP was observed in WT (open circles, $n = 13$) and *Msk2*-KO (filled circles, $n = 13$) mice. (bottom) Time-course of LTP induced by a strong tetanus (arrow) in *Msk1*- (A) and *Msk2*-KO (B) mice. Insets are sample waveforms from an animal of each genotype recorded before (grey line) and X min after the tetanus (black line). Calibration bars: X mV, X ms. Experimental conditions are as for PTP-STP, except that responses were monitored for 2h following the tetanus and that 10 consecutive responses recorded over 5 min were averaged for each mouse. All mice showed significant potentiation of the fEPSP slope after tetanic stimulation and maintained this potentiation over 2 h (A: WT (open circles, $n = 7$) and *Msk1*-KO (filled circles, $n = 7$) mice; B: WT (open circles, $n = 7$) and *Msk2*-KO (filled circles, $n = 8$) mice). Values represent means \pm SEM.

In a subset of these mice (for *Msk1* mutants and littermate controls: Fig. 6A right; for *Msk2* mutants and littermate controls: Fig. 6B right), LTP was followed for 2h. Tetanic stimulation resulted in a robust, long-lasting potentiation of the fEPSP slope lasting for the 2 h of recording in all groups (mean potentiation 95-120 min after induction vs mean response measured 30 min before induction: for *Msk1* mutants and littermate controls: time effect: $F_{1,12} = 70.854$, $p < 0.0001$; for *Msk2* mutants and littermate controls: time effect: $F_{1,13} = 16.823$, $p = 0.0012$), with a small decay over the early times for all mice that was comparable between mutants and their respective controls (for *Msk1* mutants and littermate controls: time effect: $F_{23,276} = 49.015$, $p < 0.0001$; for *Msk2* mutants and littermate controls: time effect: $F_{23,299} = 36.541$, $p < 0.0001$). Two hours after the tetanus, LTP was of similar magnitude in mutants and their respective WT littermates (for *Msk1* mutants and littermate controls: 115-120 min: WT: $17.73 \pm 2.20\%$; *Msk1*-KO: $19.56 \pm 4.57\%$; for *Msk2* mutants and littermate controls: 115-120 min: WT: $22.92 \pm 7.79\%$; *Msk2*-KO: $10.25 \pm 6.66\%$). Thus, *Msk1* deletion slightly increased PTP-STP, yet it did not alter LTP at the MPP-granule cell synapses in spite of decreased synaptic transmission. *Msk2* deletion on the other hand affected neither PTP-STP nor LTP in the DG.

BASAL AND LTP-INDUCED EXPRESSION OF CREB, pCREB, AND EGR1 IN *MSK1* AND *MSK2* MUTANT MICE

Reduced expression or activity-dependent induction of key transcription factors is often invoked as a molecular alteration underling/underlying? cognitive deficits (Alberini, 2009). Phosphorylation of the transcription factor CREB is a well-established mechanism for its activation (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001), allowing rapid regulation of genes downstream of MAPK signaling, such as the IEG EGR1. Both CREB and EGR1 are essential components of a signaling cascade required for memory consolidation, including long-term recognition memory (Jones et al., 2001; Bozon et al., 2003; Kelly et al., 2003; Penke et al., 2014), and maintenance of LTP (Bourtchuladze et al., 1994; English and Sweatt, 1997; Davis et al., 2000). To explore potential mechanisms underlying the role of MSKs in hippocampal-dependent long-term memory, we examined by quantitative western blot basal and LTP-induced expression and/or phosphorylation state of these two downstream targets of ERK–MSKs signaling. We examined the

pCREB/CREB ratio, a robust indicator of the amount of CREB activity within cells, as well as EGR1 level. In addition, as the amount of activated CREB might also vary through modulation of its expression, we also examined the level of activated CREB over actin, reflecting its prevalence.

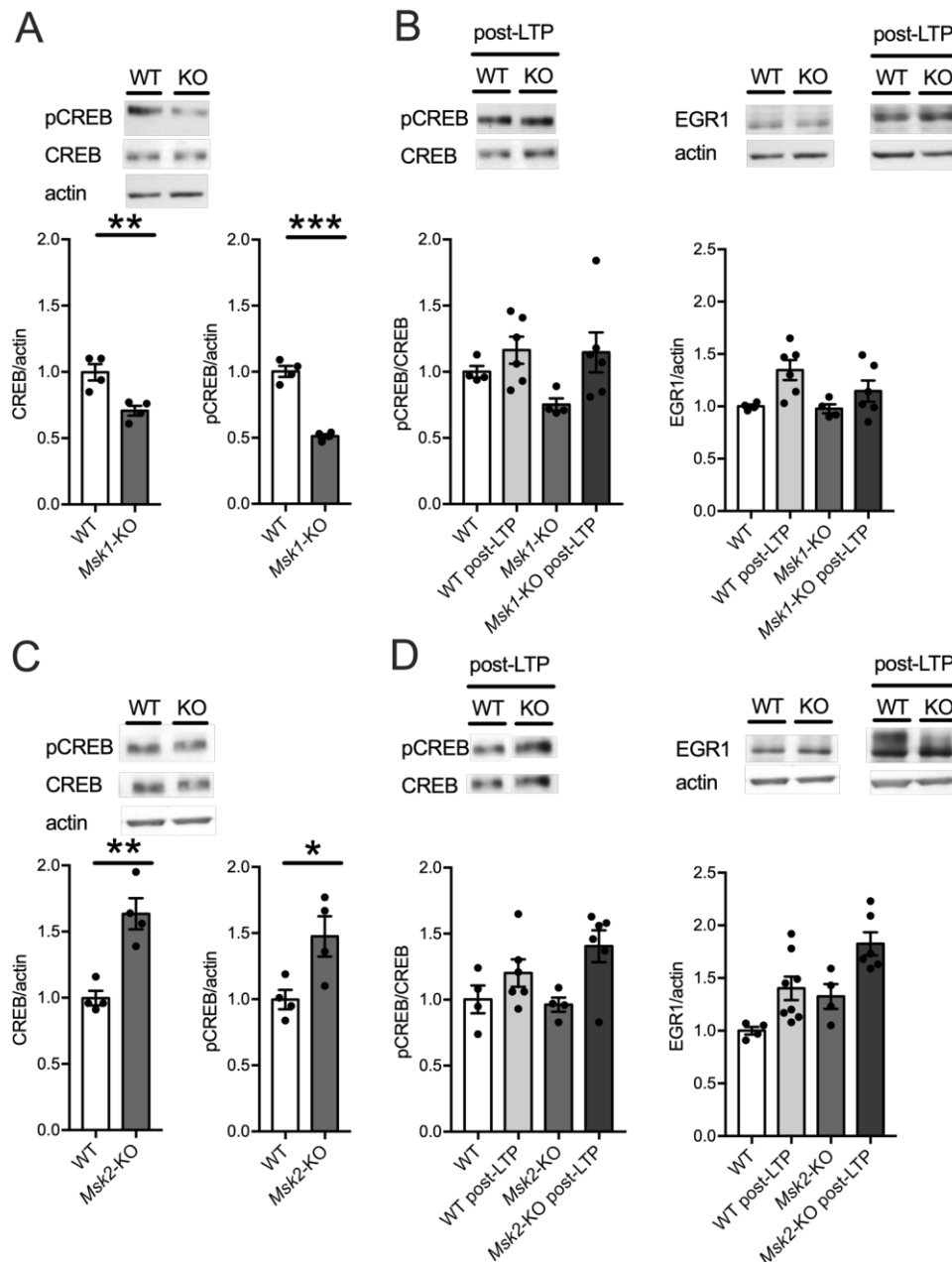


Fig. 7. Expression and LTP-induced regulation of CREB and EGR1 in the DG of *Msk1*- and *Msk2*-KO mice.

(A) Both CREB/actin and pCREB/actin ratios were decreased at basal state in the DG of *Msk1*-KO mice compared to their WT littermates. (B, left) Fifteen minutes following LTP induction the pCREB/CREB ratio was increased in DG of both WT and *Msk1*-KO mice. (C) Both CREB/actin and pCREB/actin ratios were significantly

increased in the DG of *Msk2*-KO mice compared to their WT littermates. (**D**, left) Following LTP induction, the pCREB/CREB ratio was significantly increased in DG of both WT and *Msk2*-KO mice. (**B**, right) EGR1/actin ratios in *Msk1*-KO mice and their WT littermates were not significantly different at basal state. Post-LTP, EGR1/actin ratios were increased for both WT and *Msk1*-KO mice. (**D**, right) EGR1/actin ratios were significantly increased in *Msk2*-KO mice compared to their WT littermates. These ratios were also increased 3h following LTP induction for both WT and *Msk2*-KO mice. White and gray bars correspond to basal state values in WT and KO mice respectively, and light and dark gray bars correspond to values post-LTP in WT and KO mice respectively in *Msk1*- or *Msk2*-KO mice. Representative bands are shown above the histograms. Values represent means \pm SEM (n = 4 per genotype in naïve groups and n = 6-8 per genotype in post-LTP group).

Analyses were carried out specifically in the DG of *Msk1*- (Fig. 7A,B) and *Msk2*-KO (Fig. 7C,D) mice and WT littermate controls, since the formation of long-term spatial recognition memory and LTP trigger rapid induction of EGR1 in the DG (Davis et al., 2000; French et al., 2001; Soule et al., 2008).

Compared to their WT littermates, *Msk1* mutant mice showed a decrease in both total CREB (CREB/actin, -29%; $t_6 = 4.02$, $p < 0.01$) and pCREB (pCREB/actin, -49%; $t_6 = 11.20$, $p < 0.0001$) levels in the DG (Fig. 7A) as well as 25% decrease in pCREB/CREB ratio ($t_3 = 5.351$, $p < 0.05$, compared to the theoretical mean value of the normalized WT level set at 1; fig 7B left). In contrast, in *Msk2* mutants, both total CREB and pCREB were increased compared to their WT littermates (CREB/actin, $t_6 = 4.92$, $p < 0.01$; pCREB/actin, $t_6 = 2.82$, $p < 0.05$; Fig. 7C) with a pCREB/CREB ratio unchanged (Fig. 7D, left).

We then analyzed pCREB/CREB ratio and EGR1 expression following the induction of LTP in the DG. Fifteen minutes following LTP, pCREB/CREB ratio was increased in both *Msk1*-KO and *Msk2*-KO (for *Msk1* mutants and littermate controls: LTP effect: $F_{1,16} = 5.665$, $p < 0.05$; genotype effect: $F_{1,16} = 1.272$, $p = 0.276$, ns; genotype x LTP interaction: $F_{1,16} = 0.971$, $p = 0.339$, ns; for *Msk2* mutants and littermate controls: LTP effect: $F_{1,16} = 8.475$, $p < 0.05$; genotype effect: $F_{1,16} = 0.549$, $p = 0.469$, ns; genotype x LTP interaction: $F_{1,16} = 1.219$, $p = 0.286$, ns). For EGR1 expression, basal level was unaffected by the loss of MSK1 (Fig. 7B right), while it was increased in the absence of MSK2 (genotype effect: $F_{1,18} = 10.3$, $p < 0.01$; Fig.

7D, right). As expected, EGR1 expression was upregulated in an LTP-dependent manner. Three hours following LTP induction, a significant increase in EGR1 protein was observed in all WT and *Msk* mutant mice (for *Msk1* mutants and littermate controls: LTP effect: $F_{1,16} = 8.451$, $p < 0.05$; for *Msk2* mutants and littermate controls: LTP effect: $F_{1,18} = 15.01$, $p < 0.01$).

Thus, the main consequences of *Msk*s deletion were a converse shift in basal CREB and pCREB protein expression between the effect of *Msk1* and *Msk2* deletions, with a decrease in CREB and pCREB levels in the absence of MSK1 and an increase in the absence of MSK2, and a decrease in pCREB/CREB ratio in *Msk1* mutant mice at basal state, that was unchanged in *Msk2* mutant mice. Overall, both *Msk*s-KO mice showed an increase in CREB activation and EGR1 expression following LTP, consistent with the maintenance over at least 2h of LTP in the DG of these mutant mice.

DISCUSSION

In this study, *Msk1* deletion impaired selectively long-term hippocampal-dependent object-place recognition memory, affected both synaptic transmission and short-term presynaptic plasticity at the perforant path-to-dentate granule cell synapses, and decreased CREB basal expression levels and activated CREB levels in the DG at basal state. Our findings also suggest that, in contrast to MSK1, MSK2 is not essential for recognition memory, synaptic transmission, short- and long-term synaptic plasticity at the perforant path-to-dentate granule cell synapses. *Msk2* deletion, however, was associated with an increase in CREB, pCREB and EGR1 levels at basal state.

Role of MSKs in recognition memory

Our aim was to characterize the influence of MSKs loss on recognition memory function. *Msk1/2* double knockout mice were previously shown to display impaired stress-related memory formation (Chandramohan et al., 2008), however the relative contribution of each MSK to these alterations had not yet been addressed. Altogether, our data indicate that MSK1 plays a prominent role in the consolidation of hippocampal-dependent spatial recognition memory. In contrast, loss of MSK2 does not affect performance in either the spatial or non-spatial recognition memory tasks. In earlier studies, genetic deletion of *Msk1* in mice was shown to induce a range of

cognitive alterations, including delayed spatial learning in the Barnes maze, deficient spatial search strategy in the Morris water-maze, short-term discrimination deficit in a novel object recognition task, and impaired contextual fear memory (Chwang et al., 2007; Karelina et al., 2012). Our behavioral analyses reveal dissociation in the impact of *Msk1* deletion between the spatial and non-spatial variants of the recognition memory task since it specifically impaired long-term spatial recognition memory retention, leaving simple object recognition memory unimpaired.

Earlier studies have clearly demonstrated that spatial recognition memory is more vulnerable to hippocampal dysfunction than object recognition memory (Barker and Warburton, 2011). Thus, our results add support to the idea of a stronger implication of MSK1 in hippocampal-dependent memory than in other forms of memory. Additionally, only long-term, but not short-term, spatial recognition memory was affected in *Msk1* mutants, suggesting this gene is particularly important for memory consolidation. In line with this finding, *Msk1*-KO mice were shown to exhibit altered long-term contextual fear memory (Chwang et al., 2007). In recognition memory tasks, detection of novelty depends on characteristics of the training session, including the duration of a trial as well as the number of trials and the length of the inter-trial interval. Karelina and colleagues (2012) reported impairment in short-term object recognition memory in *Msk1* mutant mice, while our results showed spared long-term object recognition memory. Our paradigm using several spaced training trials (three 5-min trials separated by 5 min intervals) is likely to have facilitated the taking of information and consequently retention, as opposed to the 10 min single training session used in the study by Karelina and co-authors, suggesting that object recognition memory resulting from a single training session would be more vulnerable than that resulting from multiple spaced training trials in *Msk1* mutants. This observation is in agreement with the delayed acquisition learning observed in the spatial version of the Barnes maze (Karelina et al., 2012).

Our study underpins an important role for MSK1 in the consolidation of long-term spatial recognition memory. Together with previous studies showing delayed spatial learning, impaired contextual fear memory, and deficient spatial search strategy (Chwang et al., 2007; Karelina et al., 2012), these findings generalize the involvement of MSK1 to various tasks placing a high demand on hippocampal function.

Role of MSKs in intracellular signaling and regulation of neuronal activity underlying information storage

Altogether the available evidence indicates that MSK1 is a component of ERK signaling required for the consolidation of hippocampal-dependent memory. To gain understanding about neurophysiological processes underlying the contribution of MSKs in hippocampal-dependent long-term memory, we examined basal synaptic transmission and different forms of synaptic plasticity in the DG of the hippocampus in the mutant mice. The DG receives cortical inputs to process and funnel cortical information to the hippocampus. Medial perforant path inputs to the DG play a key role in processing spatial information via activation of glutamate receptors, and it was suggested that spatial and non-spatial information are combined into a coherent representation of space with high levels of spatial resolution at dorsal DG granule cells (Hunsaker et al., 2007; Hunsaker et al., 2008; Albasser et al., 2010). Our present study is the first *in vivo* characterization of synaptic activity in the DG of *Msk1*- and *Msk2*-KO mice. We found that loss of *Msk1* was associated with decreased *in vivo* AMPAR-mediated synaptic transmission at MPP-granule cell synapses, whilst it was unaffected after loss of *Msk2*. To better understand how MSK1 affects basal synaptic transmission in the DG, we explored potential pre- and/or postsynaptic origins of this deficit using paired-pulse stimulus procedures. Short-term presynaptic plasticity (PPF) of fEPSPs in the DG was found reduced in *Msk1*-KO mice and unchanged in *Msk2*-KO mice, whereas paired-pulse depression and facilitation of the population spike was normal in *Msk1*-KO and slightly decreased in *Msk2* mutant mice. Choi *et al.* (2017) recently reported modification of presynaptic Ca^{2+} concentrations and a reduction of evoked Ca^{2+} influx in *Msk1* null neurons. Thus, a possible explanation for the observed decrease in basal synaptic transmission is that disruption of MSK1 signaling may reduce glutamatergic excitatory drive in the DG, possibly by altering Ca^{2+} -triggered synaptic vesicle exocytosis, a key process mediating the probability of neurotransmitter release. Future studies aiming to determine the NMDA/AMPA ratios should shed some light on the pre- and/or postsynaptic origin of the basal synaptic transmission deficit in *Msk1* mutant mice. Last, reduction in granule cell dendritic length and branching complexity (but not of the overall DG spine density) reported by Karelina and colleagues (2012) in *Msk1*-KO mice, or a possible reduction in AMPAR at the synapse could also contribute to reducing synaptic transmission.

Despite reduced excitatory glutamatergic transmission and PPF, we found no appreciable modification of theta-burst-induced LTP in *Msk1* mutant mice. Similarly, suppression of MSK2 had no effect on LTP, suggesting that neither MSK1 nor MSK2 are essential for LTP induction and its stabilization at least during the first 2 hours. Fine adjustments of the excitatory-inhibitory (E/I) balance are fundamental for proper tuning of neuronal network activity and generation of network oscillations, as well as homeostatic plasticity (Fritschy, 2008). Reduced excitatory synaptic transmission could induce a shift of the E/I balance thereby decreasing network excitability in the DG of *Msk1* mutants. This hypothesis is consistent with previous observations demonstrating that MSK1 acts as a key homeostat in the activity- and experience-dependent regulation of synaptic strength (Correa et al., 2012; Privitera et al., 2020). Determination of E/I ratio or spontaneous action potential frequency of DG neurons in *Msk1*- and *Msk2*-KO mice could allow to test this hypothesis. CREB activity acts as a molecular mediator between synaptic inputs and long-term gene transcription-dependent plasticity (Kandel, 2012; Zhai et al., 2013), and has been implicated in long-term memory (Lonze and Ginty, 2002; Carlezon et al., 2005). Differences in baseline neuronal activity in the *Msk* mutants could thus underlie their differences in long-term spatial recognition memory. In this perspective, decreasing global excitation in the *Msk2* mutant mice would be expected to elicit a reduction in excitatory synaptic transmission and CREB activity.

Appropriate circuit tuning is essential for network function in maintaining suitable activity levels in the brain necessary to ensure normal cognitive function. Alteration in DG granule cells tuning could contribute to the deficit in object-place recognition memory consolidation found in the *Msk1* mouse mutants. Recently, it was shown that appropriately tuned activity in the DG is critical for spatial object recognition performance (Kahn et al., 2019). In this study, selectively increasing or decreasing DG granule cells activity in control mice compromised their cognitive performance, suggesting a bell curve relationship between DG granule cells recruitment and behavioral performance. Moreover, these authors showed that silencing hyperactive DG cells in a mouse model of epilepsy to an appropriate activity level can restore behavioral spatial novelty detection. Thus, a testable hypothesis is that MSK1 disruption results in net changes in circuit excitability such that the altered circuit would have diverted from the optimal activity level that normally supports successful coding of information.

In the DG, MAPK kinase pathways (Kelly et al., 2003), CREB (Bozon et al., 2003) and several plasticity-related IEGs, including EGR1, are activated and required for the formation of long-term recognition memory (Jones et al., 2001; Bozon et al., 2002; Soule et al., 2008). Here we found that loss of MSK1 was associated with a decrease in the basal levels of CREB and pCREB (29% and 49%, respectively), leading to a 25% reduction of pCREB/CREB ratio in the DG. Our findings concur in suggesting that the basal level of CREB activation is crucial for memory formation since deletion of MSK1 is associated with spatial memory deficit despite normal levels of EGR1. These biochemical data suggest that MSK1 plays a dual role in modulating long-term recognition memory, a direct role on CREB activation via its phosphorylation and an indirect role via other mechanisms, such as histone acetylation. Histone acetylation was shown to enhance memory and synaptic plasticity by facilitating basal transcription at the promoters of key CREB target genes (Vecsey, 2007), while MSK1 deletion was shown to decrease histone H3 acetylation (Chwang et al., 2007). This combined requirement for histone modification and intact CREB signaling for memory formation has been suggested by Chwang et al. (2007). In addition, transcriptional repression of target genes via histone deacetylation could also contribute to the down-regulation of basal CREB expression in the DG. To determine to what extent histone acetylation contributes to the memory deficits observed in the *Msk1* mutants, it would be interesting to assess whether histone H3 acetylation state is decreased in mutant hippocampus, and more informatively the acetylation state of promoters of CREB targets genes implicated in memory formation such as *Nr4a* and *Nr4a2* (McNulty et al., 2012).

Despite reduced basal levels of CREB in *Msk1* mutant mice, LTP-induced CREB activation was preserved in the DG of the hippocampus, as was LTP-induced expression of EGR1, in line with spared DG-LTP. Mechanistically, spared expression of EGR1 following deletion of MSK1 is not entirely surprising as direct transcriptional activation of EGR1 can also occur via the ERK-mediated activation of the ELK1 pathway (Davis et al., 2000), and the *Egr1* promoter region contains multiple ELK1-associated SRE regulatory elements (Christy and Nathans, 1989). The residual expression of CREB in *Msk1* mutant mice appeared to be sufficient to sustain both normal activity-dependent CREB phosphorylation and preserved DG-LTP. In absence of MSK1, CREB phosphorylation could be achieved by other kinases such as the RSKs (Ribosomal protein S6 kinases), involved in the ERK signaling pathway

(Frödin and Gammeltoft, 1999; Romeo et al., 2012; Reyskens and Arthur, 2016). A previous *in vivo* study in a mouse model of the Coffin Lowry Syndrome showed that LTP-induced CREB phosphorylation was reduced in the DG of *Rsk2*-null mutants, indicating that RSK2 contributes to the phosphorylation of CREB (Morice et al., 2013). *Rsk2*-KO mice show phenotypic homologies with *Msk1* mutant mice, with alterations of hippocampal-dependent memory (Davis et al., 2010; Morice et al., 2013), decreased basal synaptic transmission in the DG (Morice et al., 2013) and CA1 (Mehmood et al., 2011), and reduced LTP-induced CREB phosphorylation in the DG, which does not translate into an impaired DG-LTP (Morice et al., 2013). Of note, by contrast to MSK1-loss, MSK2- (present study) or RSK2-loss is not associated with altered long-term spatial recognition memory (Poirier et al., 2007), suggesting dissimilar roles of the three kinases and/or substantial overlap of function between MSKs and RSKs.

Functionally, the apparent discrepancy between impaired spatial memory consolidation and normal LTP could be linked to the different time scales between the two processes, as spatial recognition memory retention was measured 48h after training, whereas LTP was followed for only two hours. Electrophysiology in the awake mouse would be required to follow LTP for several days. Obviously also, theta-burst stimulation in the angular bundle to induce LTP results in a much more massive and widespread type of neuronal activation than that induced by learning. Thus, even in conditions of reduced CREB expression, activation of the remaining CREB proteins and of EGR1 following theta-burst stimulation might have been sufficient to sustain DG-LTP in *Msk1*-KO mice. Chwang and colleagues (2007) reported impaired induced CREB phosphorylation in the hippocampus one hour after fear training in *Msk1*-KO mice, it would thus be interesting in future studies to explore CREB activation following object-place recognition memory training and examine the impact of CREB overexpression in *Msk1*-KO mice.

Memories for events are thought to be represented in sparse, distributed neuronal ensembles (or engrams). In a series of experiments, it was found that neurons overexpressing CREB at the time of learning are more likely allocated to the resulting engram, a mechanism used across multiple brain regions and forms of memory (reviewed in Josselyn and Frankland, 2018). In the DG, artificially increasing the excitability of a small population of neurons using CREB overexpression at the time of training resulted in preferential allocation of these more excitable neurons to

the resulting engram supporting the memory for contextual fear. In contrast, silencing these CREB-overexpressing DG neurons disrupts expression of contextual memory (Park et al., 2016). Thus, we could hypothesize that reduced CREB activity in *Msk1*-deficient mice impairs the recruitment of DG neurons during exposure to the object-place recognition memory task, leading to impaired long-term memory.

Unexpectedly, loss of MSK2 in contrast induced an increase in basal levels of total CREB and pCREB (64% and 48%, respectively), suggesting indirectly a negative regulation of CREB by MSK2 in the DG of the hippocampus. The pCREB/CREB ratio was however comparable between *Msk2*-KO and WT mice. This was associated with a slightly increased EGR1 expression and an LTP-induced increase in CREB activation and EGR1 expression comparable to that of WT littermates. In our experiments, the increased levels of CREB and pCREB, and the up-regulation of CREB and EGR1 by neuronal activity following MSK2 deletion did not translate into an increased efficacy of long-term synaptic plasticity and/or of recognition memory consolidation. Additional experiments would be needed to further explore the potential contribution of MSK2 in other functions implicating CREB and transcription-dependent plasticity in various cerebral regions. Other forms of spatial and conditioned fear memories (Benito and Barco, 2010), response to chronic exposure to drugs of abuse (Nestler, 1992; Nestler, 2004), and neurogenesis (Herold et al., 2011), which are functions altered in *Msk1* mutants (Brami-Cherrier et al., 2005; Chwang et al., 2007; Brami-Cherrier et al., 2009; Karelina et al., 2012), could be explored.

Collectively, our results reinforce the idea that MSK1, in contrast to MSK2, represents a key regulator of basal synaptic transmission and a molecular homeostat regulating synaptic strength between neurons to ensure proper neuronal network activity, thereby optimizing information storage. Our data suggest that behavioral deficits in *Msk1*-KO mice arise in part from the impaired glutamatergic transmission, the reduced short-term presynaptic plasticity, and the decreased basal level of CREB activation in the DG of the hippocampus. As this does not result in an appreciable modification of long-term synaptic plasticity in this brain region, the integration of overlapping and compensatory pathways via RSK2 operating on distinct signaling mechanisms during synaptic plasticity is hypothesized. Of note, the continuous generation and selective activation of adult new-born neurons in the DG is one of the

key processes underlying structural shaping of neural connectivity during memory stabilization (Shors et al., 2001; Bruel-Jungerman et al., 2005; Deng et al., 2009). Since this form of neuronal plasticity is decreased in the DG of *Msk1* mutant mice (Choi et al., 2012; Karelina et al., 2012), it would be interesting in further test whether it may also contribute to their long-term recognition memory deficits. Our data represent a step forward to the identification of the specific and distinct contribution of the two MSKs isoforms in hippocampal physiology and cognitive functions, which need to be carefully grasped to better understand the pathophysiology of forms of intellectual disabilities such as the Coffin Lowry syndrome and in designing potential therapeutic strategies.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

EM designed the experiments and performed behavior and electrophysiology. VE performed Western blotting. EM performed data analysis and interpretation, and drafted the manuscript. SL, SG, and VE critically revised the manuscript. SL supervised the study.

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