



## Research

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# *Zif268/Egr1* gain of function facilitates hippocampal synaptic plasticity and long-term spatial recognition memory

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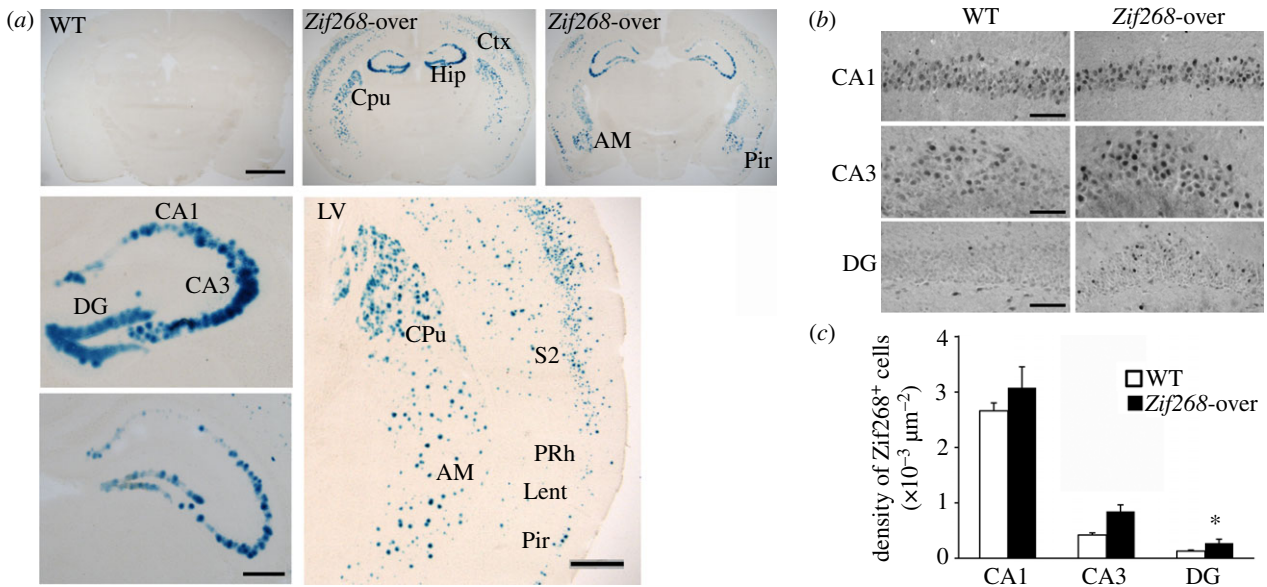
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It is well established that *Zif268/Egr1*, a member of the *Egr* family of transcription factors, is critical for the consolidation of several forms of memory; however, it is as yet uncertain whether increasing expression of *Zif268* in neurons can facilitate memory formation. Here, we used an inducible transgenic mouse model to specifically induce *Zif268* overexpression in forebrain neurons and examined the effect on recognition memory and hippocampal synaptic transmission and plasticity. We found that *Zif268* overexpression during the establishment of memory for objects did not change the ability to form a long-term memory of objects, but enhanced the capacity to form a long-term memory of the spatial location of objects. This enhancement was paralleled by increased long-term potentiation in the dentate gyrus of the hippocampus and by increased activity-dependent expression of *Zif268* and selected *Zif268* target genes. These results provide novel evidence that transcriptional mechanisms engaging *Zif268* contribute to determining the strength of newly encoded memories.

## 1. Introduction

There has nearly been a century of interest in the idea that encoding and storage of information in the brain rely on changes in the efficacy of synaptic connections within the neural networks that are activated during a learning experience, a process required to form a stored memory trace available for recall. The prevailing model for cellular consolidation underlying the laying down of memory suggests that a sequence of events including receptor activation, synapse-to-nuclear signaling and the activation of selective gene programmes and subsequent synthesis of proteins is a key mechanism underlying the enduring modification of neural networks required for the stability of memories. One critical step in this process is the activation of a class of immediate early genes (IEGs) encoding inducible transcription factors that interact with promoter regulatory elements on a host of downstream late-response genes to regulate their expression. *Zif268/Egr1* is one such IEG encoding a zinc finger transcription factor of the *Egr* family that plays a crucial role in the maintenance of hippocampal long-term potentiation (LTP) and the consolidation of several forms of memories [1]. Studies in *Zif268* knockout mice highlighted a particular sensitivity to *Zif268*-deficiency of hippocampal-dependent spatial learning and spatial recognition memory [2,3]. Consistent with this, *Zif268*-deficiency was also found to impair the formation of stable hippocampal place cell representations of novel environments [4]. *Zif268* mRNA and protein are also rapidly induced in association with LTP and in defined brain structures following learning or recall of several types of memory (see [5,6] for reviews) and this can lead to a functional increase in *Zif268* protein binding to its DNA consensus *Egr* response element (ERE) [7]. Using a gain-of-function strategy in transgenic mice, a recent study reported that enhanced neuronal expression of



**Figure 1.** Zif268 overexpression in the brain of the transgenic mice. (a) Coronal brain sections of Dox-induced expression of  $\beta$ -galactosidase in WT and Zif268-overexpressing mice. Note the strong expression of *lacZ* in CA1, CA3 and dentate gyrus (DG) areas of the hippocampus (Hip), in neocortex (Ctx), medial caudate putamen (CPu), amygdala (AM) and piriform cortex (Pir). Bottom right image shows sparse *lacZ* expression in perirhinal (PRh) and lateral entorhinal (Lent) cortices. S2, somatosensory cortex; Pir, piriform cortex; LV, lateral ventricle. Scale bars, top: 2000  $\mu$ m, bottom: 500  $\mu$ m. (b) Photomicrographs showing higher Zif268 staining in CA1, CA3 and DG of the hippocampus in Zif268-overexpressing mice. Scale bar, 50  $\mu$ m. (c) The density of Zif268<sup>+</sup> cells was significantly increased in the DG of Zif268-overexpressing mice compared with WT mice. \* $p < 0.05$ . (Online version in colour.)

Zif268 can slow down extinction of conditioned taste aversion [8]. As resistance to extinction can be taken as a sign of stronger memory formed during initial training, this raises the question of whether Zif268 overexpression can directly facilitate the formation of long-term memory.

In the current study, we therefore examined whether Zif268 overexpression can enhance the capacity for forming long-term memory in a task that does not require an explicit reinforcer. To this end, we assessed memory performance of transgenic forebrain-specific Zif268 overexpressing mice and control littermates in object and object–place recognition memory tasks, allowing us to evaluate the impact of graded spatial demand in the same paradigm. Novel object recognition memory engages the perirhinal cortex as well as the hippocampus to varying degrees depending on the experimental conditions of the task, while object–place recognition memory is more strongly dependent on hippocampal functions [3, 9–12]. Spatial exploration of objects is associated with synaptic potentiation in the hippocampus [13] and increased Zif268 expression in the dentate gyrus of the hippocampus [14]. Whereas Zif268 knockout mice are impaired in both object and object–place recognition memory, heterozygous mice carrying half the complement of Zif268 are impaired in spatial, but not object recognition memory, suggesting increasing dependence on Zif268 activity as the explicit spatial demand of the task increases [1, 15]. We therefore predicted that Zif268 overexpression might be a prevalent aid to memory of the spatial location of objects. Because Zif268 deficiency impairs LTP in the dentate gyrus of the hippocampus [1], we also examined whether Zif268 overexpression would enhance dentate gyrus LTP.

## 2. Zif268 overexpressing mice

We used inducible transgenic Zif268 overexpressing mice based on the tetracycline-controlled transactivator system (rtTA2(S)-

M2), carrying a forebrain-specific CaMKII $\alpha$  promoter-rtTA2 transgene and a transgene carrying a bitetO-promoter fused to a *LacZ* reporter gene and a Zif268 open reading frame as described previously [8]. Experiments were performed blind to the genotype and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the French National Committee (87/848). Transgenic and control wild-type (WT) littermate mice (6–12 months old) were maintained under constant temperature and lighting conditions (22°C, 12 L : 12 D cycle) and received ad libitum doxycycline (Dox)-supplemented food prepared daily (6 mg per 100 g of wet food, West-Ward Pharmaceuticals or a generous gift from Mark Nelson, Paratek Pharmaceuticals) for at least 8 days before and then throughout the duration of the experiments to induce Zif268 overexpression. Because of the limited number of double transgenic mice available, females were used for behavioural experiments (all tested the same day) and males were used for electrophysiological measures (one per day). To confirm that there was no sex-dependent effect due to differences in Zif268 expression, we measured the level of Zif268 ( $F_{3,21} = 1.53$ ;  $p = 0.235$ ), PSMB9 ( $F_{3,21} = 0.51$ ;  $p = 0.241$ ) and synapsin II ( $F_{3,21} = 1.51$ ;  $p = 0.241$ ) were similar in wild-type and Zif268-overexpressing females ( $n = 15$ ) and males ( $n = 10$ ).

With this protocol of Dox supplementation, analyses of  $\beta$ -galactosidase staining on brain sections [16] showed intense labelling in neocortex, hippocampus, medial caudate putamen, amygdala and piriform cortex of Zif268-overexpressing mice compared with WT mice on Dox (figure 1a). In allocortical regions such as the perirhinal and entorhinal cortices, labelling was sparse compared with the neocortical area (figure 1a). To examine expression of Zif268, we measured the density of Zif268 expressing cells in hippocampal subfields by immunofluorescence using a rabbit anti-Zif268 primary antibody (1/1000; Santa Cruz Biotechnology, CA, USA). Zif268 positive

cells were automatically counted under an Olympus BX60 microscope, coupled to a mapping software (MERCATOR PRO; Explora Nova, La Rochelle, France), in CA1, CA3 and dentate gyrus cell layers of the hippocampus (two sections per animal, inter-section intervals 280  $\mu\text{m}$ ) to estimate cell density (number of *Zif268*-positive cells per  $\mu\text{m}^2$ ). The volume of each layer was obtained from five sections (inter-section interval 280  $\mu\text{m}$ ) and the total number of neurons in each layer was estimated using NeuN immunohistochemistry (mouse anti-NeuN antibody, 1/2000, and goat anti-mouse antibody conjugated to Alexa 647; Molecular Probes, Eugene, OR, USA) and conventional unbiased stereological quantification methods, as described previously [17].

After Dox treatment, volumes of hippocampal subfields were indistinguishable between WT and *Zif268* overexpressing mice (CA1:  $p > 0.05$ ; CA3:  $p > 0.05$ ; dentate gyrus (DG):  $p > 0.05$ ; non-parametric Mann–Whitney comparisons, data not shown). Quantification of the number of neurons using NeuN labelling also revealed no differences between genotypes (CA1:  $p > 0.05$ ; CA3:  $p > 0.05$ ; DG:  $p > 0.05$ ; data not shown), suggesting that gross hippocampal anatomy was not affected in the transgenic mice. Analysis of basal levels of *Zif268* expression revealed a moderate (figure 1*b*), but non-significant increase in the density of *Zif268*-positive cells in CA1 and CA3 of *Zif268*-overexpressing mice ( $n = 5$ ) compared with WT ( $n = 4$ ) mice (CA1: Mann–Whitney  $p > 0.05$ ; CA3:  $p > 0.05$ ; figure 1*b*). In the dentate gyrus, however, a region where, in contrast to CA1 and CA3, basal expression is very low, there was a significant 2.07-fold increase in *Zif268*-positive cells in *zif268*-overexpressing mice (DG:  $p < 0.05$ ; figure 1*b,c*). This was confirmed by quantifying the number of *Zif268*-positive nuclei in relation to the total number of neurons. In the dentate gyrus, there was a near twofold increase in the proportion of *Zif268*-expressing neurons (WT:  $1.24 \pm 0.15\%$ ; *Zif268*-overexpressing mice (*Zif268*-over):  $2.40 \pm 0.81\%$ ;  $p < 0.05$ ), whereas in CA1/CA3, the proportion of *Zif268*-expressing neurons was much higher, but with a smaller and statistically non-significant increase in *Zif268*-overexpressing mice (WT:  $60.01 \pm 3.62\%$ ; *Zif268*-over:  $69.47 \pm 8.79\%$ , in CA1; WT:  $14.09 \pm 1.49\%$ ; *Zif268*-over:  $24.76 \pm 4.52\%$  in CA3). In all, these results show that, at the basal state, there is a substantial increase in *Zif268* expressing neurons in the dentate gyrus in our transgenic mice under Dox treatment, and only a moderate increase in hippocampal pyramidal neurons. This, in comparison with  $\beta$ -galactosidase staining, suggests a relatively rapid turnover of *Zif268* proteins in this region in the transgenic mice.

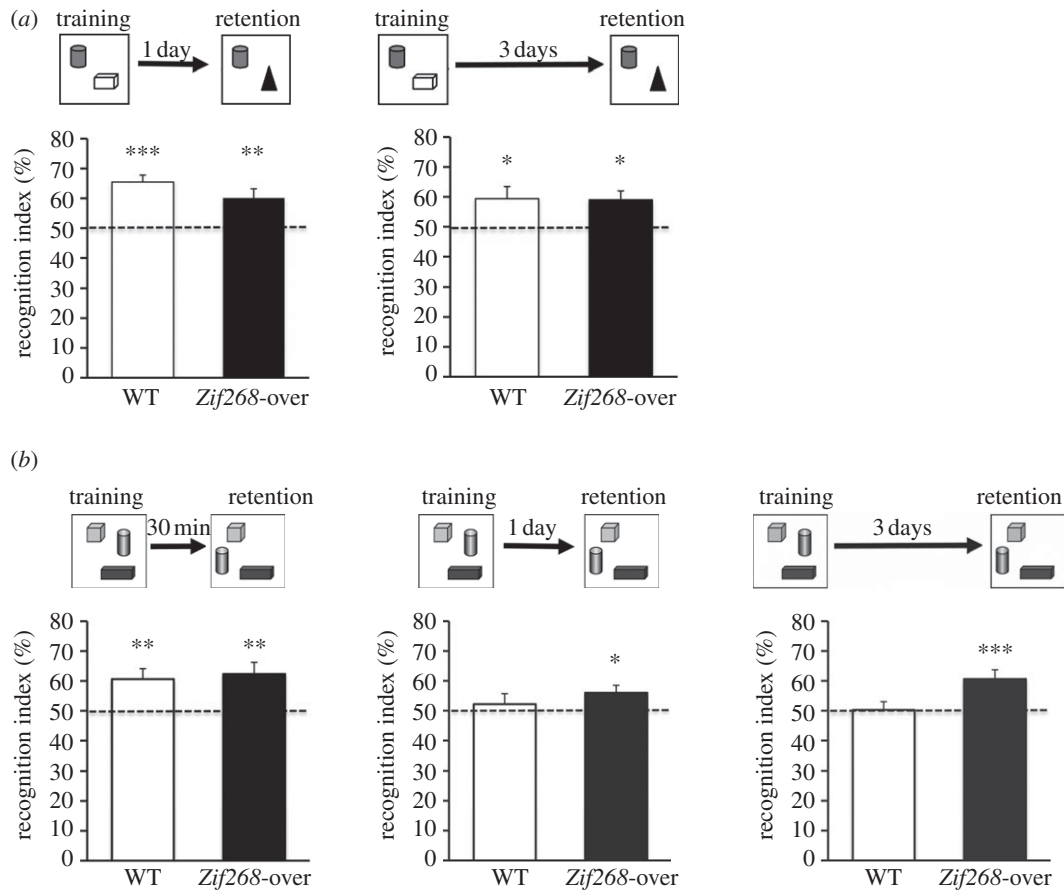
### 3. *Zif268* overexpression facilitates memory of the spatial location of objects

To determine the effect of *Zif268* overexpression on recognition memory, we first evaluated performance in an object recognition memory task using standardized procedures as previously described [1,15]. Briefly, the experimental apparatus was a black plastic square open field arena ( $45 \times 45 \times 30$  cm) with wood shavings on the floor and a cue card placed at a fixed location on the top of one of the walls of the open field to facilitate spatial mapping. It was located in a room with dim lighting and constant background noise, with a video camera mounted above the test apparatus that relayed to a video tracking system. General activity and exploratory behaviour of the animals were automatically recorded using ANY-MAZE software

(Stoelting Co., USA), and object exploration was manually scored. The criteria for exploration were based strictly on active exploration, where the mouse had both forelimbs within a circle of 4 cm around an object, head oriented towards it or touching it with its nose. Mice were handled (twice a day for 2 days) and familiarized to the empty open field (2 days) prior to the experiment. They were then allowed to explore two different objects made of wooden pieces or assembled interlocking plastic Lego pieces of different shapes and colours, for two 10-min sessions with a 10-min interval. Following a 1- or 3-day delay, one of the familiar objects was replaced with a novel object and the time spent exploring the familiar and novel objects was measured and compared with chance level. The objects and their spatial arrangement in the open field were chosen in a pseudorandom order and were counterbalanced among individuals and genotypes.

Exploratory behaviour during habituation to the open field was similar in WT and *Zif268*-overexpressing mice (total distance travelled: genotype: Student's *t*-test,  $t = 1.7$ ,  $p > 0.05$ ). Similarly, during the acquisition phase, mice of the two genotypes spent similar lengths of time exploring the two sample objects (WT: 134.7 s, *Zif268*-over: 173.9 s;  $t = 1.2$ ,  $p > 0.05$ ; data not shown), indicating no discernible effect of *Zif268* overexpression on locomotor activity or novelty-seeking behaviour. When retention was tested 1 day or 3 days after training with different sets of objects, both WT and *Zif268*-overexpressing mice explored the novel object preferentially (figure 2*a*). Statistical analyses showed that the time spent exploring the novel object was significantly above chance levels in both groups and for both retention delays, with no difference between genotypes (WT 1 day:  $t = 6.6$ ,  $p < 0.0001$ ,  $n = 13$ ; *Zif268*-over 1 day:  $t = 3.3$ ,  $p < 0.01$ ,  $n = 9$ ; WT 3 days:  $t = 2.3$ ,  $p < 0.05$ ,  $n = 12$ ; *Zif268*-over 3 days:  $t = 3.0$ ,  $p < 0.05$ ;  $n = 9$ ).

We then assessed the performance of WT and *Zif268*-overexpressing mice in an object–place recognition memory task following procedures described previously [15,18]. In this task, three different objects were present during training (consisting of two 10-min sessions with a 10-min interval) and during the test conducted 30 min, 1 day or 3 days after training, the spatial position of one of the objects was changed, in a counterbalanced manner among individuals, to a new spatial location. The time spent exploring the displaced and non-displaced objects was measured and compared with chance level (50%, averaging the time spent exploring the two non-displaced objects). During the retention test 30 min after training, mice of both genotypes explored the displaced object above chance levels (WT:  $t = 3.0$ ,  $p < 0.01$ ,  $n = 11$ ; *Zif268*-over:  $t = 3.2$ ,  $p < 0.01$ ,  $n = 9$ ; figure 2*b*), indicating similar level of short-term (30 min) object–place memory. WT mice showed no evidence for long-term object–place recognition memory, indicated by a similar level of exploration of the displaced and non-displaced objects, both at 1 day or 3 days post-training (WT 1 day:  $t = 0.6$ ,  $p > 0.05$ ,  $n = 20$ ; WT 3 days:  $t = 0.1$ ,  $p > 0.05$ ,  $n = 19$ ; figure 2*b*). Thus, with this more demanding task and a relatively short exposure protocol during acquisition, WT mice can form short-term but not long-term memory of the spatial location of objects, as previously observed with a similar training regime [18]. By contrast, *Zif268*-overexpressing mice still explored the displaced object significantly above chance, both 1 day and 3 days after training (*Zif268*-over 1 day:  $t = 2.4$ ,



**Figure 2.** Recognition memory in *Zif268*-overexpressing mice. (a) Schematic of the object recognition memory paradigm and retention performance of WT and *Zif268*-overexpressing (*Zif268*-over) mice 1 and 3 days after training. The histograms represent the recognition index expressed as the per cent time spent exploring the novel object over the total time of objects exploration. Both WT and *Zif268*-overexpressing mice spent significantly more time exploring the novel object at the 1 day ( $n = 13$  and  $n = 9$ , respectively) and 3 days ( $n = 12$  and  $n = 9$ , respectively) retention delays. (b) Schematic of the object–place recognition memory paradigm and retention performance of WT and *Zif268*-overexpressing mice 30 min, 1 and 3 days after training. Both WT ( $n = 11$ ) and *Zif268*-overexpressing mice ( $n = 9$ ) showed preferential exploration of the displaced object at the 30 min delay (left histograms). WT mice no longer showed a preference for the displaced object at the 1 day ( $n = 20$ ) or the 3 days ( $n = 19$ ) retention tests with the training protocol used in this study, whereas *Zif268*-overexpressing mice still spent significantly more time exploring the displaced object at both the 1 day ( $n = 16$ ) and 3 days ( $n = 16$ ) retention delays, indicating that *Zif268* overexpression facilitates the formation of long-term object–place recognition memory. The horizontal line represents equal exploration of the familiar and novel (a) or displaced (b) objects. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$  compared with chance.

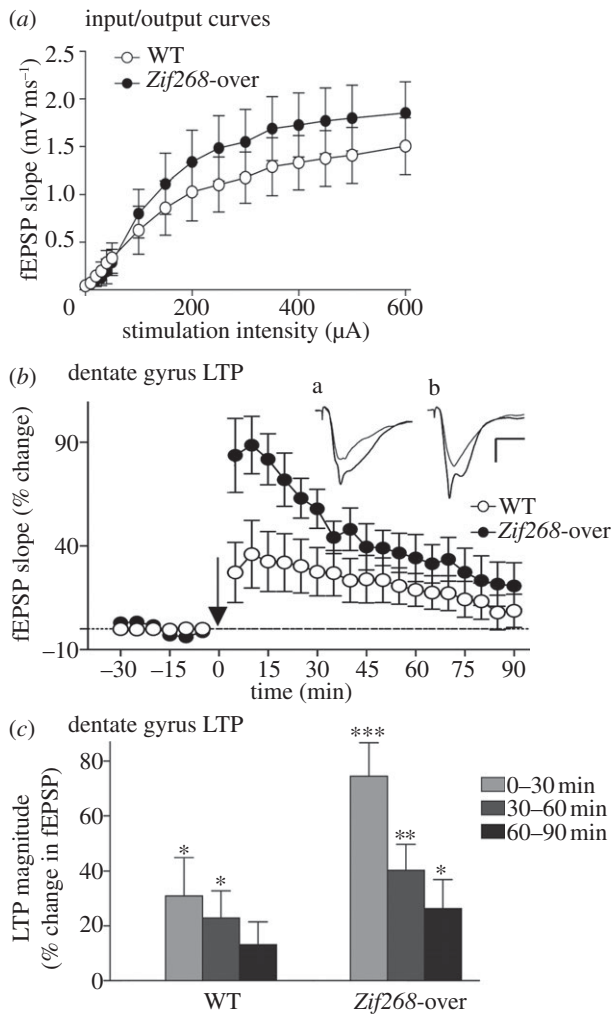
$p < 0.05$ ,  $n = 16$ ; *Zif268*-over 3 days:  $t = 3.4$ ,  $p < 0.005$ ,  $n = 16$ ; figure 2b), indicating that *Zif268* overexpression results in enhanced object–place long-term recognition memory.

#### 4. *Zif268* overexpression enhances dentate gyrus long-term potentiation

Next, we examined the effect of *Zif268* overexpression on synaptic transmission and LTP at the medial perforant path (MPP) to dentate granule cell synapses *in vivo*. Stimulation procedures and electrophysiological recordings were performed as previously described [19]. Briefly, WT and *Zif268*-overexpressing mice ( $n = 5–6$  per group) were anaesthetized with a mixture of oxygen (Air Liquid Santé, Bonneuil-sur-Marne, France) and isoflurane (CSP Translab, Cournon, France) during surgery and throughout recordings. Animals, held in a stereotaxic frame and maintained at a constant body temperature of  $37.0 \pm 0.5^\circ\text{C}$ , were implanted with concentric bipolar stimulating electrodes in the perforant path (3 mm lateral to lambda, depth approximately 1.5 mm from brain surface) and a borosilicate glass micropipette recording electrode containing a

silver wire immersed in saline lowered into the dendritic layer of the ipsilateral dentate gyrus (2 mm posterior to bregma, 1.6 mm lateral, approximately 1.5 mm from brain surface). After surgery, low-frequency baseline stimuli (60  $\mu\text{s}$  monophasic pulses, 0.033 Hz) were delivered to evoke a population field excitatory postsynaptic potential (fEPSP), stored for off-line analysis of the maximum slope of the rising phase of the fEPSP as described previously [1,19]. Analyses were performed using one- and two-way analyses of variance (ANOVA) with genotypes as between factor and intensity and time as within factors. Statistical significance was set at  $p < 0.05$ . Significant main effects were further analysed by post hoc comparisons of means using Student–Newman–Keuls tests.

After a stable response was established, input–output (I/O) curves were generated using a range of stimulus intensities (0–600  $\mu\text{A}$ ; three responses for each intensity). I/O curves in both WT ( $n = 6$ ) and *Zif268*-overexpressing mice ( $n = 5$ ) showed a typical increase in the fEPSP with increasing stimulus strength (figure 3a). The slope of the fEPSP increased significantly with intensity for all mice ( $F_{15,135} = 40.47$ ,  $p < 0.0001$ ), and although the I/O curve in *Zif268*-overexpressing mice was slightly above that of the WT mice (figure 3a), no significant difference



**Figure 3.** Synaptic transmission and plasticity in the dentate gyrus of *Zif268*-overexpressing mice. (a) The graph plots stimulus–response relationships at MPP–granule cell synapses using a range of stimulation intensities (0–600 μA) in anaesthetized WT and *Zif268*-overexpressing mice (*Zif268*-over). Each data point is an average of fEPSP slope values from three responses (abscissa in the log scale). No significant change in the I/O relationship was observed between WT (open circles,  $n = 6$ ) and *Zif268*-overexpressing mice (filled circles,  $n = 5$ ). (b,c) Dentate gyrus LTP in WT and *Zif268*-overexpressing mice. (b) Time course of LTP induced at MPP–granule cell synapses (WT: open circles,  $n = 6$ ; *Zif268*-over: filled circles,  $n = 5$ ). Responses were recorded for 30 min before and 90 min after the tetanus (arrow) to the MPP. Each data point is an average of 10 consecutive responses recorded over 5 min. All mice showed significant potentiation of fEPSP slope after tetanic stimulation. Inserts are sample waveforms from a WT (a) and a *Zif268*-overexpressing mouse (b) recorded before (grey line) and 10 min after tetanic stimulation (black line). Scale bars, 1.5 mV; 10 ms. (c) Per cent potentiation of the fEPSP over three 30 min periods post-tetanus. LTP in *Zif268*-overexpressing mice was of a higher magnitude and lasted longer than in WT mice. Significant difference from baseline: \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.005$ .

was observed (genotype effect:  $F_{1,135} = 0.45$ ,  $p > 0.05$ ; genotype  $\times$  intensity interaction:  $F_{15,135} = 1.01$ ,  $p > 0.05$ ), indicating normal basal synaptic transmission in the transgenic mice.

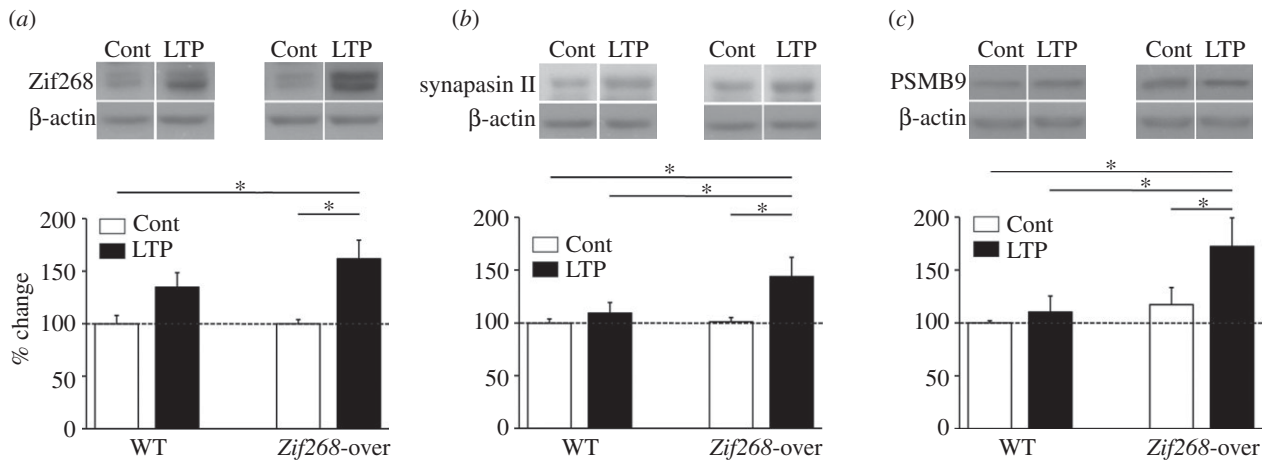
We then examined LTP in the same mice. To this end, test stimuli were delivered at 0.033 Hz for 30 min at an intensity to evoke an fEPSP slope at 50% of maximum, to establish a baseline. A tetanus was then delivered to the MPP, which consisted of six series of six trains of six stimuli at 400 Hz, 200 ms between trains, 20 s between series [1,19]. Pulse-width was doubled during the tetanus. After the tetanus, low-frequency

stimulation was resumed for 90 min. The stimulus intensity used in both groups of mice was comparable between genotypes (WT:  $235 \pm 49$  μA; *Zif268*-over:  $218 \pm 47$  μA; genotype effect:  $F_{1,9} = 0.058$ ;  $p > 0.05$ ), as was the mean fEPSP slope values during baseline (WT:  $1.14 \pm 0.14$  mV ms<sup>-1</sup>; *Zif268*-over:  $0.79 \pm 0.16$  mV ms<sup>-1</sup>; genotype effect:  $F_{1,9} = 2.875$ ;  $p > 0.05$ ). Tetanic stimulation of the MPP resulted in a robust, long-lasting potentiation of the fEPSP slope in both groups (figure 3b). Immediately after the tetanus, however, the magnitude of potentiation of the fEPSP measured during the first 5 min was significantly enhanced in *Zif268*-overexpressing mice compared with WT mice (WT:  $27.26 \pm 14.53\%$ ; *Zif268*-over:  $83.71 \pm 17.84\%$ ; genotype effect:  $F_{1,9} = 6.16$ ,  $p < 0.05$ ). Potentiation of the fEPSP remained above that of WT mice for the duration of recording, although the magnitude of LTP decreased progressively for both genotypes (time effect:  $F_{17,153} = 21.26$ ,  $p < 0.0001$ ; genotype  $\times$  time interaction:  $F_{17,153} = 5.53$ ,  $p < 0.0001$ ; figure 3b). Analysis of the averaged magnitude of fEPSP potentiation across successive 30 min periods post-tetanus (figure 3c) revealed significant effects of genotype ( $F_{1,36} = 7.57$ ,  $p < 0.01$ ) and time ( $F_{3,36} = 10.75$ ,  $p < 0.0001$ ). In WT mice, potentiation of the fEPSP was significantly above baseline for up to 60 min (0–30 min:  $F_{1,10} = 4.96$ ,  $p < 0.05$ ; 30–60 min:  $F_{1,10} = 5.33$ ,  $p < 0.05$ ), but not thereafter, whereas it remained significantly above baseline for the duration of recording in *Zif268*-overexpressing mice (0–30 min:  $F_{1,8} = 37.28$ ,  $p < 0.0005$ ; 30–60 min:  $F_{1,8} = 18.66$ ,  $p < 0.005$ ; 60–90 min:  $F_{1,8} = 6.15$ ,  $p < 0.05$ ). Thus, these results indicate that *Zif268* overexpression does not change basal synaptic transmission but increases the magnitude and prolongs the duration of LTP in the dentate gyrus *in vivo*.

## 5. *Zif268* overexpression increases activity-dependent expression of ZIF268 and target genes

LTP is associated with the activation of several intracellular signalling pathways, some of which direct rapid and transient activation of transcription factors, among them *Zif268*, leading to the expression of specific gene programmes. We therefore examined whether and to what extent LTP in *Zif268*-overexpressing mice would lead to enhanced activation of selected *Zif268* target proteins. For this, the dentate gyri ipsilateral and contralateral to the site of LTP induction were removed 3 h after LTP induction in WT ( $n = 5$ ) and *Zif268*-overexpressing ( $n = 5$ ) mice, a time point associated with activation of several LTP-associated genes [20,21]. We selected two downstream targets of *Zif268*, synapsin II and the proteasome 20S  $\beta$ -subunit PSMB9 (LMP2), a subunit of the proteasome belonging to the multisubunit catalytic core of the proteolytic machinery [22], to examine their expression following LTP. Both genes possess *Zif268* binding sites on their promoter regions [23–25]. Dentate gyrus tissue was processed for western blotting as described previously [26], using anti-synapsin II (1/15000 in TBST BSA 5%, Abcam, France) and anti-PSMB9 (1/1000 in TBST BSA 5%, Abcam, France) antibodies. We also examined *Zif268* expression using anti-*Zif268* antibodies (1/1000 in TBST BSA 5%, Cell Signalling, Ozyme, France). Protein values were normalized first to actin and then calculated as a per cent change from the contralateral side of the *Zif268* WT mice.

Overall analysis of variance (ANOVA) showed a significant difference in the expression of *Zif268* between groups



**Figure 4.** LTP-induced expression of Zif268, synapsin II and the proteasome 20S  $\beta$ -subunit PSMB9 in Zif268-overexpressing mice. (a) Zif268, (b) synapsin II and (c) PSMB9 protein expression was measured by western blotting from the dentate gyrus taken 3 h after induction of LTP (LTP side) and from the contralateral (non-stimulated) side (Cont). Data for each protein and genotype are normalized to the reference protein  $\beta$ -actin and expressed as a per cent change relative to protein levels in the control side of WT mice. There was a trend towards an increase in Zif268, but not synapsin II or PSMB9 expression after LTP in WT mice, and a higher and significant increase of all three proteins after LTP in Zif268-overexpressing mice (Zif268-over). Sample western blots of each protein and actin are represented above the histograms. \* $p < 0.05$ .

( $F_{3,16} = 6.19$ ;  $p < 0.01$ ; figure 4a). In the control, non-stimulated dentate gyrus contralateral to the site of LTP induction, there was no significant difference between genotypes (Fisher's post hoc  $p > 0.05$ ). This probably reflects the impracticality of western blotting from whole dentate gyrus tissue to detect small increases in the number of Zif268-positive neurons in this region, compared with quantification of immunolabelled neurons. Following LTP, there was a small but not significant increase in expression of Zif268 in WT mice (LTP versus control side, Fisher's post hoc  $p > 0.05$ ; figure 4a) as expected at this relatively late time point [7]. By contrast, in Zif268-overexpressing mice, the increase in Zif268 expression 3 h following LTP reached a high level, significantly above that of the control, non-stimulated sides of the WT and Zif268-overexpressing mice (Zif268-over: LTP versus control side,  $p < 0.05$ ; Zif268-over LTP side versus WT control side,  $p < 0.05$ ; figure 4a). Overall, while our Zif268-overexpressing mouse line shows a significant but moderate increase in the number of Zif268-expressing dentate gyrus neurons at basal levels (figure 1), a major feature is an enhanced capacity for activity-dependent expression of Zif268.

Analyses of synapsin II and PSMB9 expression revealed significant differences between genotypes for both proteins ( $F_{3,16} = 3.68$ ;  $p < 0.05$  and  $F_{3,16} = 3.43$ ;  $p < 0.05$ , respectively). In WT mice, there were no significant changes in synapsin II or PSMB9 protein levels after induction of LTP (WT mice: LTP versus control side, Fisher's post hoc  $p > 0.05$  for each protein; figure 4b,c). However, in Zif268-overexpressing mice, which displayed large overexpression of Zif268 after LTP, LTP resulted in a significant increase in both synapsin II and PSMB9 expression (Zif268-over: LTP versus control side, Fisher's post hoc  $p < 0.05$  for each protein; figure 4b,c), suggesting that Zif268 overexpression can boost regulation of target genes in an activity-dependent manner.

## 6. Discussion

The evidence to date suggests that rapid regulation of the expression of IEGs encoding inducible, regulatory transcription factors is a key mechanism of the genomic response underlying

synaptic plasticity and the modification of neural networks required for the stabilization of memories. In neurons activated by learning, these transcriptional events are thought to mediate the activation of selective gene programmes and subsequent synthesis of proteins, leading to stable functional and structural remodelling of the activated networks, so that the memory can later be reactivated upon recall. Over the last decades, novel insights have been gained in identifying key transcriptional regulators that can control the genomic response of synaptically activated neurons. Here, as an example of this approach, we focused on one such activity-dependent transcription factor, Zif268, known to be implicated in neuronal plasticity and memory formation. Whereas targeted deletion of the Zif268 gene results in profound impairments of several forms of long-term memory, including a fundamental memory ability, recognition memory [1,2,15,27], here we report that overexpression of Zif268 in forebrain neurons in mice is sufficient to augment the mice's ability to form a long-lasting spatial recognition memory. These findings, together with the demonstration that Zif268 overexpression can slow down extinction of conditioned taste aversion [8], clearly add to the growing evidence that Zif268 brain expression is an important factor for establishing stable long-lasting memories.

Interestingly, in previous experiments we showed that half the complement of Zif268 in heterozygous mutant mice is not sufficient to allow the formation of a memory for the spatial location of objects as heterozygous mice are impaired in this task, however it is sufficient to form a memory of the nature of the objects [15]. Mirroring this, overexpression of Zif268 leads to enhancement of the more demanding memory for the spatial location of objects, but not to improvement of object memory over and above that observed in WT mice. As discussed in detail previously, object recognition memory engages the perirhinal cortex [12] and the hippocampus with varying degree of requirement depending on the experimental conditions of the task, in particular the complexity and richness of the spatial context during objects exploration and their consequences on the ability to form spatial configurations, associative relationships between items and objects–scene relationships [3, 9–11]. The formation of object memory also recruits several of the same cellular and molecular mechanisms

in both structures (reviewed in [3,12]). Our  $\beta$ -galactosidase expression results, however, suggest moderate, if any, overexpression of *Zif268* in the perirhinal cortex. This may explain the apparent absence of enhancement of novel object recognition memory in our experimental conditions. However, it remains possible that the absence of improvement of object recognition memory, at least within the limit of 3 days after the mice first encountered the objects, is simply due to a ceiling effect, the control mice displaying robust long-term object memory at this delay. Alternatively, it is also possible that object memory is less sensitive to variations in *Zif268* expression, as exemplified by the absence of deficit in this task in *Zif268* heterozygous mice [1].

Spatial recognition memory places higher demands on hippocampal processing of information and *Zif268* heterozygous mice display as much impairment in spatial memory as *Zif268* homozygous mutant mice, while they are less impaired in tasks that do not place a high demand on hippocampal function [1], suggesting hippocampal functions are highly dependent on *Zif268* gene expression dosage. In support of this idea, it has been shown in contextual fear conditioning that partial knockdown (by approx. 66%) of *Zif268* by injection of antisense oligodeoxynucleotides in the hippocampus is insufficient to affect consolidation of contextual fear memory, but impairs its reconsolidation after recall [28], a memory process that is more vulnerable to interfering treatments than initial consolidation (see [29] for a review). The ubiquitin–proteasome system has also been involved in reconsolidation processes and during updating of memory content [30,31]. Furthermore, in a context pre-exposure facilitation paradigm, *Zif268* downregulation was shown to prevent updating of the hippocampal memory content when new information present at recall is linked to the retrieved memory [31]. These findings, together with the present results with *Zif268* overexpression, highlight the importance of *Zif268* gene expression dosage in determining the strength of memory in relation to task difficulty and cognitive demand.

Our results using conditional mice overexpressing *Zif268* in several structures of the forebrain cannot address precisely the issue of structure-specificity. However, the above findings all suggest a high sensitivity of hippocampal functions to *Zif268* gene expression dosage and thus lead to the proposal that in spatial/contextual and relational memory tasks, hippocampal *Zif268* expression levels become increasingly vital for the hippocampal component of the memory trace. In this brain area, in particular in the dentate gyrus, the extent of *Zif268* expression after LTP correlates with the persistence of LTP [32,33] and LTP in *Zif268* knockout mice cannot be maintained over 24 h, a phenotype found in both homozygous and heterozygous *Zif268* mutant mice [1]. Mirroring this, we now report that dentate gyrus LTP, but not basal synaptic transmission, is enhanced under conditions of *Zif268* overexpression, although in this case even the induction phase of LTP was enhanced, suggesting that *Zif268* overexpression may modify expression of as yet unknown molecular/cellular synaptic components involved in induction of LTP. In the continuing debate about the role of LTP mechanisms in memory, these findings provide an important complement to the suggestion that synaptic changes brought about by LTP and during memory consolidation and storage share, at least in part, common underlying molecular mechanisms.

Mechanistically, exploration of objects in an arena is associated with the slow development of NMDA receptor-

dependent synaptic potentiation in the hippocampus that can be occluded by prior induction of LTP, resulting in recognition memory deficits [13]. At the molecular level, several canonical cell-signalling cascades are activated (reviewed in [3,34]), including the MAP kinase cascade [26] known to be instrumental for LTP-induced regulation of *Zif268* [35]. Further, expression of *Zif268* is rapidly induced in the dentate gyrus of the hippocampus following spatial exploration of objects [14]. Here, we also found enhanced or prolonged LTP-induced expression both of *Zif268* itself and of two *Zif268* target genes in *Zif268*-overexpressing mice, suggesting that increasing the levels of *Zif268* increases the capacity for activity-dependent regulation of downstream gene programmes. Synapsin II contributes to regulation of transmitter release whereas PSMB9 is part of the ubiquitin–proteasome system. Although we observed a significant upregulation of these two target proteins and of *Zif268* in overexpressing mice 3 h following the induction of LTP, this is not the case in WT mice. It is possible that the time post-LTP is not the optimal window in which to detect significant changes in expression of these proteins in WT mice and that, as for *Zif268* itself, the observed changes in synapsin II and PSMB9 expression reflect a prolonged wave of expression following LTP in *Zif268*-overexpressing mice. In the absence of evidence clearly documenting the time course of regulation of these proteins following induction of LTP, we cannot rule out, however, the possibility that they may be functionally regulated by alternative signalling pathways when *Zif268* is overexpressed. Although the precise mechanisms by which *Zif268* overexpression facilitates LTP and long-term spatial recognition memory are unknown, the increased neuronal capacity to regulate *Zif268* downstream gene programmes may be one mechanism underlying the enhancement of LTP and the facilitation of the formation of a long-term recognition memory. Although several potential *Zif268* target genes bearing ERE consensus sequences on their promoter regions have been suggested [23,25,36], characterizing the selective gene programmes controlled by *Zif268* in relation to learning and memory remains a challenge for future research.

There is also accumulating evidence that transcriptional changes underlying long-term memory are supported by epigenetic modifications across diverse brain regions, including DNA methylation and posttranslational modifications of histone tails such as phosphorylation, acetylation and methylation [37]. Several findings suggest an important role of *Zif268* in experience-dependent epigenetic mechanisms that underlie memory. For example, regulation of histone methylation at the *Zif268* promoter can facilitate contextual fear memory [38], histone H4 acetylation at the *Zif268* promoter correlates with *Zif268* expression in the hippocampus [39] and histone acetylation critically modulates object recognition memory consolidation [40]. Furthermore, exploration of objects was recently shown to trigger rapid phosphorylation, acetylation and methylation of histones at the *Zif268* promoter in the hippocampus and the prefrontal cortex [41]. Blocking these epigenetic marks at the *Zif268* promoter impairs recognition memory, while their enhancement by intensive training or by transgenic intervention favours recognition memory [41]. Finally, *Zif268* can mediate epigenetic programming via DNA methylation and histone posttranslational modifications to influence downstream gene regulation [42,43]. Down-regulation of *Zif268* has been observed in ageing or certain diseases leading to cognitive deficits [44–46]. Selective intervention on these epigenetic mechanisms at the *Zif268*

promoter to enhance its expression could be one promising tool for attempting to rescue certain cognitive deficiencies.

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