Memory reconsolidation mediates the strengthening of memories by additional learning

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Memories are dynamic, rather than static, in nature. The reactivation of a memory through re-exposure to salient training stimuli results in its destabilisation, necessitating a restabilisation process known as reconsolidation, a disruption of which leads to amnesia. Here I show that one normal function of hippocampal memory reconsolidation in rats is to modify the strength of a contextual fear memory as a result of further learning.

Following their initial acquisition and consolidation, memories can be modified through further experience. For example additional learning strengthens an already-established memory trace. It is, however, not known whether such a change in memory strength depends upon the same cellular mechanisms as initial learning.

The phenomenon of memory reconsolidation, as revealed by the demonstration of an experimentally-induced retrograde amnesia for a consolidated memory in a manner that is critically dependent upon the reactivation of that memory[1], has been suggested to enable the updating of a previously-acquired memory[2,3]. However, it has not yet been demonstrated that reconsolidation is necessary to update memories in an animal model[2,4], although in some settings memory reconsolidation is only observed under conditions in which memory updating occurs[5,6]. Therefore, it remains unclear whether the modification of a memory, in particular its strengthening, depends upon reconsolidation mechanisms.

Memory reconsolidation consists of two phases, a reactivation-dependent destabilisation process, followed by the protein synthesis-dependent restabilisation phase[1]. Reconsolidation can be isolated from initial memory consolidation using the doubly dissociable mechanisms of hippocampal contextual fear memories[7], the destabilisation of which is also dependent upon synaptic protein degradation[8].

Here, a second learning trial (see **Supplementary Methods** online; all procedures were conducted in accordance with the UK 1986 Animals (Scientific Procedures) Act (Project License PPL 80/1767)) strengthened a contextual fear memory, but only following its destabilisation (**Supplementary Fig. 1**). Infusion of the protein synthesis inhibitor anisomycin into the hippocampus immediately after the second trial resulted in subsequent amnesia, consistent with previous findings in an auditory fear conditioning procedure[9]. The use of the broad-spectrum translational inhibitor, as well as having important side-effects[10], does not enable isolation of consolidation and reconsolidation mechanisms, and so it remained possible that the amnesia resulted from inhibition of both reconsolidation of the trial 1 memory and consolidation of the new trial 2 memory[9]. Hence, updating memory strength might depend upon consolidation, and not reconsolidation mechanisms.

Given that there are doubly dissociable cellular mechanisms of hippocampal contextual fear memory consolidation and reconsolidation[7], BDNF (brain-derived neurotrophic factor) being required for consolidation and zif268 (also known as EGR1, NGFI-A and Krox24) being necessary for reconsolidation, it was possible to determine whether the memory strengthening incurred by additional learning involved consolidation or reconsolidation processes. If memory strengthening involved consolidation mechanisms, both BDNF and zif268 would have to be knocked down in order to disrupt the reconsolidation of the existing memory as well as the consolidation of the new memory, and produce amnesia. However, in contrast to this hypothesis, knockdown of BDNF in the dorsal hippocampus during a second learning trial by antisense oligodeoxynucleotide (ASO) infusion, as used previously[7] and demonstrated to be functionally active by disrupting contextual fear memory consolidation (**Supplementary Fig. 2** online), had no effect upon the subsequent expression of the strengthened contextual fear memory relative to the infusion of control missense sequences (MSO), even when the concentration of oligodeoxynucleotide was doubled (**Supplementary Fig. 3** online). Instead, infusion of zif268 ASO alone into the dorsal hippocampus was sufficient to cause a subsequent severe amnesia (**Fig. 1a**). This amnesia was of the same magnitude as that following anisomycin infusion and both took several hours to emerge, as evidenced by the intact short-term memory (STM) observed 3 hours after the second learning trial, and was long-lasting for at least 7 days (**Supplementary Fig. 4** online). These observations are consistent with typical memory reconsolidation deficits[11] and, coupled with the previous demonstration that zif268 ASO infusion selectively impairs memory reconsolidation[7], support the assertion that knockdown of zif268 in the dorsal hippocampus impaired memory strengthening through the blockade of hippocampal memory reconsolidation.

While zif268-dependent reconsolidation mechanisms appear to mediate memory strengthening, the amnestic effect of zif268 ASO might be related instead to the increased absolute strength of the contextual fear memory conditioned through two learning trials, relative to that resulting from the single trial learning that functionally recruits BDNF (**Supplementary Fig. 1** online). However, when a stronger fear conditioning procedure was used, in which both trials were condensed into a single session, memory consolidation remained dependent upon hippocampal BDNF and not zif268 (**Fig. 1b**,**c** and **Supplementary Fig. 5** online). Therefore, these data reveal a double dissociation between the cellular mechanisms of initial memory consolidation (BDNF) and memory strengthening through additional learning (zif268). Moreover, the selective dependence of the two processes upon their relative cellular mechanisms is not a result of non-specific or quantitative factors.

A further contention might be that zif268 is not required for memory strengthening *per se*, but instead for synaptic plasticity in neural circuits that have been modified recently by behavioural experience. Thus the selective dependence of additional learning upon zif268 may result not from the fact that an existing memory is being updated and strengthened, but arises because some learning experience, which need not have been related, engaged the dorsal hippocampus on the previous day. To test this hypothesis, rats were first conditioned in a separate context (different operant chambers[12]), before being returned to the standard fear conditioning apparatus (context 2) for the second day of training. Infusion of zif268 ASO into the dorsal hippocampus prior to this second conditioning trial had no effect on subsequent conditioned freezing in context 2 (**Fig. 2a** and **Supplementary Fig. 6** online). In contrast, knockdown of hippocampal BDNF resulted in severe amnesia, suggesting that even when there has been recent synaptic plasticity in the hippocampus, contextual fear conditioning functionally recruits consolidation, but not reconsolidation, mechanisms. Moreover, these results provide strong evidence that memory strengthening has separable underlying mechanisms to memory acquisition/consolidation.

If the mechanisms of reconsolidation are the same as those that strengthen the memory trace, impairing memory destabilisation should prevent the modification of memory strength. It has been shown recently that hippocampal synaptic protein degradation is a critical process in the destabilisation of contextual fear memories[8]. Infusion of the proteasome inhibitor βlac into the dorsal hippocampus prevented the amnestic action of anisomycin in a standard memory reconsolidation setting. Thus whereas anisomycin infusion alone, immediately after contextual re-exposure, resulted in subsequent amnesia, the co-infusion of βlac rendered the memory invulnerable to protein synthesis inhibition[8]. Here, anisomycin alone also resulted in amnesia when infused into the dorsal hippocampus immediately after a second learning trial (**Fig. 2b** and **Supplementary Fig. 1** online). However, whereas the co-administration of βlac mitigated against the amnestic effect of anisomycin, the resultant levels of contextual freezing remained significantly lower than vehicle-infused controls. Moreover, infusion of βlac, regardless of whether it was combined with anisomycin, resulted in the failure of additional learning to strengthen the pre-existing contextual fear memory (see also **Supplementary Fig. 7** online). Therefore, preventing memory destabilisation maintained the strength of the previously-acquired memory at a constant level, further supporting the assertion that memory reconsolidation is the mechanism by which memories are strengthened through additional learning.

Given that the process of memory destabilisation is an integral step in the strengthening of memories, greater emphasis must be placed upon the mechanisms of destabilisation that are only beginning to be delineated[8,13,14]. Another important implication of the present findings is that memory reconsolidation may, in fact, be the predominant process that occurs during learning and memory in situations that involve more than a single training trial. However, given that these findings are limited to certain training parameters in a contextual fear procedure, it remains to be determined to what extent they can be generalised and whether any boundary conditions exist. Nevertheless, it remains likely that the persistence of memories acquired through repeated experience will be understood primarily through the study of memory reconsolidation, rather than initial consolidation, given their dissociable mechanisms. Moreover, these findings demonstrate that memory reconsolidation has an adaptive function in normal learning and memory, by showing that it enables the modification of memory strength.

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Figure legends

Figure 1. (**a**) Knockdown of zif268, but not BDNF, during a second conditioning session results in subsequent amnesia. Rats were fear conditioned on two consecutive days (Cond1 & Cond2), and were tested 24 hr later (Test). ANOVA revealed a significant Session x Gene x ASO interaction ($F_{2,38}$=18.73, P<0.001). Analysis of the BDNF groups alone revealed no main effect of ASO or Session x ASO interaction (F's<1), whereas analysis of the zif268 groups revealed a significant Session x ASO interaction ($F_{2,20}$=38.71, P<0.001), driven by a significant effect of ASO during the test (simple effects one-way ANOVA; P<0.05), with no differences during Cond1 and Cond2 (n=5–6 per group). Knockdown of (**b**) BDNF, but not (**c**) zif268, during a single 2-trial conditioning session results in subsequent amnesia. Rats were tested 3 hr (STM), 24 hr (LTM) and 7 d (LTM2) after conditioning. ANOVA revealed an overall significant Session x Gene x ASO interaction ($F_{2,44}$=11.11, P<0.001). Analysis of the zif268 groups alone revealed no main effect of ASO ($F_{1,12}$=1.05, P=0.32) or Session x ASO interaction ($F_{2,24}$=1.23, P=0.31), whereas analysis of the BDNF groups revealed a significant Session x ASO interaction ($F_{2,20}$=19.95, P<0.001), which was driven by a significant effect of ASO during the LTM and LTM2 tests (simple effects one-way ANOVA; P<0.05), with no effect on the STM test (n=6–7 per group). Data presented as mean ± s.e.m.

Figure 2. (**a**) Knockdown of BDNF, but not zif268, during fear conditioning to a changed context results in subsequent amnesia. Rats were fear conditioned first to CX1 (Cond1), on the next day (Cond2) to CX2, and were tested 24 hr later (Test) in CX2. While ANOVA revealed no gene x ASO interaction during Cond1 (F<1), there was an overall significant Session x Gene x ASO interaction in CX2 ($F_{1,22}$=11.31, P=0.003). Analysis of the zif268 groups alone revealed no main effect of ASO or Session x ASO interaction (F's<1), whereas the BDNF groups had a significant Session x ASO interaction ($F_{1,12}$=22.17, P<0.001), driven by a significant effect of

ASO during the LTM test (simple effects one-way ANOVA; $P<0.05$; n=6–7 per group). (**b**) Proteasome inhibition protects a contextual fear memory against both additional learning and amnesia. Rats were fear conditioned on two consecutive days (Cond1 & Cond2), and were tested 24 hr later (Test). ANOVA revealed a significant Session x ANI x βlac interaction ($F_{2,48}=26.80$, $P<0.001$). There were significant Session x βlac interactions for the ANI ($F_{2,24}=19.24$, $P<0.001$) and VEH ($F_{2,24}=9.16$, $p=0.001$) groups analysed separately, but no differences between the two groups infused with βlac (*F*'s<1). Simple effects analysis (one-way ANOVA; $P<0.05$) revealed that at Test, the βlac groups were significantly different from both the VEH and ANI alone groups, and that while the VEH and ANI alone groups changed in freezing levels from Cond2 to Test, the βlac groups did not (n=7 per group). Data presented as mean ± s.e.m.

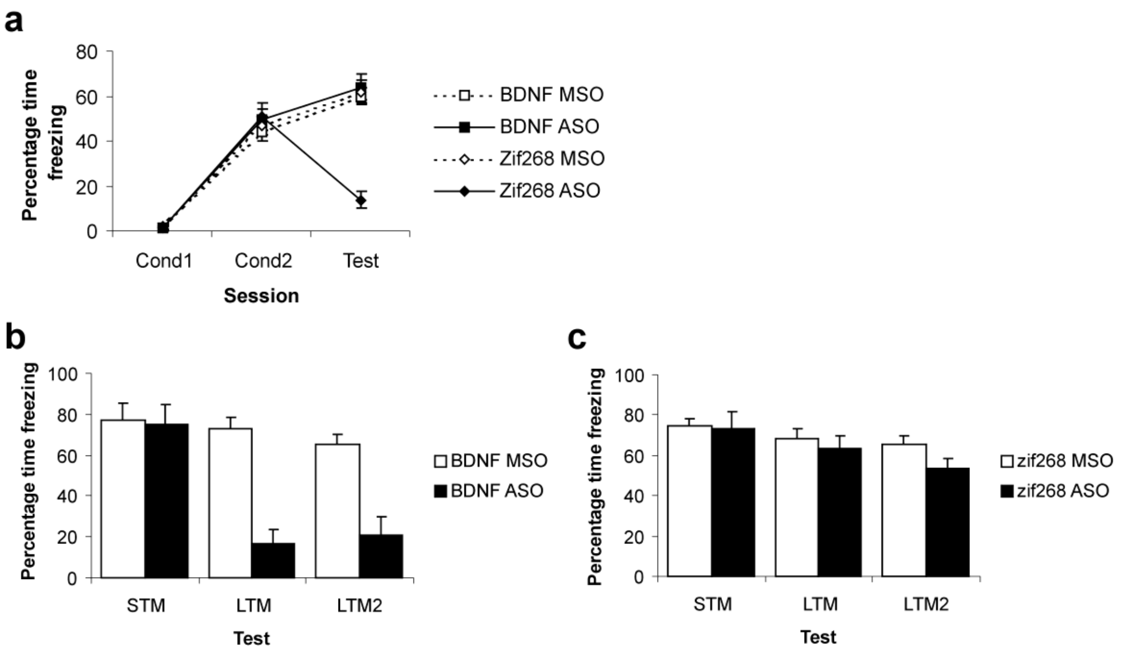


Figure-1 Lee

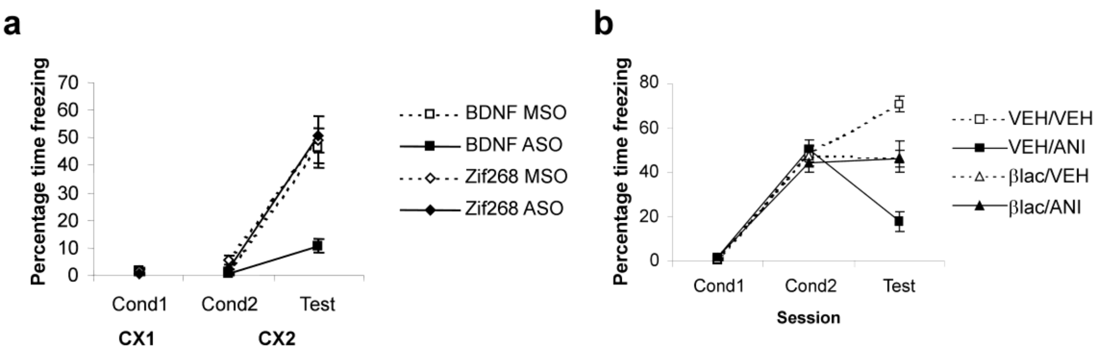
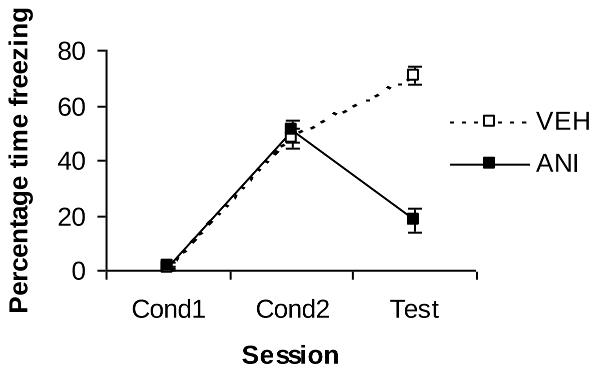
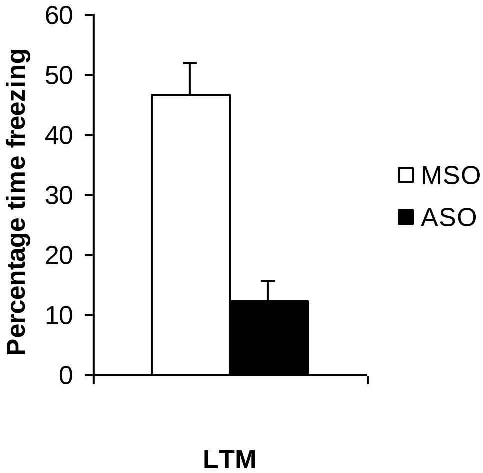


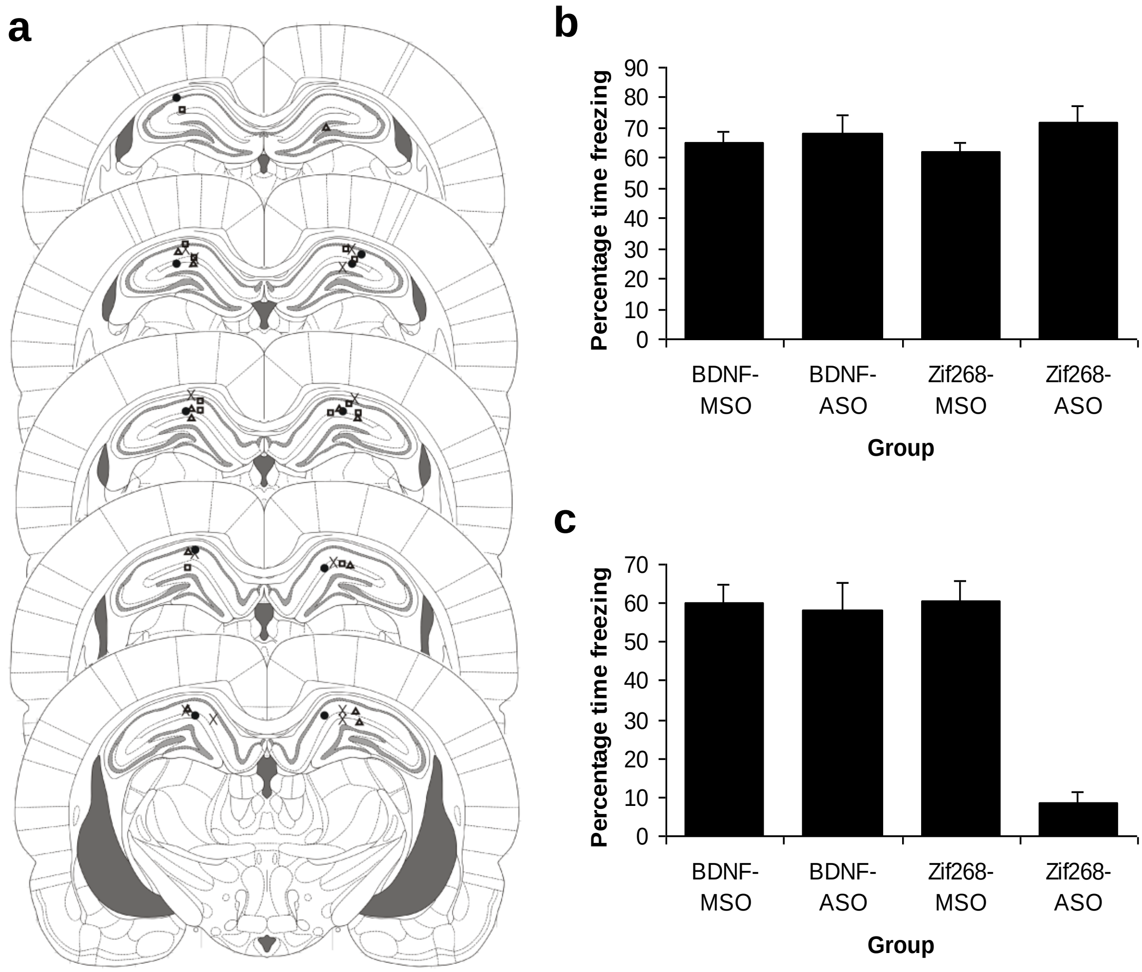
Figure-2 Lee



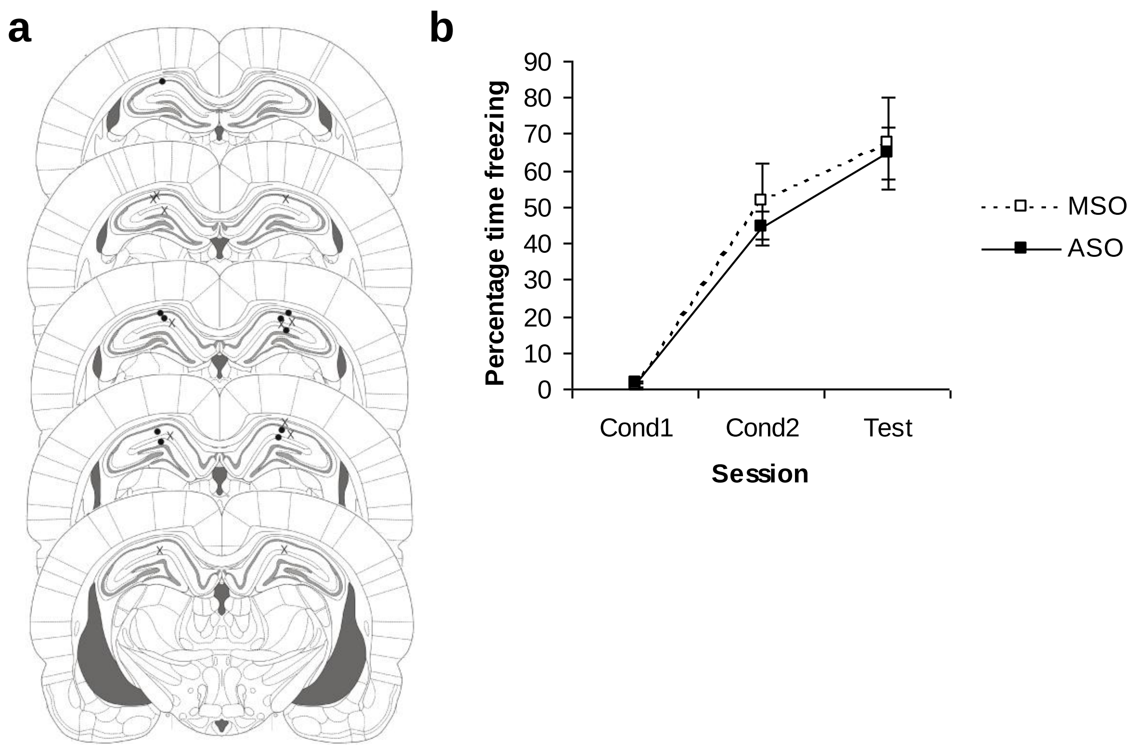
Supplementary Fig. 1. Protein synthesis inhibition after a second contextual fear conditioning session results in subsequent amnesia. Rats were fear conditioned on two consecutive days (Cond1 & Cond2), immediately after the second of which they were infused with anisomycin into the dorsal hippocampus, and then were tested 24 hr later (Test). Repeated measures ANOVA revealed an overall significant Session x ANI interaction ($F_{2,20}$=56.67, P<0.001), which was driven by a significant effect of ANI during the Test (simple effects one-way ANOVA; P<0.05), with no differences during Cond1 and Cond2. VEH controls showed a statistically significant 46.9% increase in freezing from Cond2 to Test ($F_{1,6}$=25.9, P<0.001). Data presented as mean ± s.e.m. (n=7 per group).



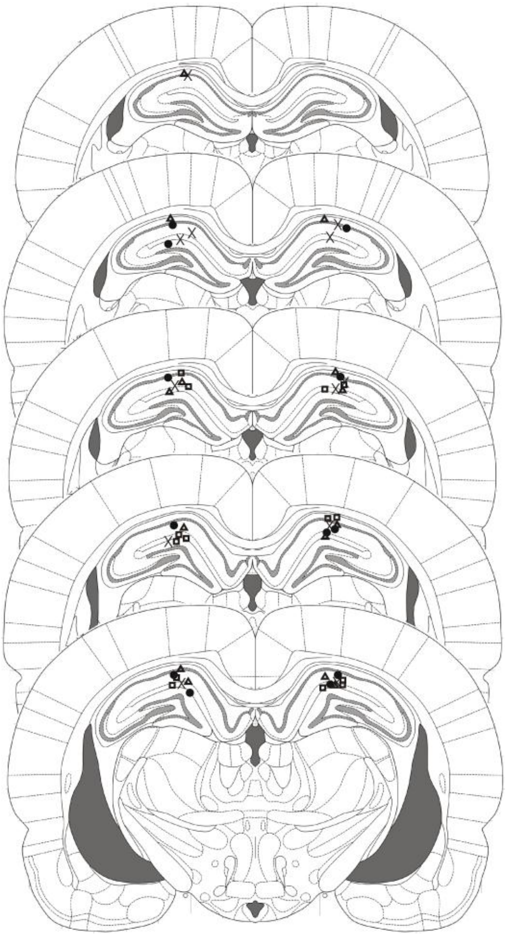
Supplementary Fig. 2. Verification of BDNF ASO efficacy. Infusion of the standard dose of BDNF ASO (1 nmol/μl) into the dorsal hippocampus 90 min before contextual fear conditioning results in severe amnesia 24 hr later (one-way ANOVA: $F_{1,13}$=20.88, P=0.001). Data presented as mean + s.e.m. (n=6 per group).



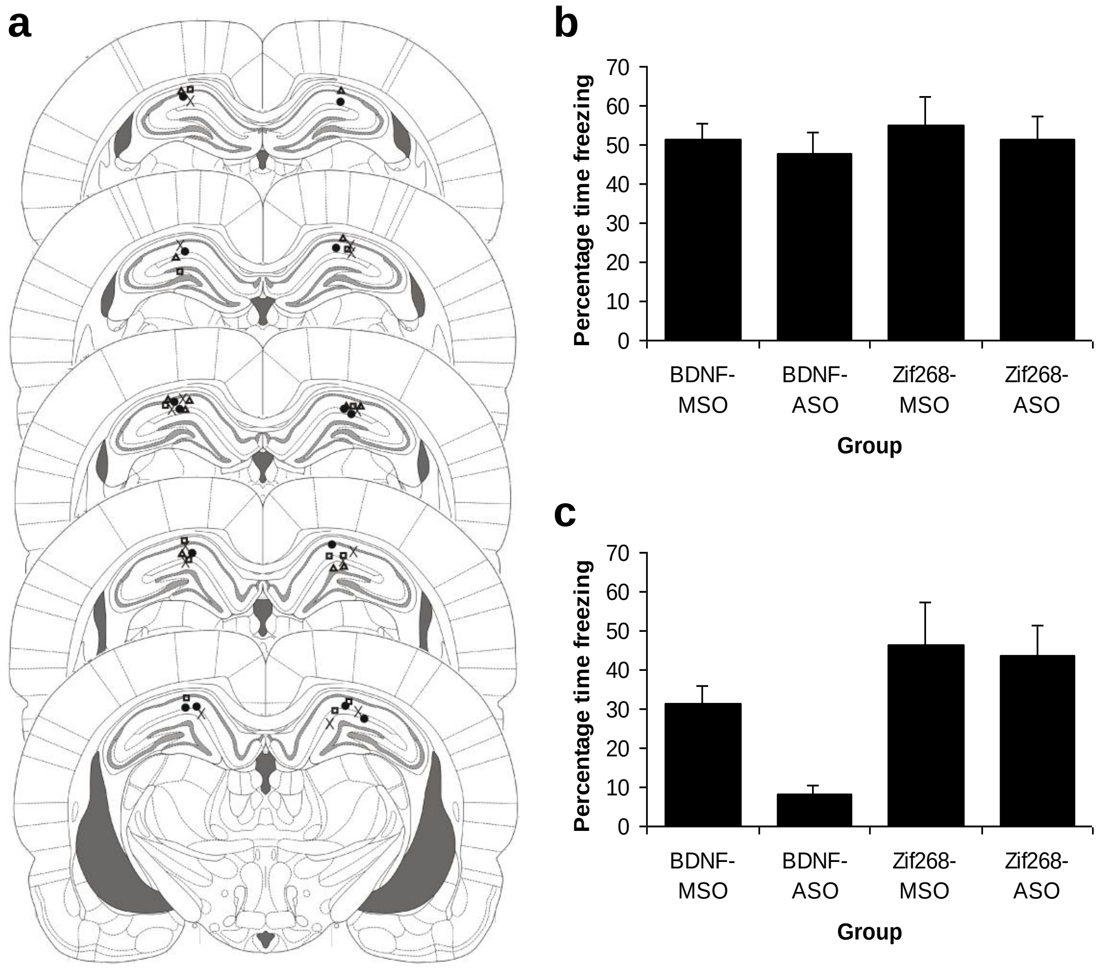
Supplementary Fig. 3. Knockdown of zif268, but not BDNF, during a second conditioning session results in subsequent amnesia (supplement to Figure 1A). (**a**) cannula placements for the rats included in the statistical analysis (●=BDNF MSO; X=BDNF ASO; □=zif268 MSO; ∆=zif268 ASO; Bregma –3.00, –3.24, –3.48, –3.72 & –3.96 mm). (**b**) no amnesia was observed 3 hr after Cond2 (one-way ANOVA: $F<1$). (**c**) rats infused with zif268 ASO remained impaired 7 d later (zif268: one-way ANOVA: $F_{1,10}=91.02$, $P<0.001$; BDNF: one-way ANOVA: $F<1$). Data presented as mean + s.e.m. (n=5–6 per group).



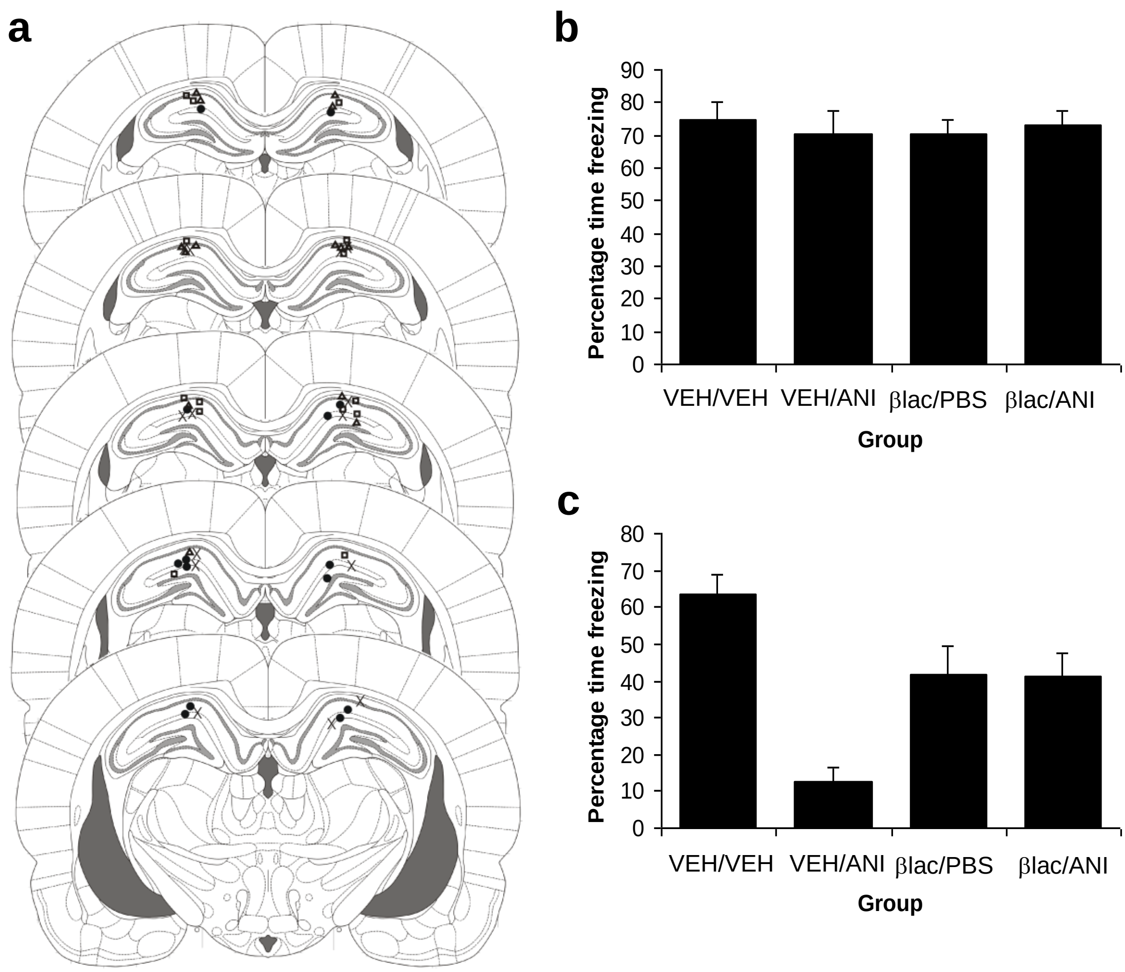
Supplementary Fig. 4. Infusion of a high dose of BDNF ASO before a second conditioning session has no effect on subsequent conditioned freezing. (**a**) cannula placements for the rats included in the statistical analysis (●=BDNF MSO; X=BDNF ASO; Bregma –3.00, –3.24, –3.48, –3.72 & –3.96 mm). (**b**) rats were fear conditioned on two consecutive days (Cond1 & Cond2), 90 min before the second of which they were infused with BDNF ASO or MSO (2 nmol/μl), and then were tested 24 hr later (Test). Repeated measures ANOVA revealed no main effect of ASO, nor a significant Session x ASO interaction (*F*'s<1). Data presented as mean ± s.e.m. (n=5–6 per group).



Supplementary Fig. 5. Cannula placements for the rats included in the statistical analysis of **Fig. 1b,c** (●=BDNF MSO; X=BDNF ASO; □=zif268 MSO; ∆=zif268 ASO; Bregma –3.00, –3.24, –3.48, –3.72 & –3.96 mm).



Supplementary Fig. 6. Knockdown of BDNF, but not zif268, during a fear conditioning to a changed context results in subsequent amnesia (supplement to **Fig. 2a**). (**a**) cannula placements for the rats included in the statistical analysis (●=BDNF MSO; X=BDNF ASO; □=zif268 MSO; Δ=zif268 ASO; Bregma –3.00, –3.24, –3.48, –3.72 & –3.96 mm). (**b**) no amnesia was observed 3 hr after Cond2 (one-way ANOVA: $F<1$). (**c**) rats infused with BDNF ASO remained impaired 7 d later (BDNF: one-way ANOVA: $F_{1,12}=24.27$, $P<0.001$; zif268: one-way ANOVA: $F<1$). Data presented as mean + s.e.m. (n=6–7 per group).



Supplementary Fig. 7. Proteasome inhibition protects a contextual fear memory against both additional learning and amnesia (supplement to **Fig. 2b**). (**a**) cannula placements for the rats included in the statistical analysis (●=PBS; X=ANI; □=βlac/PBS; Δ=βlac/ANI; Bregma –3.00, –3.24, –3.48, –3.72 & –3.96 mm). (**b**) no amnesia was observed 3 hr after Cond2 (one-way ANOVA: $F<1$). (**c**) the pattern of long-term amnesia remained the same 7 d later (one-way ANOVA: $F_{3,24}=14.01$, $P<0.001$; tukey's post-hoc analysis [$P<0.05$] revealed the βlac groups to be different from both other groups, but not to be different from each other). Data presented as mean + s.e.m. (n=7 per group).

Supplementary Text

Methods

Subjects and surgical procedures. 142 adult male Lister Hooded rats, weighing 280-320 g at the time of surgery, were implanted with chronic indwelling cannulae targeting the dorsal hippocampus as described previously[1]. At the end of the experiment, rats were perfused, and their brains were sectioned and stained to confirm cannula placements as previously described[1]. Cannula placements are shown on reproduced atlas figures[2]. 10 rats were excluded from the statistical analysis due to misplaced cannulae or failure to complete the full schedule of testing.

Infusions. Infusion procedures were as previously described[1]. Rats were habituated to the infusion procedure using the PBS vehicle on 2 occasions prior to behavioural training. Infusions (1 μl, 0.5 μl/min) were carried out of oligodeoxynucleotides (sequences and concentrations as previously used[1]), or PBS/DMSO vehicle, anisomycin (80 μg/μl), clasto-lactacystin-β-lactone (βlac; 32 ng/μl) and a combination of anisomycin and βlac prepared as previously described[3].

Behavioural procedures. Training and testing took place in 4 operant chambers as previously described[4]. The rats were subjected to a previously described contextual fear conditioning procedure[1]. For the memory strengthening experiments, two minutes after being placed singly in the chambers, the rats were exposed to a single unsignalled footshock (0.5 mA, 2 s), 60 s after which the session terminated. On the next day the rats received intra-hippocampal infusions either 90 min before (oligodeoxynucleotides) or immediately after (anisomycin/□lac) an identical training trial, and then were tested repeatedly in 2-min context re-exposure sessions at three subsequent timepoints: 3 hr (STM), 24 hr (LTM test) & 7 d (LTM2). For the stronger conditioning experiment, rats were given intra-hippocampal infusions of oligodeoxynucleotides 90 min prior to an extended 4-min conditioning session in which they received 2 unsignalled footshocks after 2 and 3 mins, and then were tested as before. Behaviour was video recorded for later analysis of conditioned freezing by an experimenter blind to the experimental conditions as previously described[1].

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