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A critical role for IGF-II in memory consolidation and enhancement

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We report that, in the rat, administering insulin-like growth factor II (IGF-II, also known as IGF2) significantly enhances memory retention and prevents forgetting. Inhibitory avoidance learning leads to an increase in hippocampal expression of IGF-II, which requires the transcription factor CCAAT enhancer binding protein β and is essential for memory consolidation. Furthermore, injections of recombinant IGF-II into the hippocampus after either training or memory retrieval significantly enhance memory retention and prevent forgetting. To be effective, IGF-II needs to be administered within a sensitive period of memory consolidation. IGF-II-dependent memory enhancement requires IGF-II receptors, new protein synthesis, the function of activity-regulated cytoskeletal-associated protein and glycogen-synthase kinase 3 (GSK3). Moreover, it correlates with a significant activation of synaptic GSK3β and increased expression of GluR1 (also known as GRIA1) α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid receptor subunits. In hippocampal slices, IGF-II promotes IGF-II receptor-dependent, persistent long-term potentiation after weak synaptic stimulation. Thus, IGF-II may represent a novel target for cognitive enhancement therapies.

Elucidating the mechanisms of memory enhancement is critical for the development of cognitive enhancement therapies. Memory strengthening and persistence depend on consolidation, a process whereby newly learned information, which is initially labile, becomes stronger and resilient to disruption[1]. This process recruits evolutionarily conserved *de novo* RNA and protein syntheses, the function of members of the cAMP response element binding protein (CREB) and CCAAT enhancer binding protein (C/EBP) transcription factor families[2–4], and correlates with synaptic structural changes[2,5]. Stable memories can again become fragile if retrieved, and undergo a process of reconsolidation that, like the initial consolidation, requires *de novo* RNA and protein synthesis, CREB and C/EBP[6,7] to re-stabilize[8]. The identity of the target genes regulated by CREB and C/EBP is still largely unknown. Studies in liver and other tissues show that C/EBP binding sites are present in promoters of IGF-II[9], a growth factor that is expressed in the brain but is still poorly characterized.

IGF-II is a mitogenic polypeptide, which together with insulin and insulin-like growth factor 1 (IGF-I, also known as IGF1) belongs to the IGF/IGFBP (IGF/IGF binding protein) system. This system is important in normal somatic growth and development, tissue repair and regeneration throughout the lifespan[10,11]. IGF-II, the less characterized member of the family, is expressed in the brain both during development and in adulthood and declines with ageing[12]. In the adult brain, it is the most abundantly expressed of the IGFs[10], and its relative concentration is highest in the hippocampus[13]. Given that IGF-II is a putative C/EBP target gene, we investigated its expression and functional role in memory formation.

C/EBPβ-dependent IGF-II expression is regulated by training

In previous studies, we showed that inhibitory avoidance (IA) training induces a significant increase in hippocampal C/EBPβ, which starts between 6 and 9 h after training, lasts for at least 28 h and returns to baseline by 48 h after training[14] (Supplementary Fig. 1). Here we tested whether C/EBPβ regulates the expression of the putative target gene IGF-II. The numeric values, and number of animals per group (*n*) of all experiments are shown in Supplementary Tables. Northern blot analysis showed that, compared to controls that were exposed to the box without foot shock and either euthanized immediately after (0 h−) or at paired time points (no shock, −), the hippocampal expression of *IGF-II* mRNA did not change at 6 and 9 h but increased significantly at 20 h and had a strong trend towards an increase at 36 h after training (Fig. 1a).

Quantitative PCR with reverse transcription (qRT–PCR) analyses of mRNA extracts confirmed the significant increase of *IGF-II* mRNA 20 h after training compared to no-shock and 0 h− controls whereas, in the same extracts, *IGF-I* mRNA remained unchanged (Fig. 1b).

Quantitative western blot analyses with an anti-IGF-II antibody that specifically recognizes IGF-II and not IGF-I (Supplementary Fig. 2) showed that hippocampal levels of IGF-II protein significantly increased at 20, but not at 72 or 96 h after training, compared to both time-matched unpaired and 0 h− controls (Fig. 1c). The unpaired control protocol temporally dissociates, within subject, context and foot shock exposure by 1 h, and does not produce long-term IA memory (Supplementary Fig. 3). Thus, IA training leads to an increase in IGF-II that temporally overlaps that of C/EBPβ[14].

We next investigated whether the IGF-II increase requires C/EBPβ. Previous studies have established that hippocampal bilateral injection of C/EBPβ antisense oligodeoxynucleotide (β-ODN), 5 h after IA training, blocks the training-dependent C/EBPβ induction and completely disrupts memory consolidation[15]. Using this injection protocol and quantitative western blot analyses we found that, compared to control scrambled ODN (SC-ODN), β-ODN completely disrupted the training-induced IGF-II increase without changing the IGF-II expression in unpaired control rats 24 h after training (Fig. 1d). Chromatin immunoprecipitation of hippocampal extracts confirmed that C/EBPβ binds *in vivo* to a C/EBPβ consensus sequence in the promoter region of the rat IGF-II exon 1 (Supplementary Fig. 4). Thus,

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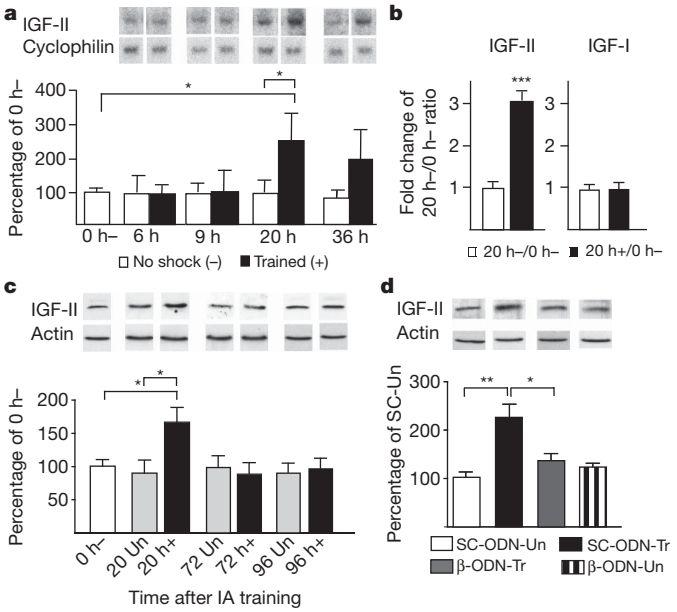

Figure 1 | C/EBPβ-dependent IGF-II expression significantly increases following training. **a**, Northern blot examples and densitometric analyses of IGF-II (cyclophilin-normalized). Data are expressed as mean percentage ±s.e.m. of 0 h− (one-way ANOVA comparing all groups $F_{8,59} = 2.46$, $P = 0.0249$, post hoc *t*-test $^{*}P < 0.05$). **b**, Real-time PCR of hippocampal IGF-II and IGF-I mRNA (18S RNA-normalized). Data are expressed as mean fold change ±s.e.m. of 20 h−/0 h− (Student's *t*-test $^{***}P < 0.0001$). **c**, Western blot analyses of hippocampal IGF-II from 0 h−, unpaired (Un) and trained (+) rats euthanized 20, 72 or 96 h later (actin-normalized). Data are expressed as mean percentage ±s.e.m. of 0 h− (one-way ANOVA comparing 0 h−, 20 Un and 20 h+ $F_{2,29} = 4.69$, $P = 0.0172$, Newman–Keuls post hoc test, $^{*}P < 0.05$). **d**, Western blot analysis of hippocampal IGF-II from trained or Un rats injected with either SC-ODN or β-ODN 5 h post-training and euthanized 24 h post-training (actin-normalized). Data are expressed as mean percentage ±s.e.m. of SC-ODN-Un (two-way ANOVA $F_{1,19} = 4.62$, $P = 0.0447$ for interaction, $F_{1,19} = 1.45$, $P = 0.2434$ for ODN-treatment, $F_{1,19} = 6.46$, $P = 0.0199$ for training-paradigm, Bonferroni post hoc $^{**}P < 0.01$;$^{*}P < 0.05$).

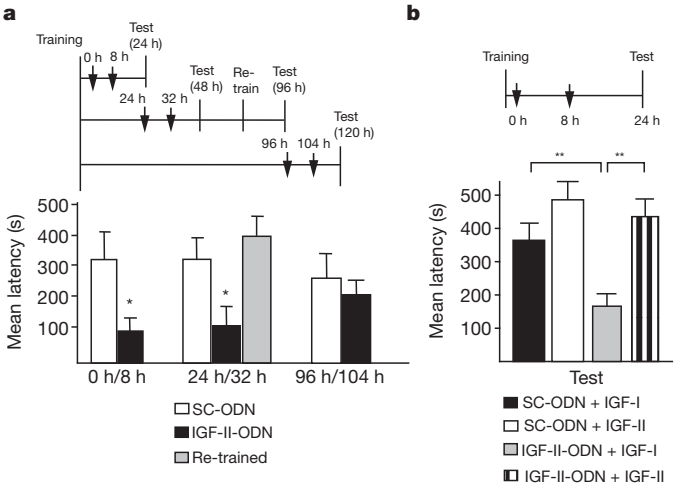

Figure 2 | Hippocampal IGF-II is required for memory consolidation. Schedules shown above figures. **a**, Mean latency ±s.e.m. of rats given double hippocampal injections (↓) of SC-ODN or IGF-II-ODN (one-way ANOVA for treatment $F_{5,47} = 2.54$, $P = 0.043$, post hoc Student's *t*-test $^{*}P < 0.05$ for 0 h/8 h and 24 h/32 h). **b**, Mean latency ±s.e.m. of rats given double hippocampal injections (↓) of SC-ODN or IGF-II-ODN with either IGF-II or IGF-I (two-way ANOVA $F_{1,33} = 4.29$, $P = 0.0468$ for interaction, $F_{1,31} = 6.34$, $P = 0.173$ for ODN-treatment, $F_{1,31} = 11.38$, $P < 0.0021$ for IGF-treatment, Bonferroni post hoc $^{**}P < 0.01$).

IA training leads to an increase in hippocampal C/EBPβ that regulates a downstream increase in IGF-II.

Limited temporal requirement of IGF–II during memory consolidation

We then investigated the functional kinetic of hippocampal IGF-II during IA memory consolidation. Bilateral injections of IGF-II ODN antisense (IGF-II-ODN) were used to selectively knockdown the IGF-II expression in the dorsal hippocampus. Injection either immediately or 8 h after training, or at both time points, showed that double, but not single, injections of IGF-II-ODN significantly disrupted memory retention at 24 h after training, compared to SC-ODN (Fig. 2a, Supplementary Fig. 5). Quantitative RT–PCR confirmed that, compared to SC-ODN, double injections of IGF-II-ODN selectively and significantly decreased the levels of *IGF-II*, but not of *IGF-I* mRNA, 16 h after training (Supplementary Fig. 6). IGF-II-ODN doubly injected at 24 and 32 h after training, compared to SC-ODN, significantly disrupted memory retention at 48 h after training (Fig. 2a) and re-training of the amnesic rats resulted in normal memory retention 24 h after re-training (Fig. 2a), thus excluding hippocampal damage or non-specific effects. However, IGF-II-ODN doubly injected at 96 and 104 h after training did not affect memory retention 24 h later (Fig. 2a). The amnesia caused by IGF-II-ODN double injections was rescued by the co-administration of recombinant IGF-II, but not IGF-I (Fig. 2b), further proving that IGF-II expression is essential for IA memory consolidation. Furthermore, whereas IGF-I had no effect (compare SC-ODN/IGF-I from Fig. 2b and SC-ODN from Fig. 2a), IGF-II seemed to enhance memory retention, although the effect was not significant, possibly because the testing latency was cut-off at 540 s.

We concluded that hippocampal IGF-II has a critical role for IA memory consolidation during a limited time window that lasts for more than one but less than 4 days.

IGF–II significantly enhances memory and prevents forgetting

Because of the tendency towards memory enhancement in our IGF-II rescue experiment (Fig. 2b), we tested whether exogenously administered IGF-II into the hippocampus immediately after training modulates memory strength. The latency cut-off time was raised to 900 s. Bilateral injections of IGF-II immediately after training significantly and persistently enhanced memory retention at 24 h and 7 days, compared to IGF-I or vehicle (Fig. 3a). This enhancement was not due to a non-specific locomotor effect (Supplementary Fig. 7).

The IGF-II-mediated memory enhancement was dose-dependent (Supplementary Fig. 8): hippocampal injections of 25 or 2.5 ng, like 250 ng, immediately after training incrementally enhanced memory retention at 24 h.

Finally, hippocampal injection of IGF-II immediately after training significantly enhanced memory retention tested 3 weeks later when the latency of vehicle-injected rats was not significantly different from acquisition, indicating that IGF-II prevents forgetting (Fig. 3b).

The IGF-II effect generalized to another memory task, contextual fear conditioning. Bilateral hippocampal injection of IGF-II immediately after contextual-auditory fear conditioning training significantly enhanced contextual fear conditioning retention 24 h later, without affecting auditory fear conditioning tested 48 h after training (Fig. 3c). No difference in baseline freezing was found between groups before foot shock delivery (Fig. 3c).

Finally, because IA consolidation also critically involves the amygdala[16], we tested the effect of bilateral IGF-II injections into the amygdala immediately after training, but found no effect at testing 24 h later (Fig. 3d). Hence, IGF-II in the hippocampus acts as a strong memory enhancer and also prevents forgetting.

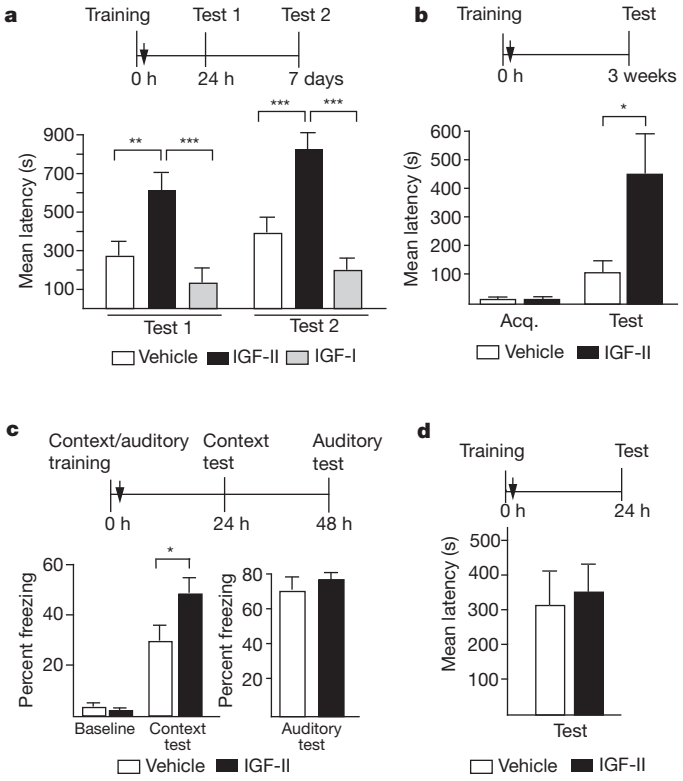

Figure 3 | Hippocampal post-training IGF-II administration enhances memory and prevents forgetting. Schedules shown above figures. **a**, Mean latency ±s.e.m. of trained rats given hippocampal injection (↓) of vehicle, IGF-II or IGF-I and tested 24 h and 7 days later (two-way ANOVA $F_{2,38} = 0.44$, $P = 0.6463$ for interaction, $F_{2,38} = 26.7$, $P < 0.0001$ for treatment, $F_{1,38} = 4.24$, $P = 0.0466$ for test, Bonferroni post hoc test $^{**}P < 0.01$, $^{***}P < 0.001$). **b**, Mean latency ±s.e.m. of trained rats given an hippocampal injection (↓) of vehicle or IGF-II (acq: acquisition latency; Student's *t*-test $^{*}P = 0.0261$). **c**, Mean percentage freezing of trained rats injected with vehicle or IGF-II (Student's *t*-test $^{*}P < 0.0434$). **d**, Mean latency ±s.e.m. of trained rats given bilateral amygdala injection (↓) of vehicle or IGF-II.

IGF-II-mediated memory enhancement: effect on reconsolidation

An established memory, resilient to disruption, becomes again labile and undergoes another protein-synthesis-dependent reconsolidation process if retrieved[17,18]. Bilateral hippocampal injection of IGF-II 24 h after training had no effect on memory retention tested at 48 h (Fig. 4a). However, if 24 h after training IGF-II was given after memory retrieval (Test 1), memory retention was significantly enhanced 24 h later (Final test, Fig. 4a).

Studies in IA and other types of learning[19–22], but not all[8,23], have shown that reconsolidation is temporally limited. IA memory undergoes protein-synthesis-dependent reconsolidation if retrieved 2 or 7 days after training but not 2 or 4 weeks after training[20]. Hence, we asked whether the enhancing effect of IGF-II is also temporally restricted, and coincides with the reconsolidation-sensitive temporal window. Bilateral hippocampal injection of IGF-II immediately after retrieval (Test 1), 2 weeks after training, did not change memory retention tested 1 day later, compared to vehicle (Final test, Fig. 4b). Hence, hippocampal IGF-II-mediated memory enhancement occurs only within the temporal window during which IA memory undergoes reconsolidation.

Mechanisms underlying IGF-II-mediated memory enhancement

IGF-II activates both IGF-I and IGF-II receptors, but with different affinity[24]. To determine whether IGF-II-mediated memory enhancement recruits one or both of these receptors, we tested the effect of

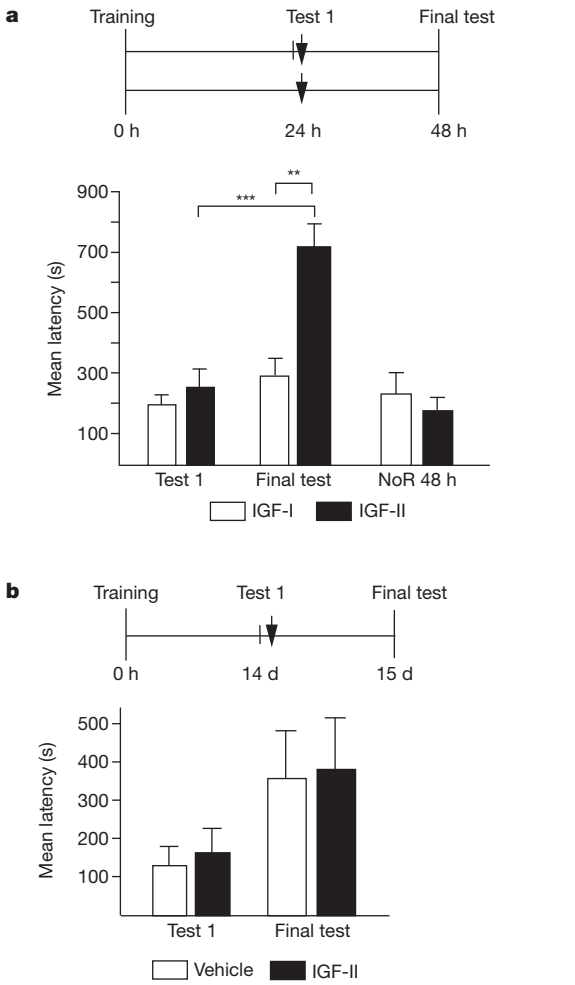

Figure 4 | Post-retrieval IGF-II administration enhances memory and the effect is temporally limited. Schedules shown above figures. **a**, Mean latency ±s.e.m. of trained rats, tested 24 h post-training and, immediately after, injected (↓) with IGF-II or IGF-I. Non-reactivated rats (NoR) were trained and injected (↓) without testing. Rats were tested 48 h post-training (two-way ANOVA $F_{1,26} = 5.67$, $P = 0.0249$ for interaction, $F_{1,26} = 9.82$, $P = 0.0042$ for treatment, $F_{1,26} = 13.67$, $P = 0.0001$ for test, Bonferroni post hoc $^{**}P < 0.01$, $^{***}P < 0.001$). **b**, Mean latency ± s.e.m. of trained rats, tested 14 days post-training and, immediately after, injected (↓) with vehicle or IGF-II; memory was tested 15 days after training.

IGF-I and IGF-II receptor (R) selective inhibitors. Specific inhibitors of IGF-IIR (anti-IGF-IIR antibody) but not IGF-IR (JB1) co-injected with IGF-II completely abolished the memory enhancement compared to respective controls (Fig. 5a). The inhibitors alone did not affect memory retention (Fig. 5a).

Similarly to the antisense experiments, compared to control IgG, a single bilateral hippocampal injection of anti-IGF-IIR antibody immediately after training did not affect memory retention (Fig. 5a), whereas double injections, immediately and 8 h after training, caused a complete amnesia 24 h after training (Fig. 5b).

We next asked whether IGF-II-mediated memory enhancement recruits new protein synthesis. Because memory consolidation *per se* requires new protein synthesis in the hippocampus, blocking protein synthesis in IGF-II-injected rats after training would not be informative. However, because new protein synthesis is not required in the hippocampus for IA reconsolidation[15], we tested the effect of protein synthesis inhibition on retrieval-dependent IGF-II-mediated memory enhancement.

Bilateral hippocampal co-injection of IGF-II and the protein synthesis inhibitor anisomycin immediately after Test 1, 24 h after training,

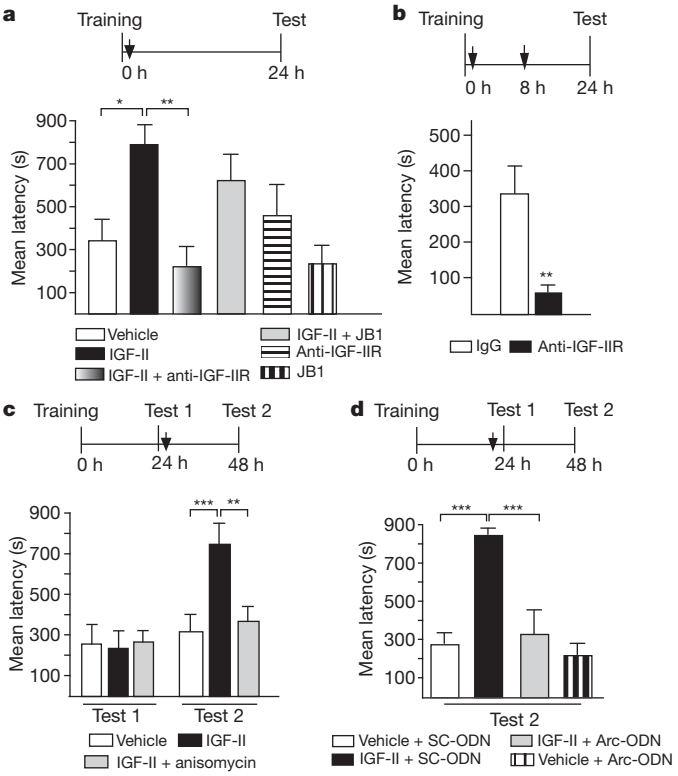

Figure 5 | The role of IGF-II receptors, *de novo* protein synthesis and Arc in memory consolidation and IGF-II-mediated enhancement. Schedules shown above figures. **a**, Mean latency ±s.e.m. of trained rats injected (↓) with vehicle, IGF-II, IGF-II/anti-IGF-IIR, IGF-II/JB1, anti-IGF-IIR or JB1 (one-way ANOVA $F_{5,40} = 3.82$, $P = 0.0023$, Newman–Keuls post hoc test $^{*}P < 0.05$ $^{**}P < 0.01$). **b**, Mean latency ±s.e.m. of trained rats given double injections of IgG or anti-IGF-IIR antibody (Student's *t*-test $^{**}P < 0.0041$). **c**, Mean latency ±s.e.m. of rats trained, tested then injected (↓) with vehicle, IGF-II or IGF-II + anisomycin (two-way ANOVA $F_{2,34} = 5.25$, $P = 0.0103$ for interaction, $F_{2,34} = 4.68$, $P = 0.0161$ for treatment, $F_{1,34} = 13.7$, $P = 0.0008$ for test, Bonferroni post hoc $^{**}P < 0.01$,$^{***}P < 0.001$). **d**, Mean latency ±s.e.m. of rats trained, tested and injected (↓) with vehicle + SC-ODN, vehicle + Arc-ODN, IGF-II + SC-ODN, or IGF-II + Arc-ODN (two-way ANOVA $F_{1,18} = 7.8$, $P = 0.0119$ for interaction, $F_{1,18} = 17.3$, $P = 0.0006$ for ODN-treatment, $F_{1,18} = 12.3$, $P = 0.0025$ for vehicle + IGF-II treatment, Bonferroni post hoc $^{***}P < 0.001$).

showed that anisomycin, compared to vehicle, completely disrupted the IGF-II-mediated memory enhancement tested 24 h later (Fig. 5c) without changing the training-induced retention levels. Hence, memory enhancement, but not reconsolidation, requires hippocampal *de novo* protein synthesis.

To begin identifying which proteins are required for the memory enhancement, we investigated the role of C/EBPβ. Bilateral hippocampal injection of β-ODN 5 h after retrieval (Test 1) did not affect the IGF-II-mediated memory enhancement tested 48 h after training (Supplementary Fig. 9). The timing of the ODN injections was based on previous kinetics studies showing maximal disruptive effect of β-ODN[7,15]. To test whether a prolonged β-ODN treatment could affect the post-retrieval IGF-II-mediated memory enhancement we injected β-ODNs at both 1 h before and 5 h after reactivation. This treatment, compared to control SC-ODN, also failed to affect the IGF-II-mediated memory enhancement (Supplementary Fig. 9), indicating that, although *de novo* protein synthesis is critical for memory enhancement, C/EBPβ is not.

We therefore hypothesized that the protein synthesis-mediated enhancement may recruit synaptic rather than cell-wide, transcriptional mechanisms. One rapidly regulated translation known to occur at activated synapses and critical for long-term plasticity and memory is that of activity-regulated cytoskeletal-associated protein (Arc)[25]. Bilateral hippocampal injection of *Arc* antisense (Arc-ODN), compared to relative SC-ODN, 1 h before retrieval (Test 1), completely blocked the post-retrieval IGF-II-mediated memory enhancement, without affecting the basal level of the memory 2 days after training (Fig. 5d).

Thus, IGF-II-mediated enhancement requires IGF-II, but not IGF-I receptors. Furthermore, retrieval-dependent IGF-II-mediated enhancement requires *de novo* protein synthesis and Arc but not C/EBPβ, indicating that it may use synaptic rather than cell-wide-regulatory mechanisms.

Memory consolidation requires the CREB-C/EBP-dependent gene cascade[2]. In IA, both CREB phosphorylation at Ser 133 (pCREB) and the expression of C/EBPβ are significantly increased in the hippocampus for more than 20 h after training[14]. We examined whether IGF-II-mediated memory enhancement following training correlates with an enhanced hippocampal activation of the CREB-C/EBP pathway. Quantitative western blot analyses confirmed that training significantly increased both pCREB and C/EBPβ levels in the hippocampus 20 h later[14] (trained-vehicle vs naive-vehicle, Fig. 6a). Compared to vehicle, IGF-II treatment immediately after training resulted in only a tendency towards a further increase in both markers (Fig. 6a). Thus, IGF-II-mediated memory enhancement does not correlate with significant enhancement in the activation of the CREB-C/EBP cascade, strengthening our hypothesis that IGF-II-regulated mechanisms may be synaptic rather than cell-wide. We therefore investigated the synaptic expression levels of GluR1 and GluR2 (also known as GRIA2) AMPA receptor subunits. Synaptic GluR1 AMPA receptors levels have been shown to increase rapidly following IA training and have a critical role in consolidation[26,27]. Furthermore, AMPA receptor subunit synaptic trafficking is known to accompany both long-term potentiation (LTP) and long-term depression (LTD)[28].

Quantitative western blot analyses of synaptoneurosomal extract (see Supplementary Fig. 10 for the biochemical characterization) revealed that there was an increase in synaptic GluR1 30 min after training compared to naive, which, however, was not significant, probably due to the relatively low shock intensity used (Fig. 6b). Importantly, synaptic GluR1 levels were significantly increased in trained rats treated with IGF-II compared to vehicle. This increase was completely abolished by anti-IGF-IIR antibody. On the other hand, GluR2 levels remained unchanged across groups.

Previous studies reported that AMPA receptor trafficking and dendritic expression of GluR1 in neurons are regulated by GSK3 (ref. 29) and, interestingly, IGF-II has been implicated in GSK3 regulation[30]. As depicted in Fig. 6c, the IGF-II-mediated significant increase of GluR1 was paralleled by a significant synaptic activation of GSK3β (measured by its dephosphorylation at Ser 9, ref. 31), which was also completely abolished by anti-IGF-IIR antibody. Furthermore, whereas blocking hippocampal GSK3 function with pretraining injection of the inhibitor SB216763 completely disrupted IA memory retention (data not shown), the same treatment delivered immediately after retrieval (Test 1) selectively eliminated the IGF-II-mediated enhancement tested 2 days after training (Test 2) without affecting memory reconsolidation (Fig. 6d).

Thus, IGF-II-dependent memory enhancement requires the activation of GSK3β and correlates with increased synaptic expression of GluR1.

IGF–II facilitates long–term potentiation (LTP)

To determine whether the effect of IGF-II was generalized to long-term synaptic plasticity, we tested the effect of IGF-II on hippocampal LTP, which is widely regarded as a cellular correlate of long-term memory[32]. IGF-II was applied to acute hippocampal slices and both LTP in the Schaffer collateral pathway and basal synaptic transmission were investigated. As shown in Fig. 6e, a weak high-frequency stimulation (wHFS) elicited a transient synaptic potentiation that decayed to baseline within 100 min after induction (slope = 109.4 ± 9.7%, calculated

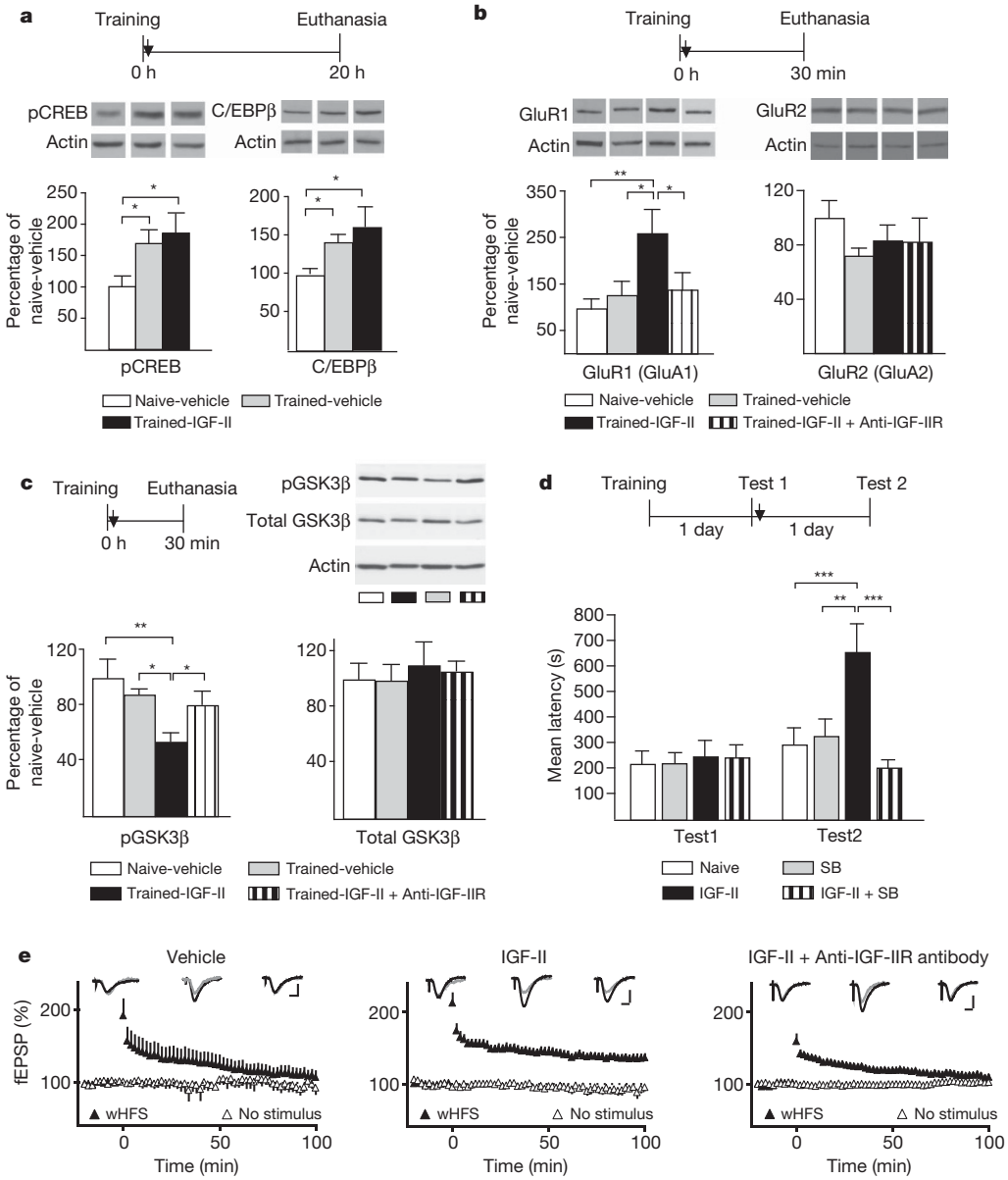

Figure 6 | Mechanisms of IGF-II-mediated memory enhancement. IGF-II promotes LTP. a, Western blot analysis of hippocampal pCREB and C/EBPβ from naive or trained rats injected (↓) with vehicle or IGF-II and euthanized 20 h later (actin-normalized). Data are expressed as mean percentage ±s.e.m. of naive-vehicle (one-way ANOVA, pCREB: $F_{2,20} = 4.3$, $P = 0.0287$, C/EBPβ: $F_{2,19} = 5.7$, $P = 0.0117$, Newman–Keuls post hoc test, $^{*}P < 0.05$). **b**, Western blot analysis of hippocampal GluR1 and GluR2 from naive or trained rats injected (↓) with vehicle, IGF-II, IGF-II + anti-IGF-IIR antibody (actin-normalized). Data are expressed as mean percentage ±s.e.m. of naive-vehicle (one way ANOVA $F_{3,19} = 4.24$, $P = 0.0188$, Newman–Keuls post hoc test $^{*}P < 0.05$, $^{**}P < 0.01$). **c**, Western blot analysis of hippocampal pGSK3β and GSK3β from the same extracts as in **b** (actin normalized). Data are expressed as percentage ±s.e.m. of naive-vehicle (one-way ANOVA $F_{3,19} = 4.93$, $P = 0.130$, Newman–Keuls post hoc test $^{*}P < 0.05$,$^{**}P < 0.01$). **d**, Mean latency ±s.e.m. of rats trained, tested and injected (↓) with vehicle, IGF-II, SB216763 (SB) or IGF-II + SB (two-way ANOVA $F_{3,56} = 4.44$, $P = 0.0072$ for interaction, $F_{3,56} = 5.07$, $P = 0.0035$ for treatment, $F_{1,56} = 9.12$, $P = 0.0038$ for test, Bonferroni post hoc $^{**}P < 0.01$,$^{***}P < 0.001$). **e**, Time-courses of field excitatory postsynaptic potentials (fEPSPs) in area CA1 stratum radiatum are shown with sample traces obtained during the baseline period, 2 min and 100 min after the delivery of weak high frequency stimulation (wHFS) (grey traces, no wHFS; black traces, wHFS). Scale bars, 0.5 mV (*y*-axis) and 5 ms (*x*-axis), respectively. Left panel, in vehicle-treated slices, wHFS induced only transient potentiation that returned to baseline levels within 100 min. Middle panel, in the presence of IGF-II, the same protocol induced stable LTP (Student's *t*-test $P < 0.05$). Right panel, in slices pretreated with antibodies against the IGFII receptor, IGF-II failed to facilitate the induction of stable LTP.

as the average of the final 10 min of recording normalized to the full baseline period for each slice). When this weak stimulus was delivered in the presence of IGF-II, stable LTP was expressed (slope = 135.2 ± 6.6% of baseline). This enabling effect was completely blocked in slices that were pretreated with anti-IGF-IIR antibody (112.05 ± 4.7%) (Fig. 6e). Neither IGF-II nor the anti-IGF-IIR antibody affected basal synaptic transmission (Supplementary Figs 11 and 12).

Discussion

Our study shows that memory retention can be enhanced, LTP promoted and forgetting prevented by the administration of IGF-II, a growth factor physiologically regulated following learning. IGF-II is endogenously upregulated following learning as a C/EBPβ target gene and required in the hippocampus for memory consolidation during the first 1-2 days after training but not at later times, extending previous

conclusions[15] that the transcription- and translation-dependent phase of IA consolidation in the dorsal hippocampus lasts for more than one, but less than 4 days.

The effect of IGF-II as memory enhancer is temporally restricted to active phases induced by either learning or memory retrieval, generalized to different types of hippocampal-dependent memories and occurs with an acute treatment in low doses.

The training-related IGF-II-dependent memory enhancement is restricted to a temporal window that lasts less than a day. However, at later times, the enhancing effect re-emerges if IGF-II is given in combination with memory retrieval, which is known to reactivate the memory and induce reconsolidation[17]. The IGF-II effect following retrieval is also temporally limited and, in fact, restricted to a temporal window that overlaps with the reconsolidation sensitive period of IA[7,15,20]. Both retrieval-induced memory fragility and IGF-II-dependent enhancement require new protein synthesis but in different brain regions: the former in the amygdala[7], the latter in the hippocampus. Hence, during the first 1–2 weeks after training, IA memory is in a sensitive period during which, if in an active state, it can be either significantly weakened or enhanced. These findings strengthen our previously proposed hypothesis that reconsolidation is a phase of a lingering consolidation process[17,33]. We speculate that the retrieval-induced memory fragility mediated by amygdala mechanisms may be critical for promoting memory enhancement mediated by hippocampal mechanisms and that the temporal boundary of the sensitive period may reflect the hippocampal-cortical redistribution of memory storage[34,35] or the multiple trace distribution of memory[36].

Intriguingly, the effect of IGF-II as memory enhancer is selectively mediated by IGF-II and not IGF-I receptors. IGF-IIR is identical to the mannose-6-phosphate (M6P) receptor[11] and has a role in lysosomal enzyme trafficking, clearance and endocytosis-mediated degradation of IGF-II and possibly in transmembrane-receptor-mediated signal transduction[11] but, in general, little is known about its function in the brain.

IGF-II-mediated memory enhancement is not paralleled by significant activation of pCREB or C/EBPβ and does not functionally require C/EBPβ, but critically depends on GSK3 and Arc and is accompanied by a significant increase in synaptic GSK3β activation and GluR1 expression. Because C/EBPβ is significantly upregulated for more than 28 h after training[14], it is possible that this induction is sufficient to also mediate the memory enhancement. Alternatively, the enhancement may use mechanisms either downstream of C/EBPβ or distinct from those mediating consolidation. Thus, the IGF-II-dependent enhancement might not recruit the activation of new cells, but rather uses those that have been transcriptionally 'marked' by training and target synaptic mechanisms, possibly those at activated synapses. One of these mechanisms might be GSK3-regulated GluR1 synaptic mobilization, a hypothesis in line with previous reports of functional links between dendritic trafficking of GluR1 and GSK-3 (ref. 29), as well as between GluR1 and Arc expression, synaptic plasticity and memory consolidation[25]. We cannot exclude that IGF-II-dependent memory enhancement may occur via recruitment of new cell activation, which however would be independently from the activation and function of CREB-C/EBPβ.

Thus, IGF-II may be a novel target for cognitive enhancement therapies.

METHODS SUMMARY

Inhibitory avoidance, contextual and auditory fear conditioning: all behavioural procedures were performed as described in refs 15,37,38.

Cannulae implants, hippocampal and amygdala injections were done as described in refs 37,39. Chromatin immunoprecipitation (ChIP) was done as described in ref. 40. Western, northern blot and PCR analyses were carried out as described in ref. 37. Electrophysiology methods were conducted as described in ref. 41.

Statistical analysis: one- or two-way analysis of variance (ANOVA) followed by the Newman–Keuls or Bonferroni post hoc test, and Student's *t*-test were used.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permission information are available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondences and requests for materials should be sent to C.M.A. (Cristina.Alberini@mssm.edu).

METHODS

Animals. Long Evans adult male rats (Harlan) weighing between 200 and 250 g at the beginning of the experiments were used. Rats were housed individually on a 12 h light-dark cycle with *ad libitum* access to food and water. All experiments were done during the light cycle between 9 a.m. and 6 p.m. All rats were handled for 2–3 min per day for 5 days before any behavioural procedure. All protocols complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mount Sinai School of Medicine Animal Care Committees.

Inhibitory avoidance (IA). IA was carried out as described previously[15,37]. The IA chamber (Med Associates) consisted of a rectangular Perspex box divided into a safe compartment and a shock compartment. The safe compartment was white and illuminated, whereas the shock compartment was black and dark. Foot shocks were delivered to the grid floor of the shock chamber via a constant current scrambler circuit. The apparatus was located in a sound-attenuated, non-illuminated room. During training sessions, each rat was placed in the safe compartment with its head facing away from the door. After 10 s, the door separating the compartments was automatically opened, allowing the rat access to the shock compartment; the rats usually enter the shock (dark) compartment within 10–20 s of the door opening. The door closed 1 s after the rat entered the shock compartment, and a brief foot shock (0.6 mA for 2 s for all experiments except for those of Fig. 6a which was done at 0.9 mA) was administered. Latency to enter the shock compartment was taken in seconds as acquisition. The rat was then returned to its home cage and tested for memory retention at the designated time-point(s). Retention tests were done by placing the rat back in the safe compartment and measuring its latency to enter the shock compartment. Foot shocks were not administered on the retention tests, and testing was terminated at 540 s or 900 s as indicated in the figures.

Controls consisted of rats that remained in their home cage (naive), rats exposed to the training apparatus without foot shock (−), or rats exposed to the training apparatus and to foot shock 1 h later (unpaired, Un).

In reactivation (reconsolidation) experiments, rats were trained as described and, at the indicated time points, were tested. This test reactivated the memory. Immediately after or at the designated time-points, rats were injected with the indicated compounds and subsequently tested again for retention.

During testing, the experimenter was blind to the treatments given.

Contextual and auditory fear conditioning. Fear conditioning was carried out as described previously[38]. Rats were conditioned in contextual fear conditioning chamber (CFC), which consisted of a rectangular Plexiglass box (30.5 × 24.1 × 21.0 cm) with a metal grid floor (Model ENV-008 Med Associates). All rats were pre-exposed to this chamber for 5 min. On the next day, rats were placed in the CFC chamber for 120 s and then presented with 30 s of the auditory cue consisting of a 5 kHz 75 dB tone that co-terminated with a 0.6 mA 2 s foot shock. One hundred twenty seconds after the first foot shock, another 30 s auditory cue was presented that also co-terminated with another 0.6 mA 2 s foot shock. Rats were returned to their home cage 120 s after the second foot shock. Freezing levels during the first 148 s (before the presentation of the first foot shock) was recorded, scored and reported as baseline freezing. Freezing was defined as lack of movement except for breathing. Twenty-four hour later, rats were placed back in the CFC chamber and their freezing levels recorded for 5 min and scored. Twenty-four hours after CFC test, rats were placed in a different context (the illuminated IA box) for 120 s before being presented with three 30 s auditory cues. The three 30 s auditory cues were separated by 120 s. Freezing levels during the cue presentations was recorded and scored by an experimenter who was blind to the treatment conditions.

Cannulae implants and hippocampal and amygdala injections. Hippocampal and amygdala injections were given as described previously[15,37,39]. Rats were anaesthetized with ketamine (65 mg per kg, intraperitoneally (i.p.)) and xylazine (7.5 mg per kg, i.p.), and stainless-steel guide cannulae (22-gauge) were stereotactically implanted to bilaterally target the hippocampus (4.0 mm posterior to the bregma; 2.6 mm lateral from midline; and 2.0 mm ventral). For amygdala injections, 26-gauge guide cannulae were implanted to target the basolateral amygdala bilaterally (2.8 mm posterior to bregma; 5.3 mm lateral from midline; and 6.25 mm ventral). The rats were returned to their home cages and allowed to recover from surgery for 7 days. At the indicated time points before or after training or retrieval, rats received bilateral injections of compounds as specified. All injections are indicated by arrow in the experimental schedule. All hippocampal injections were carried out in 1 μl per side, whereas all amygdala injections were done in 0.5 μl per side. Hippocampal injections used a 28-gauge needle and amygdala injections used 33-gauge needles that extended 1.5 mm beyond the tip of the guide cannula and connected via polyethylene tubing to a Hamilton syringe. The infusions were delivered at a rate of 0.33 μl min^{-1} using an infusion pump. The injection needle was left in place for 2 min after the injection to allow complete dispersion of the solution.

To verify proper placement of cannula implants, at the end of the behavioural experiments, rats were anaesthetized and perfused with 4% paraformaldehyde in PBS. Their brains were post-fixed overnight in the same fixative with 30% sucrose. Forty-micrometer coronal sections were cut through the hippocampus, stained with cresyl violet, and examined under a light microscope. Rats with incorrect cannula placement were discarded from the study.

Antisense ODNs and relative scrambled sequences (SC-ODNs) were injected at 2 nmol μl^{-1} in all antisense experiments. Sequences: C/EBPβ antisense (β-ODN: 5′-CCAGCAGGCGGTGCATGAAC-3′), C/EBPβ scrambled (SC-ODN: 5′-TCG GAGACTAAGCGCGGCAC-3′); IGF-II antisense (IGF-II-ODN: 5′-CCCAT TGGTACCTGAAGTTG-3′); IGF-II scrambled (SC-ODN: 5′- CGCCTTGT GATACGACTTAG-3′); Arc antisense (Arc-ODN: 5′-GTCCAGCTCCATCT GCTCGC-3′); and Arc scrambled (SC-ODN: 5′-CGTGCCCTCTCGCAGCT TC-3′). Vehicle: phosphate-buffered saline (PBS, pH 7.4). The antisense for C/EBPβ has been previously shown to knockdown C/EBPβ in the hippocampus[15]. The antisense for IGF-II mRNA was specific for the sequence that includes the translational start site and was previously used successfully to knockdown IGF-II in other tissues[42]. The antisense for Arc has been previously shown to block Arc protein expression and long-term memory consolidation when injected into the hippocampus[43]. The respective SC-ODNs, which served as control, contained the same base composition but in a random order and show no homology to sequences in the GenBank database. All ODNs were phosphorothioated on the three terminal bases of both 5′ and 3′ ends to produce increased stability. Both ODNs were reverse-phased-cartridge-purified and obtained from Gene Link.

Recombinant IGF-I and IGF-II were purchased from R&D Systems and were dissolved in 0.1% bovine serum albumin (BSA) in 1× PBS. All experiments with recombinant IGF-II or IGF-I were carried out with 250 ng per injection, except for the dose–response curve (250, 25 or 2.5 ng) and for those in Fig. 5a where 25 ng was used.

The IGF-I receptor (IGF-IR) antagonist JB1 (Bachem Biosciences) was dissolved in PBS. JB1 was injected at 20 ng μl^{-1}, a concentration that has been used successfully to block IGF-1 activity in various tissues, including the brain[44,45]. Anti-IGF-II receptor antibody (anti-IGF-IIR, R&D Systems) was dissolved in 1× PBS and injected at 5 ng μl^{-1}. This concentration blocked 95% of IGF-II receptor in an *in vitro* binding assay (R&D).

Anisomycin (Sigma Aldrich) was dissolved in 0.9% saline pH 7.4. and injected at 125 μg μl^{-1}. This dose blocks more than 80% of protein synthesis in the dorsal hippocampus for up to 6 h (ref. 46).

GSK3 inhibitor SB216763 was purchased from Sigma and was dissolved in 1% DMSO in PBS and injected at 1 ng μl^{-1}. This dose has been shown to block GSK3β activity (as measured by its dephosphorylation levels) in the brain[47].

Synaptoneurosomal preparation and western blot analysis. Synaptoneurosomal preparation was adapted from ref. 48. Briefly, dorsal hippocampi were rapidly dissected in ice-cold cortical dissection buffer followed by homogenization in buffer containing 10 mM HEPES, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, phosphatase and protease inhibitor cocktails (Sigma). A glass-Teflon homogenizer was used and homogenates were filtered through 100 μm nylon mesh filter and 5 μm nitrocellulose filters sequentially. Synaptoneurosomes were obtained by centrifugating the filtrate at 1,000*g* for 10 min. Synaptoneurosomal fraction was enriched for PSD-95 and N-methyl-D-aspartic acid (NMDA) receptor subunit NR-1 (Supplementary Fig. 10).

Western blot analysis was done as reported previously[15,37]. Hippocampal total extracts from rat were obtained by Polytron homogenization in cold lysis buffer with protease and phosphatase inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 2 mM NaF, 2 mM $Na_4P_2O_7$, 4 U ml^{-1} aprotinin, 2 mM DTT, 1 mM EGTA, 1 μM microcystin, 1 mM benzamidine). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of total protein (10–20 μg per lane) were resolved on denaturing SDS–PAGE gels and transferred to Hybond-P membranes (Millipore) by electroblotting. Membranes were dried and then reactivated in methanol for 5 min and then washed with three changes of water. The membrane was then blocked in 3% milk/PBS or according to manufacturers' instruction for 1 h at room temperature, then incubated with either anti-IGF-II (1/500, Millipore), or anti-actin (1/5,000, Santa Cruz Biotechnology) antisera in PBS overnight at 4 °C. Anti-phospho-CREB (1/1,000), anti-GluR1 (1/2,000), anti-GluR2 (1/2,000), anti-PSD95 (1/5,000) and anti-NR1 (1/1,000) antibodies were purchased from Millipore. Anti-C/EBPβ antibody was purchased from Santa Cruz Biotechnology (1/1,000). pGSK3β and GSK3β antibodies were purchased from Cell Signaling (1/1,000). pGSK3β was normalized to actin and GSK3β. The colloidal gold total protein stain was purchased from Bio-Rad. The membranes were washed, treated with a secondary horseradish peroxidase-labelled donkey anti-rabbit antibody (1/4,000, GE Healthcare) for 1 h, washed again and incubated with HRP-streptavidin complex and ECL detection reagents

(GE Healthcare). Membranes were exposed to HyBlotCL (Denville Scientific), and quantitative densitometric analysis was performed using NIH ImageJ.

Real time quantitative RT–PCR. Hippocampal total RNA was extracted with TRIzol (Invitrogen) and reverse-transcribed using SuperScript II RNase H minus RT (Invitrogen). Real-time PCR was done with an ABI Prism 7900HT (Applied Biosystems). Five hundred picograms of the first-strand cDNA was subjected to PCR amplification using a QuantiTect SYBR Green PCR kit (Qiagen). IGF-II primers (forward: 5′-CCCAGCGAGACTCTGTGCGGA-3′; reverse, 5′-GGAA GTACGGCCTGAGAGGTA-3′); IGF-I primers (forward: 5′-CCTGGGCTTT GTTTTCACTTCGG-3′; reverse: 5′-CACAGCTCCGGAAGCAACACTCA-3′). Forty cycles of PCR amplification were performed as follows: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension for 30 s at 72 °C. Three PCR assays with triplicates were performed for each cDNA sample. 18S rRNA (forward, 5′-CGCCGCTAGAGGTGAAATTCT-3′; reverse, 5′-CAGACCTCC GACTTTCGTTCT-3′) was used as internal controls. Data were analysed with Sequence Detector System version 2.0 software (Applied Biosystems). The cycle threshold method (C_T, see Applied Biosystems User Bulletin Number 2, P/N 4303859) was chosen to determine the relative quantification of gene expression in trained and control rats.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described in ref. 40. The rat hippocampi were dissected and minced into ~1 mm pieces, and immediately cross-linked in 1% formaldehyde for 17 min at room temperature rotating. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and incubated for 7 min. The tissue was washed five times in cold PBS containing protease inhibitor (Roche Applied Sciences) and then frozen on dry ice. The chromatin was solubilized and extracted by adding 500 µl of lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM EDTA), followed by sonication. The homogenate was diluted in 1.1 ml ChIP dilution buffer (1.1% Triton X-100, 167 mM NaCl, 16.7 mM Tris-HCL pH 8.1, 1.2 mM EDTA, 0.01% SDS). The homogenate was used for C/EBPβ ChIP. Magnetic Protein A beads (30 µl, EZ-Magna ChIP A kit; Millipore) and 5 µg of C/EBPβ antibody was added to the homogenate. The mixture was incubated rotating overnight in 4 °C. The wash, elution, and reverse cross-link to free DNA were all performed according to the manufacturer's protocol (EZ-Magna ChIP A kit).

Specific primers were designed to amplify the proximal promoter region of approximately 150 bp 5′ of exon one of rat IGF-II (GenBank: X17012.1), which contains a putative C/EBP binding site. Putative C/EBP binding site was predicted using an on-line program AliBaba 2.1. Similar C/EBP binding sites have been identified in other species[49,50]. Primer sequences used: forward 5′-GGTTCCCC ACGTTAGGCTTGGAT-3′; reverse 5′-TTGCGGCCCTGGGAATGAGTG-3′. A standard thirty-five cycle PCR was performed as followed: denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension for 30 s at 72 °C. The PCR reaction was resolved on a 2% agarose gel and sequenced. Sequencing confirmed the identity of the fragment. DNA sequencing was performed by W. M. Keck Facility at Yale University.

Northern blot analysis. Northern blot analyses were performed as described previously[14]. The rat IGF-II probe consisted of a 224-bp fragment corresponding to nucleotides 1145–1368 of the IGF-II sequence in GenBank accession number NM-031511. The same membrane was stripped and rehybridized with a full-length rat cyclophilin probe that was used as a loading control. Probes were labelled with random oligonucleotide primers (Prime-It II kit, Stratagene) and [α-^{32}P]dCTP (Amersham). Quantitative densitometry analysis was performed using NIH Image J. Data were expressed as mean percentage ±s.e.m. of the 0 h− (100%) control mean values.

Electrophysiology methods. Brains were removed from isoflurane-anaesthetized male Long-Evans rats (6–8 weeks old), and chilled in ice-cold ACSF (in mM: 1.25 NaH2PO4,1.3 MgSO4, 2.5 CaCl2, 3.5 KCl, 15 glucose, 24 NaHCO3, and 118 NaCl) bubbled with 95% O_2/5% CO_2 (pH 7.35). Acute transverse slices of dorsal hippocampus (400-µm thick) were recovered in an interface chamber at room temperature, as described previously[41]. Slices were individually transferred to a recirculating submersion recording chamber and superfused with ACSF at 30–32 °C. Field EPSPs (fEPSPs) were recorded with an ACSF-filled pipette (2–4 MΩ) positioned in stratum radiatum of area CA1, and Schaffer collateral inputs were stimulated with 50 µs monophasic pulses using a bipolar concentric electrode placed in area CA3. Weak HFS, which normally induces only transient synaptic potentiation[41], consisted of two 1-s trains of 100 Hz pulses, delivered 20 s apart, with stimulus intensity set at 20% of the spike threshold.

IGF-II, freshly prepared from stock to a final concentration of 1 nM, was introduced 20 min before HFS was delivered and was present for the remainder of the experiment. The antibody against the IGF-II receptor was used at a final concentration of 16 or 50 µg ml^{-1}, prepared fresh before use. As these two concentrations produced indistinguishable results, the data were pooled for analysis and presentation. The antibody was introduced at least 30 min before HFS was delivered, and remained in the superfusate for the rest of the experiment. The numbers for the electrophysiology experiment are: vehicle (wHFS: $n = 4$; no stim.: $n = 4$), IGF-II (wHFS: $n = 5$; no stim.: $n = 6$), IGF-II + anti-IGF-IIR antibody (wHFS: $n = 5$; no stim.: $n = 5$).

Statistical analysis. One- or two-way analysis of variance (ANOVA) followed by either the Newman–Keuls, or Bonferroni post hoc test, or Student's *t*-test was used for statistical analyses. For the electrophysiology experiments, EPSP slopes were compared by a Student's *t*-test.

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