A hippocampal insulin-growth factor 2 pathway regulates the extinction of fear memories

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**Extinction learning refers to the phenomenon that a pre-
viously learned response to an environmental stimulus,
for example, the expression of an aversive behaviour upon
exposure to a specific context, is reduced when the stimu-
lus is repeatedly presented in the absence of a previously
paired aversive event. Extinction of fear memories has
been implicated with the treatment of anxiety disease
but the molecular processes that underlie fear extinction
are only beginning to emerge. Here, we show that fear
extinction initiates upregulation of hippocampal insulin-
growth factor 2 (*Igf2*) and downregulation of insulin-
growth factor binding protein 7 (*Igfbp7*). In line with
this observation, we demonstrate that IGF2 facilitates
fear extinction, while IGFBP7 impairs fear extinction in
an IGF2-dependent manner. Furthermore, we identify
one cellular substrate of altered IGF2 signalling during
fear extinction. To this end, we show that fear extinction-
induced IGF2/IGFBP7 signalling promotes the survival
of 17–19-day-old newborn hippocampal neurons. In
conclusion, our data suggest that therapeutic strategies
that enhance IGF2 signalling and adult neurogenesis
might be suitable to treat disease linked to excessive
fear memory.**

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Introduction

The precise molecular clockwork that regulates memory
function is far from being understood. Such research is,
however, of great relevance since memory disturbances are
key features of various neurological disorders. Extinction of
fear memory is a particular form of cognitive function, that is
of special interest because of its involvement in the treatment
of anxiety and mood disorders (Myers and Davis, 2007;
Fischer and Tsai, 2008; Quirk and Mueller, 2008; Pape and
Pare, 2010; Radulovic and Tronson, 2010). Here, extinction
training is central as a therapeutic strategy to inhibit excess
fear. Fear is normally a protective mechanisms aimed to
promote an adaptive response to danger. However, extreme
extrinsic events can force the system beyond its limits. Such a
homeostasis breakdown often leads to excessive fear and
mental illnesses like anxiety and mood disorders, which
severely affect the life of patients and are an increasing
burden to our societies (Sotres-Bayon *et al*, 2006; Bremner
et al, 2008; Fischer and Tsai, 2008; Hartley and Phelps, 2010).
During extinction training, fear inhibition is achieved by
repeated re-exposure to the fear-eliciting stimulus in the
absence of any aversive event. This normally leads to a
gradual reduction of fear, namely the extinction of fear
memories. To understand the molecular mechanisms under-
lying fear extinction is, therefore, of great clinical importance.

In the laboratory, fear extinction is often studied using the
Pavlovian fear conditioning (FC) paradigm. In the contextual
version of this paradigm, a single exposure of rodents to a
novel context followed by an electric foot-shock elicits the
acquisition of conditioned fear. On the basis of associative
learning, the animals display an inborn aversive freezing
behaviour upon re-exposure to the conditioned context.
This form of contextual FC is hippocampus dependent and
leads to a long-lasting fear memory (Fischer *et al*, 2004, 2007;
Frankland *et al*, 2006; Matynia *et al*, 2008). During extinction
training, animals are repeatedly re-exposed to the condi-
tioned context without receiving the foot-shock again
(extinction trial, E), which eventually results in the decline
of the aversive freezing behaviour (Sananbenesi *et al*, 2007).

Experimental data obtained across species demonstrated
that the amygdala, the medial prefrontal cortex and the
hippocampus are critically involved in fear extinction
(Phelps and LeDoux, 2005; Myers and Davis, 2007;
Bremner *et al*, 2008; Fischer and Tsai, 2008; Quirk and
Mueller, 2008; Ponomarev *et al*, 2010; Shin and Liberzon,
2010). The most common theories suggest that fear extinction
involves a combination of associative processes such as new
learning and also non-associative mechanisms involving
decreased responsiveness to the fear-eliciting stimulus
(Myers and Davis, 2007). The molecular processes that
underlie fear extinction are only beginning to emerge.

In this study, we observe that contextual fear extinction
training induced the upregulation of hippocampal insulin-
growth factor 2 (*IGF2*) and downregulation of insulin-growth

factor binding protein 7 (*IGFBP7*), which attenuates the biological function of IGFs. We demonstrate that IGF2 facilitates fear extinction, while IGFBP7 impairs fear extinction in an IGF2-dependent manner. Our data, furthermore, suggest that one important cellular substrate of altered IGF2 signalling during fear extinction is adult neurogenesis. In conclusion, our data indicate that therapeutic strategies to enhance IGF2 signalling and the survival of newborn hippocampal neurons could be suitable therapeutic avenue to treat neuropsychiatric disorders that involve excessive fear memory.

Results

Fear extinction regulates hippocampal Igf2 and Igfbp7 levels

We employed mice to investigate fear extinction in the hippocampus-dependent contextual FC paradigm (Sananbenesi *et al*, 2007). To this end, male C57BL/6J mice were exposed to the FC box (context) followed by an electric foot-shock, which elicits the acquisition of conditioned contextual fear. For extinction training, animals were repeatedly re-exposed to the conditioned context on consecutive days (24 h interval) without receiving the foot-shock again (extinction trial, E). This procedure eventually results in the decline of the aversive freezing behaviour (Figure 1A; Sananbenesi *et al*, 2007). Mice that were exposed to the conditioning context without receiving FC training served as control groups. Since pilot experiments showed that the expression of *cFos* do not significantly differ among mice that were exposed to the context either twice or five times this control group was pooled for gene array analysis and in subsequent experiments. To gain a better understanding of the molecular processes underlying fear extinction, we performed a genome-wide analysis of the hippocampal transcriptome during fear extinction. In the employed paradigm, fear extinction is a gradual process. To capture the long-

itudinal course of fear extinction, we decided to perform hippocampal microarray analysis at two time points: (1) after the first extinction trial (E1) when animals display high levels of aversive freezing behaviour and (2) at the extinction trial on which the freezing behaviour was significantly reduced when compared to E1. This extinction trial, in the case of this experiment E5, we termed 'extinction trial low freezing' (E_{LF}) (Figure 1A). Please note that we will use the term E_{LF} only for experiments in which we need to define a time point for molecular analysis. Mice that were exposed to the conditioning context without receiving FC training served as control groups. Previous data showed that the immediate early gene FBJ osteosarcoma oncogene (*cFos*), a marker for neuronal activity, was transiently upregulated at the beginning but towards the end of extinction training (Tronson *et al*, 2009). Therefore, we decided to perform the microarray at the time point 1 h after E1 and E_{LF}. This experimental approach is obviously a compromise and does not capture all critical time windows potentially implicated with contextual fear extinction. We are aware that microarray studies are prone to false positives and especially data obtained from complex biological systems need to be interpreted cautiously. Yet, we reasoned that our approach might provide a realistic chance to identify genes that may have an important role in fear extinction and thus open up new avenues for further experiments.

We found that 137 genes, implicated with biological processes such as cell proliferation, neuronal projection or neurogenesis, were differentially expressed during fear extinction (Figure 1B and C; Supplementary Table S1). To confirm the gene array data, we analysed hippocampal expression of genes that were chosen to represent signalling cascades affected during fear extinction by quantitative real-time PCR (qPCR) using the same mRNA samples used for the microarray (Supplementary Figure S1) and in samples from an independent experiment (Figure 1D). Hippocampal lysates
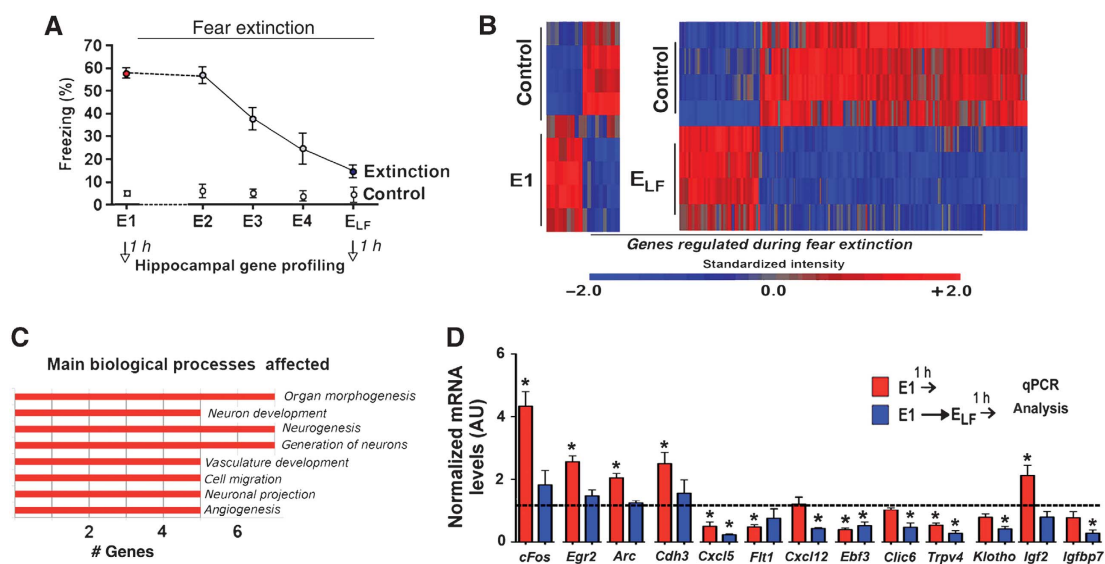

Figure 1 Fear extinction regulates hippocampal gene expression. (**A**) Experimental design. Mice ($n = 5$/group) were subjected to contextual fear conditioning (FC) followed by extinction training on consecutive days. Hippocampal tissue was prepared for gene array analysis 1 h after E1 and E_{LF}. Mice that were subjected to the conditioning context but did not undergo fear extinction served as control groups. (**B**) Heat map showing differential gene expression during fear extinction. (**C**) GO-term analysis of genes regulated during fear extinction. (**D**) The expression of selected genes upon fear extinction was analysed via qPCR. *$P < 0.05$ versus control; E, extinction trial; E_{LF}, extinction trial low freezing; ($n = 5$/group); error bars indicate s.e.m.

obtained 1 h after mice were exposed to FC training served as an additional control group (Supplementary Figure S2). Consistent with our microarray results, we observed a strong upregulation of *cFos* expression 1 h after FC and E1. Notably, *cFos* induction significantly decreased upon E_{LF} exposure, when compared to E1 (Figure 1D). A similar trend was observed for other immediate early genes such as early growth response 2 (*Egr2*) and activity-regulated cytoskeleton-associated protein (*Arc*) as well as chromodomain helicase DNA binding protein 3 (*Chd3*) (Figure 1D). We also confirmed differential expression for a number of genes that were linked to insulin-growth factor (*Igf*) signalling. To this end, the *Igf2*, *Igfbp7* and *Klotho* genes were exclusively regulated upon fear extinction (Figure 1D).

While our microarray data point to a number of interesting signalling cascades that certainly merit further investigation in the present study we decided to focus on hippocampal IGF2 signalling. This decision was based on the fact that two genes of this pathway were regulated in an opposite manner. Namely, *Igf2* was upregulated after E1 and *Igfbp7 was* downregulated after E_{LF}. However, since IGFBPs attenuate the function of IGFs (Baxter, 1991), such a regulation would ultimately result in increased hippocampal IGF signalling. Especially IGF1 (Trejo *et al*, 2008; Llorens-Martín *et al*, 2009, 2010), but most recently also IGF2 (Chen *et al*, 2011) have been implicated with cognitive function, but its role in fear extinction has not been investigated previously. In summary, these data strongly suggested a role of *Igf2* and *Igfbp7* in fear extinction that merit further investigation.

First, we decided to investigate the regulation of *Igf2* and *Igfbp7* during fear extinction in greater detail. We subjected additional groups of mice to our fear extinction paradigm and analysed hippocampal *Igf2* and *Igfbp7* expression 1, 3 and 24 h after exposure to E1 and E_{LF} (Figure 2A). We observed that *Igf2* expression was significantly increased 1 and 3 h after exposure to E1 but did not change upon exposure to E_{LF} (Figure 2B). Elevated *Igf2* levels after E1 exposure are not due to the FC procedure (Supplementary Figure S3). The expression of *Igfbp7* did not change after E1 but was strongly downregulated after exposure to E_{LF} (Figure 2B). This effect was not simply due to the passing of time because *Igfbp7* expression did not change in mice that were exposed to FC and E1 but sacrificed 4 days later without further extinction training (Supplementary Figure S3). In an additional experiment, we also analysed the expression of *Igf2* and *Igfbp7* in specific hippocampal subregions and found that the differential expression of *Igf2* and *Igfbp7* was most prominent in the hippocampal dentate gyrus (Figure 2C; Supplementary Figure S4) where significant levels of IGF2 and IGFBP7 protein are produced (Figure 2D). The expression of other key molecules of the hippocampal IGF signalling pathway including *Igf1*, *Igf1 receptor*, *Igfbp4* and *Igfbp5* was not altered during extinction training (Supplementary Figure S5). In conclusion, these data establish that fear extinction training regulates hippocampal *Igf2* and *Igfbp7* levels. However, changes in mRNA level do not always translate into altered protein amount. Without a corresponding change in IGF2 and IGFBP7 protein levels such a pathway may, however, not have any biological relevance in contextual fear extinction. To address this question, we analysed the levels of IGF2 and IGFBP7 protein after fear extinction via quantitative immunoblot. Mice were exposed to our fear

extinction paradigm and lysates from the hippocampal cornu ammonis (CA) and dentate gyrus regions were prepared 3 h after E1 and E_{LF}. We chose the time point 3 h since changes in protein levels usually follow changes in mRNA expression. In line with the gene expression data, we observed that IGF2 protein levels increased 3 h after E1 exposure in the hippocampal dentate gyrus but not in the CA region (Figure 2E and F). The levels of IGFBP7 decreased 3 h after E_{LF} in the dentate gyrus but not in the CA region (Figure 2E and F). In conclusion, our data suggest that the extinction of contextual fear memory involves altered hippocampal IGF2/IGFBP7 function.

IGF2 and IGFBP7 regulate fear extinction

On the basis of the above-described data, we decided to further investigate the role of IGF2 and IGFBP7 during fear extinction. IGF2 and IGFBP7 are secreted proteins that act in an autocrine or paracrine manner (Broughton and Partridge, 2009). As such, we reasoned that a suitable approach to study the role of those proteins would be to manipulate their levels during fear extinction via intrahippocampal injection of recombinant proteins or blocking antibodies. Therefore, microcannulae were implanted into the dentate gyrus of mice. After all mice had recovered from surgery they were subjected to FC followed by extinction training (Figure 3A). To test whether decreasing physiological IGF2 levels in the hippocampus would affect fear extinction mice were injected with an IGF2 blocking antibody (IGF2ab; Ostrovsky *et al*, 2009) immediately after each extinction trial. When compared to the vehicle group, fear extinction was severely impaired in IGF2ab-treated mice (Figure 3B). Extinction training was stopped when the vehicle group reached E_{LF} criteria. Importantly, mice were able to extinct fear memories once IGF2ab treatment was stopped (Figure 3C). Moreover, prolonged IGF2ab treatment did not cause obvious hippocampal damage or long-lasting memory impairment (Supplementary Figure S6). Next, we analysed if increasing hippocampal IGF2 levels would affect fear extinction. The experiment was performed as described above but instead of IGF2ab, mice were injected with IGF2 while the other group received vehicle solution. Fear extinction was significantly facilitated in IGF2-treated mice (Figure 3D). In conclusion, those results show that IGF2 is required for successful fear extinction. Notably, inhibition of physiological IGF1 signalling via intrahippocampal injection of IGF1 blocking antibody (IGF1ab) did not affect fear extinction. Similarly, administration of IGF1 had no effect on fear extinction and did not rescue IGFBP7-mediated impairment of fear extinction (Supplementary Figure S7). These data point to a specific role of IGF2 signalling in fear extinction and also suggest that injection procedure itself does not affect fear extinction.

Next, we employed the same experimental strategy to analyse the role of IGFBP7 in fear extinction. Microcannulae were implanted into the dentate gyrus of mice. Immediately after each extinction trial, mice were injected with IGFBP7 or vehicle. Animals that were treated with IGFBP7 displayed severely impaired fear extinction when compared to the vehicle group (Figure 4A). Once IGFBP7 treatment was stopped, mice were able to extinct fear memories (Figure 4B). Prolonged IGFBP7 treatment did not cause obvious hippocampal damage or long-lasting memory impairment (Supplementary Figure S6). Next, we
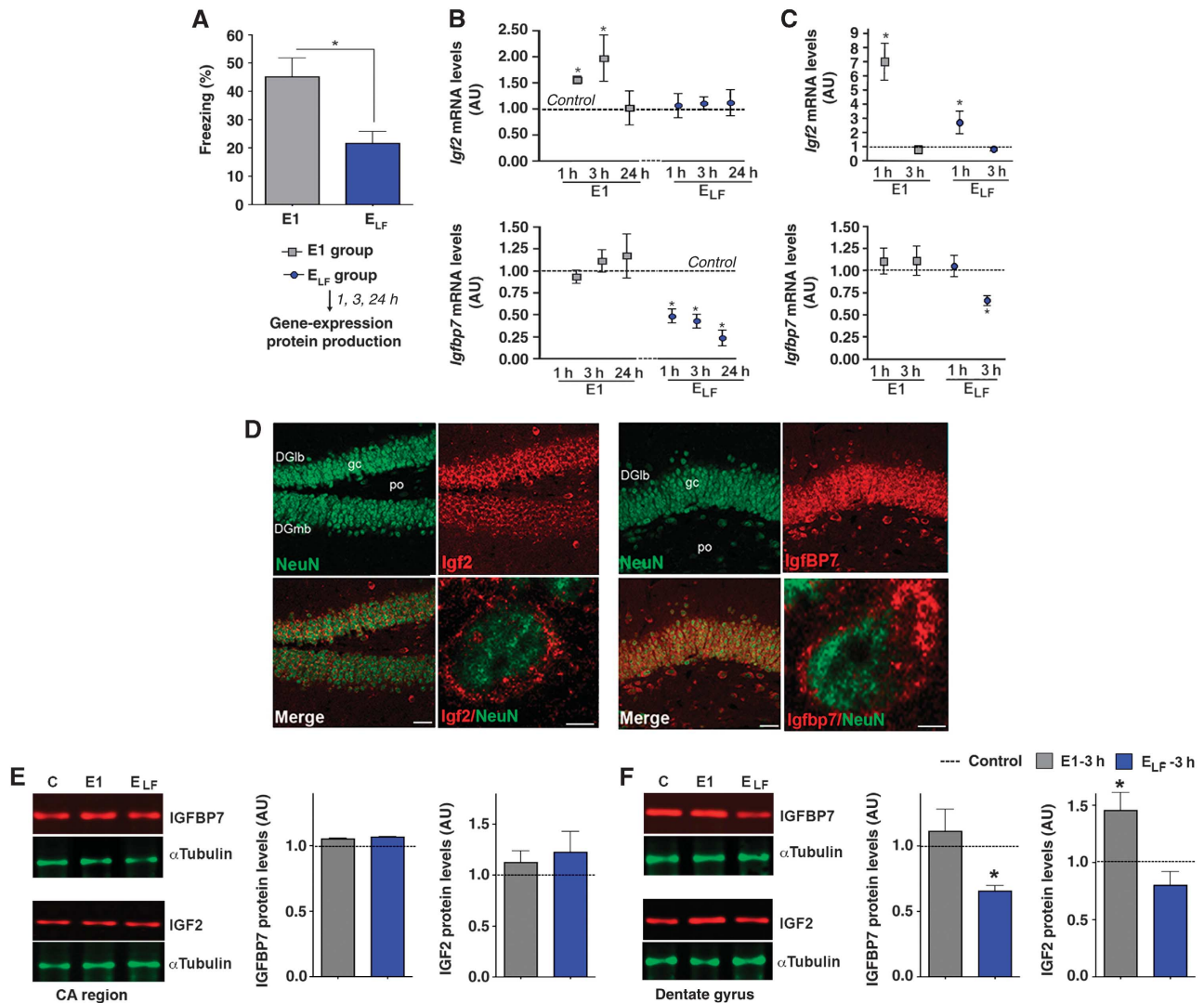
Figure 2 Fear extinction regulates hippocampal *Igf2/Igfbp7* expression. (**A**) Mice were subjected to fear extinction. Behavioural analysis confirmed that freezing behaviour was significantly reduced upon E_{LF} exposure, when compared to E1. Mice that were subjected to the conditioning context but not subjected to fear extinction served as control (*$P < 0.05$ versus E1; $n = 15$/group). (**B**) qPCR analysis of *Igf2* and *Igfbp7* expression in the total hippocampus at different time points after fear extinction training (*$P < 0.05$ versus control; $n = 5$/group). (**C**) qPCR analysis of *Igf2* and *Igfbp7* expression in the dentate gyrus (*$P < 0.05$ versus control; $n = 5$/group). (**D**) Representative images showing immunostaining for IGF2 and IGFBP7 protein levels in the dentate gyrus. Scale bars low magnification images: 50 μm; high magnification: 2 μm. (**E**) Left panel: Representative immunoblot analysis of IGF2 and IGFBP7 protein levels in the CA region 3 h after exposure to E1 or E_{LF}. Right panel. Quantification ($n = 5$/group). (**F**) Left panel: Representative immunoblot analysis of IGF2 and IGFBP7 protein levels in the dentate gyrus region 3 h after exposure to E1 or E_{LF}. Right panel. Quantification (*$P < 0.05$ versus control, $n = 5$/group). DGlb, dentate gyrus lateral blade; DGmb, dentate gyrus medial blade; E, extinction trial; E_{LF}, extinction trial low freezing; gc, granular cell layer of the dentate gyrus; Po, polymorph layer of the dentate gyrus; error bars indicate s.e.m.

employed an additional experimental group in which IGFBP7 was co-injected with IGF2. While in agreement with our previous results, IGFBP7-treated mice showed severely impaired fear extinction, this effect was completely reversed in mice that were co-injected with IGFBP7 and IGF2 indicating that administration of additional IGF2 could overcome the effect of IGFBP7 (Figure 4C). Notably, administration of IGF1 was not able to rescue the effect of IGFBP7 (Supplementary Figure S7). None of the treatments affected locomotor activity (Supplementary Figure S8). Although we cannot exclude that IGF2 and IGFBP7 affect fear extinction also via independent mechanisms, our data strongly support the view that a hippocampal IGF2/IGFBP7 signalling pathway mediates fear extinction.

Fear extinction promotes the survival of 17–19-day-old newborn hippocampal neurons

Having established that a hippocampal IGF2/IGFBP7 pathway regulates the extinction of fear memories we wondered about the cellular substrate targeted by this pathways. Since IGF signalling is implicated with various cellular functions (Broughton and Partridge, 2009) it is likely that multiple cellular mechanisms account for the observed effect of IGF2 and IGFBP7 on fear extinction. We hypothesized that one candidate mechanism by which IGF2/IGFBP7 signalling affects the extinction of fear memories could be the regulation of adult neurogenesis. This is based on three findings: (1) on the fact data mining using GO-term analysis identified neurogenesis as a key biological process affected by fear
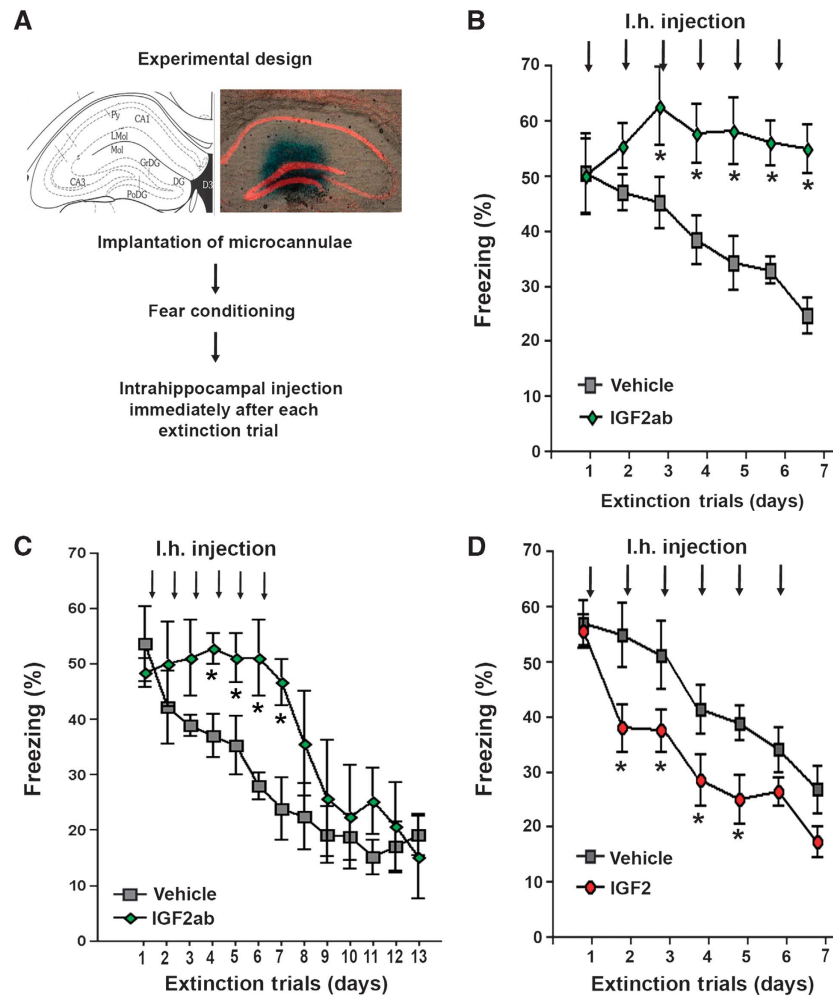
Figure 3 Hippocampal IGF2 signalling regulates fear extinction. (**A**) Experimental design. The image shows the position of the injection cannulae as visualized by methylene blue injection. (**B**) Fear extinction was significantly impaired in mice that were injected immediately after each extinction trial with IGF2ab when compared to the vehicle group (*$P < 0.05$ versus vehicle, $n = 10$/group). (**C**) In an independent experiment, we found that IGF2ab-treated mice were able to successfully extinct fear memories once IGF2ab treatment was stopped. (**D**) Fear extinction was significantly enhanced in mice that were injected immediately after each extinction trial with IGF2 when compared to the vehicle group (*$P < 0.05$ versus vehicle, $n = 10$/group). Py, pyramidal cell layer; rad, stratum radiatum; Lmol, lacunosum moleculare; CA1, subfield CA1; CA3, subfield CA3; DG, dentate gyrus. Error bars indicate s.e.m.
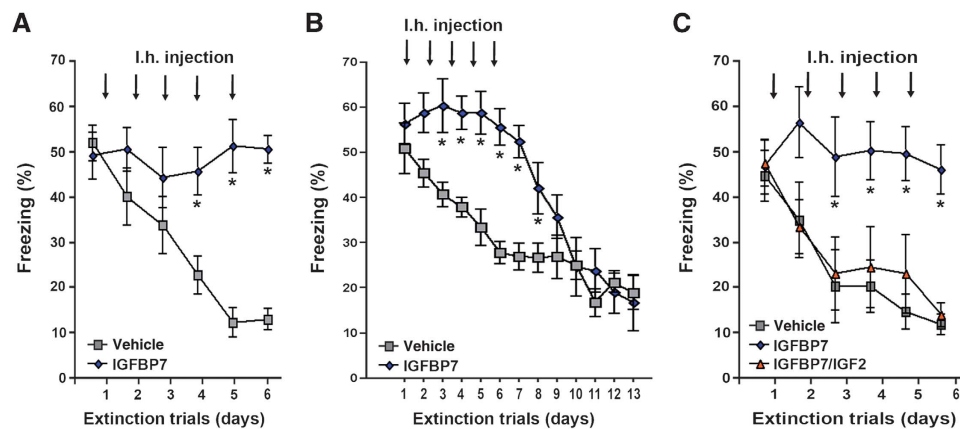

Figure 4 Hippocampal IGFBP7 signalling regulates fear extinction. (**A**) Fear extinction was significantly impaired in mice that were injected immediately after each extinction trial with IGFBP7 when compared to the vehicle group (*$P < 0.05$ versus vehicle) ($n = 10$/group). (**B**) In an independent experiment, we observed that once IGFBP7 treatment was stopped, those mice were able to successfully extinct fear memories. (**C**) Mice were subjected to fear extinction and subsequently injected with IGFBP7, IGFBP7 and IGF2 or vehicle. IGF2 injection rescued the effect of IGFBP7 (*$P < 0.05$ versus vehicle) ($n = 10$/group). Error bars indicate s.e.m.

extinction in our gene array analysis (Figure 1C); (2) although little is known on the specific role of IGF2 (Lehtinen *et al*, 2011) and IGFBP7, IGF signalling has been implicated with cell proliferation and adult neurogenesis (Leventhal *et al*, 1995; Aberg *et al*, 2003; Bateman and McNeill, 2006; Broughton and Partridge, 2009; Lee and Son, 2009); and (3) a recent study demonstrated that adult hippocampal neurogenesis is required for fear extinction (Deng *et al*, 2009).

Adult neurogenesis is a complex process that consists of three main phases: the proliferation of neuroprogenitor cells, differentiation into neurons and the survival of the newly generated neurons (Kempermann *et al*, 2004; Jessberger *et al*, 2008; Zhao *et al*, 2008). In the hippocampus, newborn cells originate from proliferating neuronal progenitor cells in the subgranular zone. The rate of proliferation can be measured by labelling dividing cells with the base analogue Bromodeoxyuridine (BrdU) that can subsequently be detected by immunostaining. However, we found that exposure of mice to our extinction paradigm did not affect the proliferation of hippocampal neuroprogenitor cells (Supplementary Figure S9). Therefore, we designed experiments to test whether fear extinction affects the survival of newly generated neurons. Within 1–4 weeks after birth, adult neurons undergo a critical period of maturation in which they display greater plasticity than fully mature neurons (Schmidt-Hieber *et al*, 2004; Tashiro *et al*, 2007; Deng *et al*, 2010). During this phase, the survival of those immature cells can be affected by environmental factors (Kempermann *et al*, 2004; Zhao *et al*, 2008; Lucassen *et al*, 2010). To investigate the effect of fear extinction on neuronal survival, we labelled dividing cells by BrdU injection. All experimental groups were sacrificed 42 days after the first BrdU injection and the number of surviving neurons was analysed by co-immunostaining of BrdU and Calbindin, a well-established marker for mature granule cells (Figure 5A). After BrdU injection, mice were separated into three experimental groups that underwent fear extinction and reached E_{LF} criteria (Figure 5B). Importantly, the groups of mice were exposed to fear extinction at different time points after BrdU injection. To this end, groups were exposed to the first extinction trial when labelled newborn cells were either 10–12 days (Figure 5C), 17–19 days (Figure 5D) or 31–33 days (Figure 5E) old. The survival of newly generated adult neurons was analysed by counting the number BrdU/Calbindin-positive cells in comparison with a control group that did not underwent FC or extinction training. Mice that were exposed to FC but did not underwent fear extinction served as additional control groups (Figure 5C–E).

Interestingly, FC significantly decreased the survival of 10–12-day-old newly generated neurons and this effect was not affected by extinction training (Figure 5C). While adult neurogenesis has been implicated with various types of memory formation (Zhao *et al*, 2008), it has been reported that depending on the experimental paradigm FC can impair hippocampal neurogenesis (Pham *et al*, 2005). Similarly, FC decreased the survival rate 17–19-day-old neurons. In striking contrast, the number of surviving BrdU/Calbindin-positive cells was significantly increased in mice that underwent fear extinction (Figure 5D). Similar data were obtained when BrdU/Calretinin-positive cells were analysed immediately after the end of fear extinction training (Supplementary Figure S10). Notably, neither FC nor fear extinction had any

effect on 31–33-day-old adult-generated neurons (Figure 5E). In line with these data, we found that the number of active caspase-3-positive neurons, indicating apoptotic cell death, was significantly lower in the dentate gyrus of mice that underwent extinction training and reached E_{LF} criteria when compared to mice subjected to FC without subsequent extinction training (Figure 5F and G). These data suggest that fear extinction specifically enhances the survival of 17–19-day-old hippocampal neurons. At first, it may appear counterintuitive that those immature neurons should contribute to extinction learning. However, previous findings suggesting that such immature newborn neurons can participate in neuronal plasticity (Schmidt-Hieber *et al*, 2004; Tashiro *et al*, 2007; Deng *et al*, 2009, 2010). To provide further evidence that in our experimental settings 17–19-day-old newborn neurons are able to participate in neuronal network plasticity we analysed if those cells can induce cFOS levels during fear extinction. cFOS is a well-established marker for neuronal activity, that is upregulated after exposure to E1 and has been used previously to assay neuronal plasticity in immature newborn neurons (Jessberger and Kempermann, 2003). Notably, we were to show that 17–19-day-old newborn display increased cFOS expression upon exposure to fear extinction (Figure 5H and I) further supporting the view that those neurons are able to participate in neuronal activity related to extinction learning. In conclusion, our data establish that fear extinction initiates a signalling pathway that promotes survival of immature 17–19-day-old hippocampal neurons.

On the basis of these data, we were able to design experiments that allowed us to investigate the role of IGF2 and IGFBP7 on neuronal survival during fear extinction. Although an autocrine mode of action cannot be excluded, our finding that fear extinction can be manipulated by intrahippocampal injection of recombinant IGF2, IGF2ab or IGFBP7 (see Figures 3 and 4) indicates that IGF2 and IGFBP7 mediate this effect in a paracrine manner. This is in line with the fact that IGF2 and IGFBP7 are secreted proteins and our finding that 17–19-day-old newborn neurons express insulin-growth factor 1 receptor (IGF1r) (Supplementary Figure S11) the main receptor through which IGF2 mediates biological function, since IGF2 receptor normally attenuates IGF2 signalling (Brown *et al*, 2009). However, IGF2r has recently been implicated with memory consolidation (Chen *et al*, 2011). Interestingly, in the context of fear extinction inhibition of IGF1r impaired fear extinction while inhibition of IGF2r had no effect (Supplementary Figure S11). To this end, mice were injected with BrdU and subjected to FC followed by extinction training 14 days later. One group of mice was injected immediately after each extinction trial with IGF2ab while a control group received vehicle solution (Figure 6A). As shown before, administration of IGF2ab severely impaired fear extinction (Figure 6B). In the same group of mice, neuronal survival was analysed 42 days after the first BrdU injection. Notably, neuronal survival was significantly reduced in IGF2ab-treated mice when compared to the vehicle group (Figure 6C). Importantly, the injection procedure itself did not seem to severely affect neuronal survival, as indicated by the fact that similar number of newborn cells were detected in the FC/E1-E_{LF} group (Figure 5D) and the vehicle groups shown in Figure 6C and E.

Next, we analysed the effect of elevated IGF2 levels on the survival of 17–19-day-old newborn neurons during fear
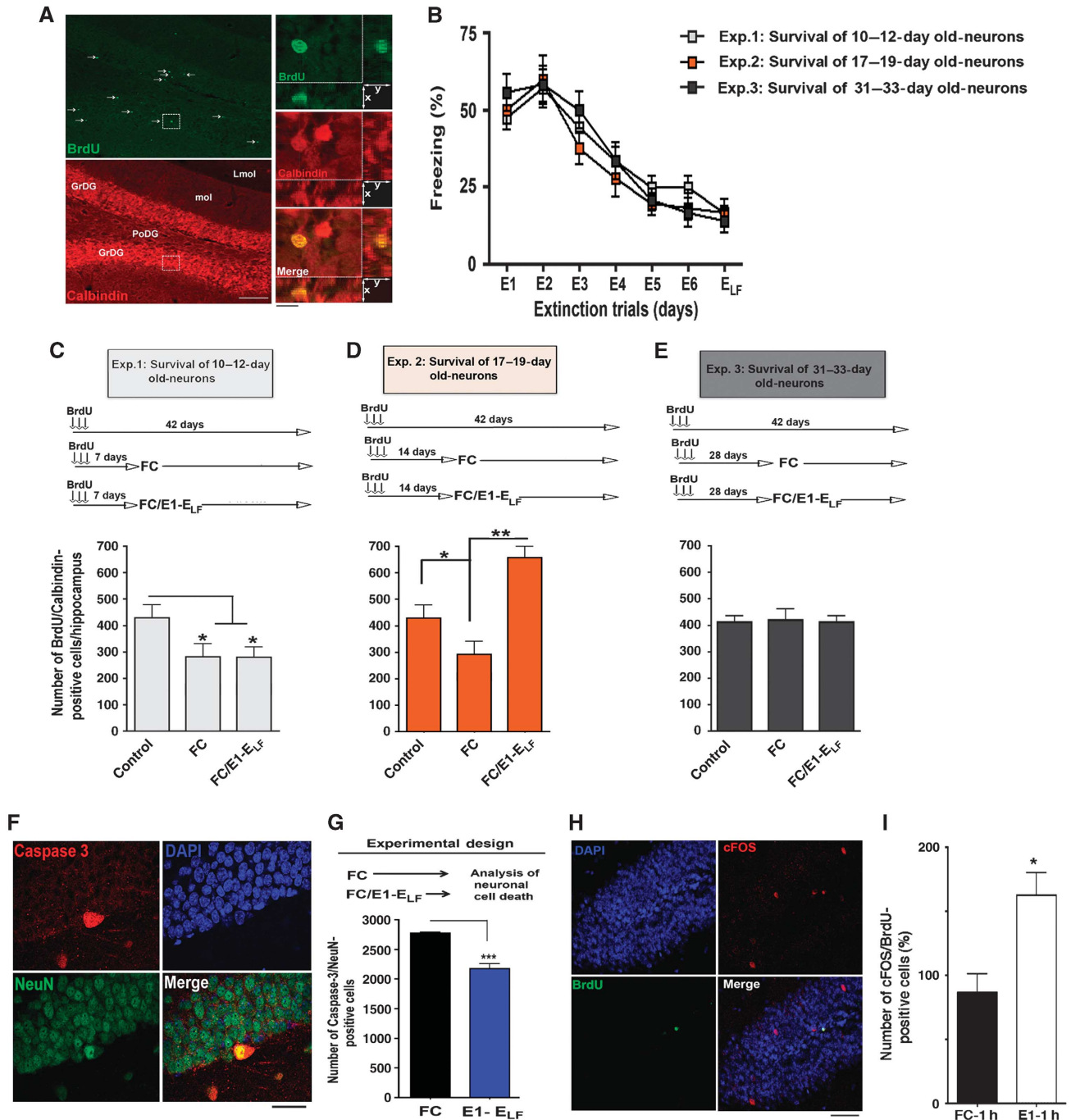
Figure 5 Fear extinction promotes the survival of immature newly generated neurons. (**A**) Representative images showing BrdU/Calbindin-positive cells that indicate maturation of newborn hippocampal neurons. Arrows indicate BrdU-positive cells. Scale bar low magnification images: 100 μm. Scale bar high magnification images: 20 μm. (**B**) Fear extinction, as indicated by the gradual decline of aversive freezing behaviour throughout extinction trials, was similar among the experimental groups that were used to analyse the survival of newly generated hippocampal neurons during fear extinction ($n = 8$/group). (**C–E**) Upper panels show the experimental design to analyse the effect of fear extinction on 10–12-day-old (**C**), 17–19-day-old (**D**) or 31–33-day-old (**E**) hippocampal neurons. Lower panels display the quantification of BrdU/Calbindin-positive cells under each experimental condition. Note that successful fear extinction significantly enhanced the survival of 17–19-day-old newly generated neurons when compared to the control (*$P < 0.05$) or fear conditioning group (**$P < 0.01$). (**F**) Representative images showing active caspase-3-positive cells, indicative of apoptotic cell death, in the hippocampal dentate gyrus region. Scale bar: 20 μm. (**G**) Mice were subjected to fear conditioning or fear extinction training. The number of active caspase-3-positive cells in the dentate gyrus was significantly reduced in mice that underwent extinction training when compared to mice that were only exposed to fear conditioning (***$P < 0.0001$). (**H**) Representative image showing a cFOS-positive 17–19-day-old neuron in the hippocampus of a mouse 1 h after exposure to E1. Scale bar: 50 μm. (**I**) Mice were subjected to fear conditioning or fear extinction training. The number of cFOS/BrdU-positive cells was analysed 1 h after exposure to E1 or 1 h after FC. The data are normalized to the FC group ($P = 0.0154$). GrDG, granular cell layer of the dentate gyrus; Lmol, lacunosum moleculare. Error bars indicate s.e.m.
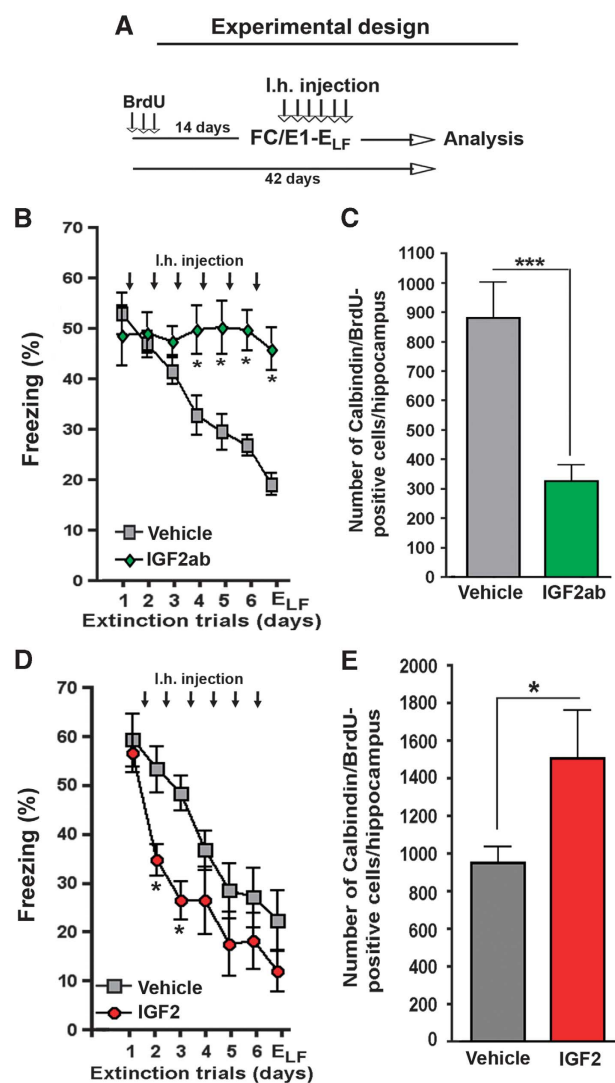
Figure 6 IGF2 promotes the survival of immature neurons during fear extinction. (**A**) Experimental design. Mice were injected with BrdU on three consecutive days and subjected to fear extinction training 14 days later. Immediately after each extinction trial, mice were injected with vehicle IGF2 or IGF2ab. Neuronal survival was analysed 42 days after the first BrdU injection by counting BrdU/Calbindin-positive cells. (**B**) Fear extinction was significantly impaired in mice injected intrahippocampally with an IGF2ab (*$P < 0.05$). (**C**) Survival of 17–19-day-old neurons was impaired in IGF2ab-treated mice (***$P < 0.0001$ versus vehicle). (**D**) Fear extinction was significantly facilitated in the IGF2-treated mice (*$P < 0.05$ versus vehicle). (**E**) Survival of 17–19-day-old neurons was enhanced in IGF2-treated mice (*$P < 0.05$; **$P < 0.01$). $n = 8$/group; Error bars indicate s.e.m.

extinction. Similar to our previous findings, fear extinction was significantly enhanced in the IGF2-treated group (Figure 6D). Notably, when compared to vehicle-treated mice the survival of 17–19-day-old adult-generated neurons was significantly enhanced in the IGF2 group (Figure 6E). In conclusion, these data suggest that during fear extinction IGF2 signalling contributes to the survival of 17–19-day-old newborn hippocampal neurons.

Next, we analysed the role of IGFBP7 on neuronal survival during fear extinction. Mice were injected with BrdU and subjected to fear extinction 14 days after the first BrdU injection. Immediately after each extinction trial, mice were
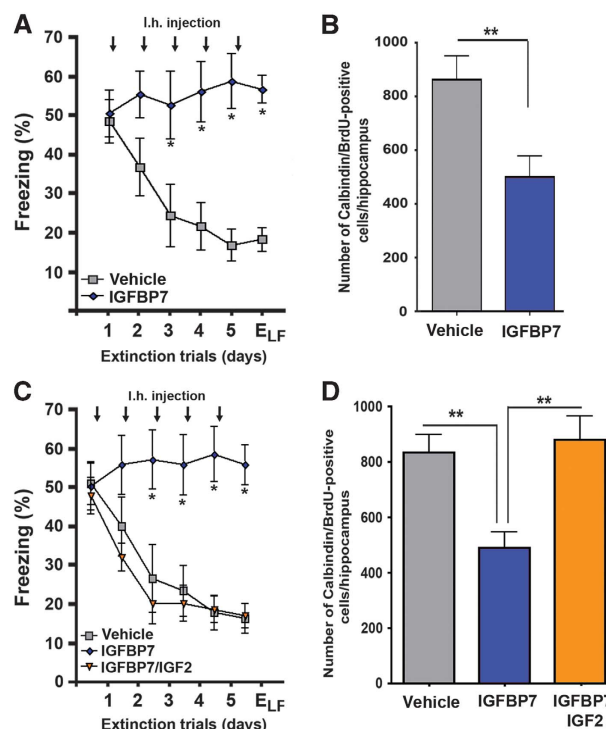

Figure 7 IGFBP7 regulates the survival of immature neurons during fear extinction in an IGF2-dependent manner. (**A**) Using the same experimental approach as shown in Figure 6A, we found that fear extinction was significantly impaired in mice injected intrahippocampally with IGFBP7 (*$P < 0.05$ versus vehicle). (**B**) Survival of 17–19 days was impaired in IGFBP7-treated mice (**$P < 0.001$ versus vehicle). (**C**) Mice were injected with either vehicle, IGFBP7 or co-injected with IGFBP7 and IGF2. While fear extinction was impaired in IGFBP7-injected mice, co-injection with IGF2 was able to rescue this effect. (**D**) IGF2 rescued IGFBP7-mediated impairment of neuronal survival during fear extinction (**$P < 0.001$ versus Igfbp7/vehicle). $n = 8$/group; Error bars indicate s.e.m.

injected with IGFBP7 or vehicle. As shown previously, mice that were treated with IGFBP7 displayed severely impaired fear extinction (Figure 7A). In comparison with the vehicle group, IGFBP7-treated mice displayed reduced survival of 17–19-day-old neurons (Figure 7B). Interestingly, IGFBP7-mediated impairment of fear extinction (Figure 7C) and the survival of 17–19-day-old newborn neurons (Figure 7D) were reversed in mice that were co-injected with IGFBP7 and IGF2.

In conclusion, our data show that fear extinction critically requires elevated hippocampal IGF2 signalling. Under physiological conditions, this is achieved by upregulation of *Igf2* and downregulation of *Igfbp7*. We furthermore provide evidence that one of the cellular substrates affected by altered IGF2/IGFBP7 signalling during fear extinction is adult neurogenesis.

Discussion

In this study, we employ an unbiased, genome-wide approach to investigate hippocampal gene expression during fear extinction. Our aim was to identify interesting novel pathways for further analysis of the mechanisms that

underlie contextual fear extinction. As such, the experimental design of our microarray study is certainly a compromise that did not allow us to detect all possible transcriptome changes associated with fear extinction, neither was it designed to dissect mechanisms of fear extinction from those linked to reconsolidation (Monfils *et al*, 2009). A more detailed analysis of the gene expression after different extinction trials and additional time points will certainly reveal further important candidate pathways. Nevertheless, among the identified genes that showed differentially expression, genes involved with insulin signalling were of particular interest. This is because we found that the *Igf2* gene was transiently upregulated after exposure to E1, whereas the *Igfbp7* mRNA decreased after E_{LF}. Since IGFBPs attenuate the function of IGFs (Oh *et al*, 1996; Yamanaka *et al*, 1997; Wang *et al*, 2008; Suzuki *et al*, 2010) these data suggested that, despite the fact that *Igf2* and *Igfbp7* are regulated in opposite directions, fear extinction might involve elevated hippocampal IGF2 signalling. Moreover, while IGF2 signalling has been implicated with cognitive function (Chen *et al*, 2011), to the best of our knowledge its role in fear extinctions has not been addressed so far. In addition, previous data showed that *Igf2* and *Igfbp7* can be regulated in response to environmental stimuli (Jiang *et al*, 2008; Tomimaru *et al*, 2009) and established that both proteins are present in hippocampal neurons (Rodriguez *et al*, 2007; Wajapeyee *et al*, 2008; Csoregh *et al*, 2009). In summary, those considerations prompted us to select *Igf2* and *Igfbp7* for further analysis. The role of other interesting target genes identified in our microarray study is currently ongoing. A more detailed analysis of *Igf2* and *Igfbp7* expression during fear extinction revealed that *Igf2* upregulation and *Igfbp7* downregulation were most prominent in the hippocampal dentate gyrus region. In line with increased gene expression during fear extinction, we detected a significant upregulation of IGF2 protein levels in the dentate gyrus but not in the CA regions. Similarly, quantitative immunoblot analysis showed extinction-dependent downregulation of IGBP7 protein levels in the dentate gyrus but not in the CA region of mice exposed to extinction training. While the precise mechanisms by which *Igf2* and *Igfbp7* are regulated remain to be investigated, our data suggested the possibility that hippocampal IGF2/IGFBP7 signalling could have an important role in contextual fear extinction.

To test a role for IGF2 signalling during fear extinction directly, we designed experiments to either decrease or enhance hippocampal IGF2 signalling. We found that inhibiting physiological IGF2 signalling by intrahippocampal injection of an IGF2 blocking antibody (Ostrovsky *et al*, 2009) impaired fear extinction. Conversely, administration of recombinant IGF2 protein into the dentate gyrus of mice facilitated fear extinction. These data are in agreement with the upregulation of IGF2 during fear extinction. Notably, mice that received intrahippocampal IGFBP7 injection displayed severely impaired fear extinction, which is in line with the fact that during successful fear extinction IGFBP7 levels decrease. Importantly, the effect of IGFBP7 administration on fear extinction was rescued by IGF2 injection, suggesting that both proteins act in the same pathway. In line with the fact that IGF2 can mediate its biological action via GF1 receptor, blocking IGF1 receptor impaired fear extinction. Interestingly, IGF1 had no effect on fear extinction and also failed to rescue the effect of IGFBP7, suggesting that under physiological conditions the action of hippocampal IGF1 might be tightly controlled.

The fact that we were able to manipulate fear extinction by direct injection of IGF2, IGF2ab or IGFBP7 into the hippocampus of mice is in line with the paracrine action of those proteins (Bateman and McNeill, 2006). As such, our data suggest a scenario in which the fine-tuning of hippocampal IGF2 signalling, that achieved via upregulation of *Igf2* and downregulation of *Igfbp7*, is required for successful fear extinction.

IGF signalling has been implicated with a number of cellular substrates, such as cell morphology or survival, that could potentially contribute to the observed effect on fear extinction (Leventhal *et al*, 1995; Aberg *et al*, 2003; Bateman and McNeill, 2006; Rodriguez *et al*, 2007; Chao and D'Amore, 2008; Scolnick *et al*, 2008; Broughton and Partridge, 2009; Lee and Son, 2009). In search for a cellular substrate of altered IGF2 signalling during fear extinction, we decided to focus initially on the role of adult neurogenesis. This was based on the fact, that GO-term analysis of the genes identified in our microarray study pointed to neurogenesis as a key biological process affected by fear extinction. Notably, this is in line with a recent study showing that the specific elimination of immature adult-born hippocampal neurons impairs contextual fear extinction in mice (Deng *et al*, 2009). In this study, it was found that the depletion of immature 1–4-week-old newborn neurons significantly impaired fear extinction. While this finding at first may appear surprising, it is of note that within 1–4 weeks after birth newly born hippocampal neurons undergo a critical period of maturation (Zhao *et al*, 2008). Importantly, such immature neurons are known to display enhanced plasticity (Schmidt-Hieber *et al*, 2004) and their survival can be affected by environmental stimuli (Kempermann *et al*, 1997; van Praag *et al*, 1999; Ge *et al*, 2005; Zhao *et al*, 2008; Lucassen *et al*, 2010). In line with these data, we found that fear extinction training specifically promotes the survival of 17–19-day-old hippocampal neurons while the proliferation of neuroprogenitor cells was not affected. Together with the Deng *et al* study, these data strongly suggest that contextual fear extinction critically involves the survival of immature newborn hippocampal neurons which can have an immediate role in data processing within the hippocampal circuitry. To this end, one of the generally accepted functions of the dentate gyrus is its role in pattern separation (Kempermann *et al*, 2004; McHugh *et al*, 2007; Zhao *et al*, 2008; Clelland *et al*, 2009). It has been suggested that a new event activates a subset of dentate gyrus neurons which affects a specific set of CA3 cells creating a specific CA3 pattern and that this procedure can be modulated by the recruitment of newborn neurons (Kempermann *et al*, 2004; Aimone *et al*, 2006, 2009). Therefore, it is intriguing to speculate that fear extinction specifically recruits 17–19-day-old adult-generated neurons to create a novel pattern that is, however, still linked to the original fear memory trace. As such, the specific recruitment of immature newly generated neurons into the original fear memory trace may affect the way this memory trace is retrieved (Supplementary Figure S12).

Notably, we found that the survival of 17–19-day-old neurons is regulated by hippocampal IGF2/IGFBP7 signalling during fear extinction. To this end, facilitation of fear extinction via intrahippocampal IGF2 injection correlated with enhanced survival of 17–19-day-old newly generated neu-

rons. In turn, inhibition of physiological IGF2 signalling by intrahippocampal injection of IGF2ab, which impaired fear extinction, also impaired the survival of 17–19-day-old neurons. These data are in line with the fact that IGF2 can promote cell survival (Chao and D'Amore, 2008; Lehtinen et al, 2011). In contrast to the action of IGF1 (Llorens-Martín et al, 2009), a role for IGF2 in adult neurogenesis has not been reported before suggesting that IGF2 mediates fine-tuning of the IGF system under physiological conditions. This is in line with the fact that IGF1 but not IGF2 function is critical for proper brain development (Bateman and McNeill, 2006; Broughton and Partridge, 2009). In addition, mice that were treated with IGFBP7 displayed decreased neuronal survival and severely impaired fear extinction. IGFBP7 also attenuates the action of IGF1 and VEGF signalling (Tamura et al, 2009) which promotes adult neurogenesis (Cao et al, 2004; Heine et al, 2005). As such, the effect of IGFBP7 on fear extinction-dependent neuronal survival might be mediated via multiple targets. Nevertheless, the effect of IGFBP7 administration on fear extinction and neuronal survival was rescued by IGF2 injection, suggesting that IGFBP7 mediates the observed actions, at least in part, in an IGF2-dependent manner.

It is likely that in addition to neuronal survival hippocampal IGF2/IGFBP7 signalling regulates fear extinction also via other cellular substrates, a possibility that remains to be investigated. Nevertheless, in support of the data presented in this study a role for adult neurogenesis in hippocampal memory erasure (Feng et al, 2001) and fear extinction (Deng et al, 2009) has been demonstrated recently. Moreover, chronic administration of Fluoxetine, a drug that increases neurogenesis and is used to treat anxiety and mood disorders including PTSD (Santarelli et al, 2003; Bremner et al, 2008; Pollak et al, 2008; Takemura and Kato, 2008), also facilitates contextual fear extinction (Siegmund and Wotjak, 2007).

In conclusion, our data show that fear extinction critically requires elevated hippocampal IGF2 signalling. Under physiological conditions, this is achieved by upregulation of Igf2 and downregulation of Igfbp7. We furthermore provide evidence that one of the cellular substrates affected by altered IGF2/IGFBP7 signalling during fear extinction is adult neurogenesis. To this end, fear extinction specifically increases the survival of 17–19-day-old neurons in an IGF2/IGFBP7-dependent manner. We suggest, that strategies that enhance IGF2 signalling, potentially via inhibition of IGFBP7, could be a suitable therapeutic avenue to promote extinction in the context of anxiety diseases.

Materials and methods

Animals
Adult male C57Bl/6 wild-type mice (3 months of age) were housed under standard conditions with free access to food and water. All experiments were carried out in accordance with the animal protection law and were approved by the District Government of Germany.

Contextual fear extinction
Behaviour testing was performed by an experimenter that was blind to the treatment as described previously (Fischer et al, 2004; Sananbenesi et al, 2007) using TSE Systems apparatuses and software as well as Freeze Scan software (Clever Systems). In brief, FC was carried out with a computerized fear conditioning system (TSE, Bad Homburg, Germany) using a computer connected to a

control unit containing a shock and a tone generator. Animals were allowed to explore the training cage for 3 min followed by a mild electric shock (2 s, 0.7 mA). Context-dependent freezing, defined as the absence of movements other than those required for breathing, was assessed 24 h later. Extinction of contextual fear was performed on consecutive days (24 h intervals) consisting of re-exposure to the training context in a non-reinforced manner for 3 min. In all experiments, this extinction training protocol led to a significant reduction of freezing behaviour. However, as described before the dynamics of fear extinction can vary among experiments (Sananbenesi and Fischer, 2009) and thus treatment groups should only be compared to the given control group.

Locomotor activity
Mice were subjected to a 3-min exposure to a context. The distance travelled (in cm) during this 3-min context exposure is used as a measurement of the locomotor activity (Supplementary Figure S8).

Cannulation and drug injection
Microcannulae were inserted into the dorsal hippocampus as previously described (Sananbenesi et al, 2007). In brief, bilateral microcannulae were inserted into the dentate gyrus region of the hippocampus: anteroposterior −1.70 mm relative to bregma; lateral ± 1 mm; dorsoventral 2 mm from skull. Stock solutions were IGF2 (Sigma, Munich, Germany; 1 µg/µl in 0.1% BSA in sterile PBS), IGF2 blocking antibody (R&D Systems, 1 µg/µl in PBS), Anti-IGF2 receptor antibody (R&D Systems, 20 ng/µl in PBS), IGF1 (Sigma, 1 µg/µl in 0.1% BSA in sterile PBS), IGF1 blocking antibody (R&D Systems, 1 µg/µl in PBS), IGF1 receptor antagonist JB1 (Bachem Biosciences, 80 ng/µl in PBS), IGFBP7 (R&D Systems, 0.5 µg/µl in 0.1% BSA in sterile PBS). Drugs were injected bilaterally into the hippocampus (0.25 µl, 0.25 µl/min). As such, the total amount of these drugs injected in the brain was 250 ng (IGF2), 250 ng (IGF2 blocking antibody), 5 ng (Anti-IGF2 receptor antibody), 250 ng (IGF1), 250 ng (IGF1 blocking antibody), 20 ng (IGF1 receptor antagonist JB1) and 125 ng (IGFBP7).

BrdU injections and staining
BrdU (Sigma) was prepared as a stock solution of 5 mg/ml in sterile filtered 0.9% NaCl. Mice received four intraperitoneal injections during the same day (every 2 h) of BrdU each at 50 mg/kg of body weight at the desired time point to evaluate neuronal proliferation. Mice received during three consecutive days three intraperitoneal injections of BrdU per day, every 4 h, each at 50 mg/kg of body weight to assess neuronal survival. Animals were sacrificed either 24 h after the last injection to evaluate neuronal proliferation (Doublecortin/BrdU), 19 days after the last injection to check neuronal differentiation (Calretinin/BrdU), 42 days after the first BrdU injection to assess neuronal survival (Calbindin/BrdU) or 19 days after the first injection to check neuronal activity (cFos/BrdU). Double labelling of BrdU together with doublecortin (proliferation), Calretinin (differentiation), Calbindin (survival) and cFOS (neuronal activity) was assessed with a fluorescence microscope and a Leica confocal laser-scanning microscope (510 LSM; lasers HENE 543 NM and ARGON 458/488 NM). Sections were coded for confocal imaging that was performed by a rater blind to the experimental conditions.

Immunohistochemistry
Immunostaining was performed as described previously (Fischer et al, 2004; Sananbenesi et al, 2007; Peleg et al, 2010) and analysed using a Leica SP2 confocal microscope. Antibodies were used in the following concentrations: BrdU (mouse monoclonal diluted 1:1000; Millipore, Billerica, USA), NeuN (mouse monoclonal diluted 1:1000; Chemicon International, USA), active Caspase 3 (rabbit polyclonal 1:300; Abcam, Cambridge, UK); IGF2 (rabbit polyclonal 1:200; Abcam, Cambridge, UK); IGFBP7 (rabbit polyclonal 1:200; Abcam); IGF1r (rabbit polyclonal 1:300; Abcam); doublecortin (rabbit polyclonal 1:1000; Abcam); Calretinin (rabbit polyclonal 1:500; Abcam); Calbindin D-28k (rabbit polyclonal 1:300; Swant, Bellinzona, Switzerland); cFOS (sc-52) (rabbit polyclonal 1:2000; Santa Cruz Biotechnology, USA); Map-2 (rabbit polyclonal 1:1000; Synaptic Systems, Göttingen, Germany); Synaptophysin (clone SVP-38) (rabbit polyclonal 1:1000; Sigma); Cy3 labelled (goat anti-rabbit 1:500; Jackson ImmunoResearch, USA) and Alexa488 labelled (donkey anti-mouse 1:500; Invitrogen, USA).

Quantification of cell numbers

Stereological analysis of the number of cells was performed on one-in-nine series of 40 μm free-floating coronal sections which were analysed by confocal microscopy to count cells expressing the indicated marker throughout the whole rostrocaudal extent of the dentate gyrus, as described (Gould *et al*, 1999; Lie *et al*, 2005; Taupin, 2007). We calculated the total estimated number of cells within the dentate gyrus by multiplying the average number of positive cells for each marker per section by the total number of 40 μm sections comprising the entire dentate gyrus. We performed the quantifications for Fos$^+$, BrdU$^+$ and BrdU$^+$/Fos$^+$ similar to Kee *et al* (2007). In brief, BrdU/cFos double labelling was performed using a confocal microscope with a ×40 objective to quantify the phenotype Fos$^+$, BrdU$^+$ and BrdU$^+$/Fos$^+$ cells through the entire (one-in-nine series of 40 μm free-floating coronal sections) anterior–posterior extent of the dentate gyrus. We quantify the different cell phenotypes in the granule cell layer and including the subgranular zone.

Immunoblotting

Lysates for immunoblotting were prepared as previously described (Fischer *et al*, 2007). Brain tissue was homogenized in TX-buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% NP-40, 0.1% SDS), subjected to the Bioruptor (Diagenode) for 15 min (High, 30 s ON, 30 s OFF) before centrifugation at 12 000 r.p.m. for 10 min. The supernatant was used for immunoblotting. Immunoblots were performed using fluorescent secondary antibodies and data were quantified using an Odyssey Imager (Licor). It was made sure that responses were within the linear range. Antibodies were used in the following concentrations: for IGF2 (rabbit polyclonal 1:1000; Abcam), for IGFBP7 (rabbit polyclonal 1:1000; Abcam) and for α-Tubulin (clone B-5-1-2) (mouse monoclonal 1:1000, Sigma).

Quantitative real-time PCR

Tissue from total hippocampus was dissected and prepared for qPCR analysis as described before (Peleg *et al*, 2010). The dissection of dentate gyrus and CA regions was performed under a stereomicroscope (Motic) as described previously (Hagihara *et al*, 2009). qPCR was performed using a Roche 480 light cycler. cDNA was generated using the Transcriptor High Fidelity cDNA Synthesis kit (Roche) and qPCR for individual genes was performed using the Roche Universal probe library (UPL). Data were normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (Hprt).

DNA microarray

The microarray study was carried out as mono-colour experiment. Total RNA was isolated from mice 1 h after exposure to FC and 1 h after exposure to E_{LF}. Mice that were subjected to the conditioning context without receiving the foot-shock served as control group. Total RNA was Cy3 labelled according to Agilent's Low RNA Input Fluorescent Linear Amplification Kit and hybridized to Agilent Whole Mouse Genome 4 × 44K G4122F microarrays according to the manufacturer's protocol. Quantity and Cy-dye incorporation rates of the generated target material were assessed using a NanoDrop ND-100. Post processing washes were done according to the Agilent Technologies SSPE protocol (v2.1), replacing wash solution 3 by acetonitril, followed by immediate scanning using an Agilent G2505B scanner. Intensity data were extracted using Agilent's Feature Extraction (FE) software, version 9.5.3.1 and analysed using the Limma (Smyth, 2004) package of Bioconductor

(Gentleman *et al*, 2004). The microarray data analysis consists of the following steps: (1) between-array normalization, (2) fitting the data to a linear model and (3) detection of differential gene expression. VSN normalization (Huber *et al*, 2002) was applied to the intensity values as a method for between-array normalization, to assure that the intensities had similar distributions across arrays. To estimate the average group values for each gene and assess differential gene expression, a simple linear model was fit to the data, and group-value averages and standard deviations for each gene were obtained. To find genes with significant expression changes between groups, empirical Bayes statistics were applied to the data by moderating the standard errors of the estimated values (Smyth, 2004). *P*-values were obtained from the moderated *t*-statistic and corrected for multiple testing with the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). For each gene, the null hypothesis, that there is no differential expression between degradation levels, was rejected when its adjusted *P*-value was <0.05. GO association analysis was carried out using the topGO package of Bioconductor (Alexa *et al*, 2006).

Statistical analysis

The data were analysed by unpaired Student's *t*-test and one-way or two-way ANOVA (Analysis Of Variance). Errors are displayed as standard error of the mean (s.e.m.).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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