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A Hippocampal Insulin-Growth-Factor 2 Pathway Regulates the Extinction of Fear Memories

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   25 February 2011

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by two referees and their comments are provided below. As you can see both referees find the analysis interesting. However, there are also significant concerns raised with the present analysis, including that important controls are missing. The issues raised would have to be resolved before further consideration here.

Should you be able to address the raised concerns in full, then we would be willing to consider a revised manuscript. Please note that acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to resolve the main concerns at this stage. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor

The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this study, Agis-Balboa et al. carried out a genome-wide microarray analysis in mouse hippocampal extracts after the first and or several fear extinction trials and consequently investigated the role of two transcripts found to be regulated in extinction, specifically IGF-2 and IGFBP7. They show that extinction of contextual fear memory results in upregulation of hippocampal IGF2 and downregulation of IGFBP7 3 hours after the end of extinction. The authors then showed that intra-hippocampal administrations of IGF2 enhance whereas administrations of IGF2ab or IGF7BP block extinction. Lastly, the authors report that fear extinction specifically promotes survival of 17-19 day-old hippocampal neurons. They conclude that IGF-2 signaling and adult neurogenesis maybe suitable to treat diseases associated with excessive fear memory.
The study presents some interesting correlation, however there are several main issues concerning data interpretations and controls that need to be addressed.

Major issues:
1- The microarray hybridization results are not clearly explained. Given the high error probability of these techniques several replicas should be used. How many replicas were used per experiment?

2- The PCR data performed to confirm the array results are not clear. Were the same hippocampal extracts that were previously used for array hybridizations also used for these PCR or were these independent extracts?

3- The results of microarrays, data analysis and conclusions are presented in a very partial manner. They should be either removed or presented in full.

4- The study focuses on investigating IGF-2. However, a large part of the literature cited in the manuscript refers to IGF-1 and its effects on plasticity or neurogenesis. IGF-2 and IGF-1 may have distinct roles in these functions. The literature citations should be corrected.

5- As IGFBP7 may interact with either IGF-1 or IGF-2, the regulation of both factors should be investigated. How is the concentration of IGF-1 in the E1 and ELF samples? The authors conclude that IGFBP7 down-regulation interacts with in the IGF-2 up-regulation but the two changes may act through different partners, as also mentioned in the discussion. Therefore the conclusions proposed are not sufficiently supported by the presented data.
The premise of the study is that IGFBP-7 plays an opposite role in modulating fear memories than IGF-II. The authors make a point to say that IGFBPs attenuate the actions of IGFs. However, the paper they cite in this regard (Baxter 1999) does not discuss IGFBP-7, only IGFBP1-4. Moreover, IGFBP-7 is thought to bind to IGFs with a much lower affinity than the other IGFBPs, and with even less affinity to IGF-II than to IGF-I (Oh Y 1998). Therefore, to convincingly conclude that IGF-II and IGFBP-7 are indeed interacting, the interaction will have to be shown directly. Additionally, in light of the fact that IGFBP-7 binds more readily to IGF-I, the authors should show whether IGF-I interacts with IGFBP-7 to modulate fear extinction as well.

6- The controls for anti-IGF-II antibody injections should be a non-specific antibody not just vehicle. Controls for IGFBP7 should be an inactive protein in the same concentration.

7- To block IGF-II, the authors use an IGF-II antibody. The paper they cite (Ostrovsky 2009) did not use this antibody functionally. Thus, another method to block IGF-II should confirm their data. Additionally, considering that the neurogenesis data hinges on the binding of IGF-II to the IGF-IR, the authors should determine whether the IGF-IR is required for extinction and for the IGF-II-mediated facilitation of extinction.

8- To block IGF-II, the authors use an IGF-II antibody. The paper cited for this functional use

(Ostrovsky 2009) did not use this antibody as a blocking antibody. Thus, another method to block IGF-II should be use to confirm the data or it should be shown that the antibody is functionally blocking. Additionally, considering that the neurogenesis data hinges on the binding of IGF-II to the IGF-IR, the authors should determine whether the IGF-IR is required for extinction and for the IGF-II-mediated facilitation of extinction.

9- Figure 1D - controls seem to be missing here, or data are interpreted incorrectly. Is there one control group that the FC, E1, and ELF animals are being compared to? If so, then the control group is not controlled for time for the three different groups as they are sacrificed at different time points. If they are each compared to a separate, time-matched control, then they should be presented separately and cannot be compared to one another (i.e. "cFos induction significantly decreased upon ELF exposure, when compared to E1.

10- Figure 2E and 2F - again, controls are not well explained, and appear to be missing. When were the control group sacrificed? Is there a control for a decrease in protein over time, rather than as a result of extinction?

11- Figure 3B - demonstrating conclusively that this is extinction via a reminder shock would be important.

12- Figure 3C - this figure seems to contradict what the authors stated previously as the function of IGFBP-7, namely that it attenuates the actions of IGF-II. If that were the case, then IGFBP-7 injected with IGF-II should prevent IGF-II from facilitating extinction, not the other way around.

13- It should be excluded that treatments have effect on locomotor function.

14- It is puzzling that survival of new neurons is decreased after new fear learning. If new leaning critically involves survival of newly generated neurons, why should they decrease?

15- It is stated that IGF-I receptor is the main receptor through which IGF-II exerts its biological functions. The statement is incorrect. IGF-II exerts its functions via both IGF-II receptors (toward which it has higher affinity) as well as IGF-I receptors.

16- Concerning neurogenesis, the authors refer to literature that is distant from the subject of their study. Out of the five articles that they discussed as motivating them to look at IGF-II and neurogenesis, only one actually showed any role of IGF-II, as the others all referred to IGF-I or insulin. Indeed, in that one article (Leventhal, 1995), the authors show that IGF-II promotes cell growth of a neuroblastoma cell line. The literature should be carefully revised and correctly cited.

Minor issues

-In the abstract the authors state that: "This type of memory function (extinction) is of great clinical importance because the extinction of fear memory is an essential mechanism to treat anxiety disorders". Although extinction is indeed employed in clinical practice, this is an overstatement. It is used to treat phobias and OCD. However, it is not "an essential mechanisms to treat anxiety disorders" in general. I suggest changing the text.

- The manuscript needs editing

Referee #2 (Remarks to the Author):

In this manuscript, the authors investigate the role of different cellular substrates in fear extinction learning. The extinction of fear memories, as observed by the authors, coincides with an up-regulation of IGF2 and a down-regulation of IGFBP7 indicating a possible role of these two substrates in the facilitation of extinction learning. Through the use of microcannulae implantations into the hippocampus, the authors show that inhibiting IGF2 or increasing IGFBP7 leads to an inability of animals to extinct fear memories while adding IGF2 facilitated the extinction of fear memories. The extinction of fear memories was also shown to promote the survival of 17-19 day old newborn neurons. The experiments used in this manuscript were both well designed and effective

and although it has previously been shown that IGF2 plays a critical role in cognitive behavior, this paper highlights specifically the role IGF2 plays in contextual fear conditioning extinction learning. There are, however, some major concerns with the manuscript.

1. One main concern of the paper is the variability of the control animals during extinction trials. Particularly in the microcannulae implantation experiments, the vehicle-treated controls in all of the experiments vary quite a bit. For example, the control animals in fig 4A appear to extinct the fear memory much faster than control animals in fig 4B. By day 5 of extinction trials, fig 4A animals are at roughly 12% freezing whereas fig 4B animals are at roughly 33%. This is also observed between experiments. In fig 7 the control animals appear to extinct the fear memory very fast compared to the other control animals of fig 3, fig 4, and fig 6. Assuming that the fear extinction protocols are the same between experiments, this observation should be explained. Most importantly, the authors should state whether the experimenters were blind to the groups while conducting the behavioral tests.

2. The second main concern is with the last experiment where the authors look at the involvement of 17-19 day old neurons in fear extinction. On pages 10 and 11, the authors discuss cfos expression of these neurons after fear extinction but there is no data to support this claim aside from one picture (fig. 5H). Quantification with cfos expression in these 17-19 day old neurons needs to be included. The discussion section also speculates about the involvement of these immature neurons in fear extinction and the authors should include data to support this statement. One experiment could be to follow the same paradigm as fig 5D except sacrifice the animals immediately after ELF , stain for cfos and look at co-localization of cfos and BrdU. This would provide evidence that the 17-19 day old neurons are activated during extinction.

Minor concerns

1. In the manuscript, the term ELF is defined by "the extinction trial on which the freezing behavior was significantly reduced" but it is confusing (fig 1A) why E3 or E4 are not ELF. Both E3 and E4 seem to be significantly reduced from the original freezing percentage. The term needs to be more clearly stated.

2. In fig 2B, it is interesting to note the mRNA upregulation and downregulation of Igf2 and Igfbp7 at E1 and ELF but it would be helpful to include more time points in between. At which extinction trial does the upregulation of Igf2 stop? At what extinction trial does the downregulation of Igfbp7 start?

3. In fig 2E, the IGF2 protein level at ELF appears to be below control levels. Is this significant? If so, please explain.

4. For the BrdU survival experiments, the number of surviving BrdU/Calbindin cells in control animals for figs 6 and 7 are almost twice as high as surviving BrdU/Calbindin cells in controls of fig 5C-5E. The difference between the experiments is that animals in figs 6 and 7 received intrahippocampal injections whereas animals in fig 5 did not. Given that the BrdU survival paradigms were the same for each experiment, are the intrahippocampal surgeries somehow increasing BrdU cell survival in control animals?

5. On page 11, the authors state... "Our finding that fear extinction can be manipulated by intrahippocampal injection of recombinant IGF1, IGF2ab or IGFBP7 (see Fig. 3&4) shows that IGF2 and IGFBP7 mediate this effecting a paracrine manner." The data from these experiments do not exclude the possibility that IGF2 and IGFBP7 are acting in an autocrine manner.

6. On page 9, the authors state... "Adult neurogenesis is a complex process that consists of two main phases." It would be more appropriate to say 3 main phases: proliferation, differentiation into neurons, and survival of newborn neurons.

7. Include at what age the animals were tested.

8. Include confocal images of BrdU/calretinin staining.

9. There are a number of typos and grammatical errors within the text and figures.

Please find attached the revised version of our manuscript entitled "A HIPPOCAMPAL INSULIN-GROWTH-FACTOR 2 PATHWAY REGULATES THE EXTINCTION OF FEAR MEMORIES" (EMBOJ-2011-77125).

We are grateful that both referees appreciated the significance of our work and are thankful for their insightful comments.

While the questions raised by referee 2 could be adressed in a reasonable time window, referee 1 aksed for some very time consuming additional experiments. We were now able to adress all of the referees concerns. Thus, in the revised version of our mansucript we present 5 novel supplemental figures and various additional panels within the existing figures. All changes made to the text are underlined.

We hope that this substantially revised version of our manuscript will be suitalbe for publication in EMBO J.

Please find below the point-to point rebuttal.

Response to referees.

Referee 1:

Referee 1, points 1-3:

This referee raises 3 points with repespect to the gene-array data presented in our work

1. "The microarray hybridization results are not clearly explained. Given the high error probability of these techniques several replicas should be used. How many replicas were used per experiment?"

2. "The PCR data performed to confirm the array results are not clear. Were the same hippocampal extracts that were previously used for array hybridizations also used for these PCR or were these independent extracts?"

3 "The results of microarrays, data analysis and conclusions are presented in a very partial manner. They should be either removed or presented in full"

We thank this referee for his/her insightful comments. The gene-array was perfomed according to standart procedures as described before (Peleg et al., 2010 Science, 328) using 5 biological replicates/group.
With respect to point 2, this is indeed a very crucial question. We first assessed whether differential expression of genes that were identified by the gene-array could be confirmed via qPCR analysis using the same mRNA samples as used for the array. We did not present this data in the previous version of the manuscript because our study was not desinged as a sole gene profiling experiment and we believed it was of more biological relevance to confirm differential expression of selected genes in an independent experiment. However, we now include qPCR analysis using the same mRNA samples used in the gene-array as a novel supplemental Figure 1 and describe this data on page 6 line 5-6 of the revised mansucript.
We also fully agree that gene-array data is often prone to false positive results. Especially, with respect to point 3 we like to reiterate that we used the gene array approach, being fully aware of the potential experimental pitfalls, to identify interesting genes that would be suitalbe for subsequent analysis. We are convinced that the data presented in our manuscripts verifies such an approach. Thus we argue that the gene-array data should remain within the manuscript, since otherwise the

rational for studying IGF2/IGFBP7 signaling during fear extinction is not obvious. We now explain the this issue in greater detail on page 5, lines 3-4 from bottom and point out potential limitations in greater detail (See also legend of novel Fig S1). If requested by the editor, we could merge the novel Fig.1 with novel supplemental figures S1 & S2 and move this data to supplemental material.

Referee 1, point 4:

This referee says: "*The study focuses on investigating IGF-2. However, a large part of the literature cited in the manuscript refers to IGF-1 and its effects on plasticity or neurogenesis. IGF-2 and IGF-1 may have distinct roles in these functions. The literature citations should be corrected.*"

We apologize for this misunderstanding. Much more literature is available on the role of IGF1 in various cellular processes including adult neurogenesis and cogntive function. We are fully aware that IGF1 and IGF2 may serve distict functions, yet they belong to the same biological signaling pathway. Thus we reasoned that the knowledge on the role of IGF signaling in general may help us to design experiments to study the role of IGF2 in fear extinction. We now explain this issue in greater detail. See page 6, lines 2-5 from bottom.

Referee 1, point 5:

He/she mentions: "*As IGFBP7 may interact with either IGF-1 or IGF-2, the regulation of both factors should be investigated. How is the concentration of IGF-1 in the E1 and ELF samples? The authors conclude that IGFBP7 down-regulation interacts with in the IGF-2 up-regulation but the two changes may act through different partners, as also mentioned in the discussion. Therefore the conclusions proposed are not sufficiently supported by the presented data.*
The premise of the study is that IGFBP-7 plays an opposite role in modulating fear memories than IGF-II. The authors make a point to say that IGFBPs attenuate the actions of IGFs. However, the paper they cite in this regard (Baxter 1999) does not discuss IGFBP-7, only IGFBP1-4. Moreover, IGFBP-7 is thought to bind to IGFs with a much lower affinity than the other IGFBPs, and with even less affinity to IGF-II than to IGF-I (Oh Y 1998). Therefore, to convincingly conclude that IGF-II and IGFBP-7 are indeed interacting, the interaction will have to be shown directly. Additionally, in light of the fact that IGFBP-7 binds more readily to IGF-I, the authors should show whether IGF-I interacts with IGFBP-7 to modulate fear extinction as well."

Although the data presented in the original manuscript in our opinion provides strong evidence that IGFBP7 affects fear extinction, at least in part, in an IGF2-dependent manner we now conducted a number of additional experiments in order to adress this referees concerns.

1. We measured the expression of Igf1, Igf1 receptor, Igfbp4 and Igfbp5 (which are prominently expressed in the mouse hippocampus; see Allen brain atlas) during fear extinction. We now show that in contrast to Igf2, expression of these genes was not altered upon extinction training. This data is now presented as a novel supplemental figure 5 and discussed within the text of the revised manuscript. See page 7, lines 13-15 and novel supplemental figure 5 of the revised manuscript and novel

2. We now adresses the role of IGF1 during fear extinction directly via two independent experiments. First we injected an IGF1 blocking antibody (IGFab) into the hippocampus of mice that underwent fear extinction training. IGF1ab did not affect fear extinction, suggesting that in contrast to IGF2 endogenous IGF1 plays a minor role during fear extinction. Second, IGF1 was injected into the hippocampus of mice that were subjected to fear extinciton training. In contrast to the the role of IGF2, increasing IGF1 levels during fear extinciton had no effect. This data is now presented as a novel supplemental figure 7 and suggests that IGF2 but not IGF1 plays the major role in the extinction of fear memories. See alsopage 8 lines 1-3 from bottom and page 9 lines 1-2 from top of the revised manuscript.

3. We tested whether IGF1 could rescue the effect of IGFBP7 on fear extinction. In contrast to IGF2, IGF1 could not rescue IGFBP7-mediated impairment of fear extinction. This data is now presented within a novel supplemental figure 7B, page 8 lines 1-3 from bottom and page 9 lines 1-2

from top of the revised manuscript.

4. We also add more specific references on the role of IGFBP7 in IGF signaling. See page 6 lines 2-5 from bottom.

Referee 1, point 6:

The referee says. "*The controls for anti-IGF-II antibody injections should be a non-specific antibody not just vehicle. Controls for IGFBP7 should be an inactive protein in the same concentration."*

We now provide novel data showing that intrahippocampal injection of IGF1 and IGF1ab does not affect fear extinction (see response to point 5). Thus, it is unlikely that the effect of IGF2 and IGFBP7 injections is due to unspecific effects of the injection procedure. In addition, the fact that IGF2 and IGFBP7 injection affect fear extinction in a opposite manner, either facilitate or enhance fear extinction, also argues against an unselective effect of the injection procedure. We discuss this issue now in greater detail on page 8, lines 1-3 from bottom and page 9 lines 1-2 from top of the revised manuscript. (See also response to point 5 raised by this referee).

Referee 1, point 7:

He/she says. "*To block IGF-II, the authors use an IGF-II antibody. The paper they cite (Ostrovsky 2009) did not use this antibody functionally. Thus, another method to block IGF-II should confirm their data. Additionally, considering that the neurogenesis data hinges on the binding of IGF-II to the IGF-IR, the authors should determine whether the IGF-IR is required for extinction and for the IGF-II-mediated facilitation of extinction."*

The IGF2 antibody IGF2ab used in our study blocks the bioactivity of IGF2 as indicated in the antibody datasheet (see Material and Methods section). Moreover, we now provide novel data showing that IGF2 but not IGF1 is required for fear extinction, further demonstrating the specificity of our findings. In addition we provide novel data showing that blocking hippocampal IGF1 receptor prevents fear extinction. We describe these issue now in greater detail on page 8, lines 1-3 from bottom and page 9 lines 1-2 from top, page 12, lines 14-17 and novel supplemental figure S11.

Referee 1, point 8:

He/She say: "*To block IGF-II, the authors use an IGF-II antibody. The paper cited for this functional use (Ostrovsky 2009) did not use this antibody as a blocking antibody. Thus, another method to block IGF-II should be use to confirm the data or it should be shown that the antibody is functionally blocking. Additionally, considering that the neurogenesis data hinges on the binding of IGF-II to the IGF-IR, the authors should determine whether the IGF-IR is required for extinction and for the IGF-II-mediated facilitation of extinction."*

This comment is identical to the concern raised under point 7, we understand that this is a mistake due to "copy & paste" editing.

Referee 1, point 9 & 10:

Referee 1 makes two comments with respect the control groups. These comments are related and thus will be answered together:

"*Figure 1D - controls seem to be missing here, or data are interpreted incorrectly. Is there one control group that the FC, E1, and ELF animals are being compared to? If so, then the control group is not controlled for time for the three different groups as they are sacrificed at different time points. If they are each compared to a separate, time-matched control, then they should be presented separately and cannot be compared to one another (i.e. "cFos induction significantly decreased upon ELF exposure, when compared to E1."*

"*Figure 2E and 2F - again, controls are not well explained, and appear to be missing. When were the control group sacrificed? Is there a control for a decrease in protein over time, rather than as a*

result of extinction?"

We apologize for any misunderstanding and describe this issue now in greater detail. As for the control group used in Figs 1 & 2, all mice were sacrificed at the same time, since experiments were started accordingly. Since in pilot experiments we did not observe signifcant differences in cFos expression of mice that were subjected to the conditioning context either twice (resembling E1) or 5 times (resembling E5) we pooled this control group in for the gene array experiment. As for the FC groups we do have a separate control group to which the FC data had been normalized. We now present the FC data separately within the novel Fig. S2.

Most importantly, in former Fig S1 (now Fig. S3 of the revised manuscript) we showed that the downregulation of IGFBP7 (mRNA & protein) during fear extinction is not due the passing of time but depends on extinction training.

We now also present novel data to show that the expression of IGF2 mRNA and protein is not due to context exposure or FC training but specifically requires fear extinction training. This data is now presented as a novel panel in novel Fig S2 C&D (Fig S1 in the previous mansucript).

We dicuss this issue now in greater detail on page 5, lines 5-8 & lines 3-6 from bottom.

Referee 1, point 11:

He/She say: *"Figure 3B - demonstrating conclusively that this is extinction via a reminder shock would be important."*

With respect to Figure 3B, this data shows that intrahippocampal injection of IGF2ab impaires fear extinction. Thus, these animals show already high freezing behavior and a reminder shock treatment (that would not further increase freezing behavior in this case) would not yield new insight.

We have previously shown that the employed fear extinction paradigm does not erase the fear memory trace by using a reminder shock procedure that could reinstate freezing behavior (e.g. see Fischer et al., J Neurosci 2004, 24; Sananbenesi et al., Natute Neurosci, 2007, 10). Thus we are confident that our experimental paradigm is suitable to investigate fear extinction.

Referee 1, point 12:

This referee says: *"Figure 3C - this figure seems to contradict what the authors stated previously as the function of IGFBP-7, namely that it attenuates the actions of IGF-II. If that were the case, then IGFBP-7 injected with IGF-II should prevent IGF-II from facilitating extinction, not the other way around."*

We appreciate this comment, however we have to interpret the data we obtained. We show that IGFBP7 impaires fear extinction. Because blocking endogenous IGF2 but not IGF1 (see novel supplemental figure 7) impaires fear extinction this data indicates that IGFBP7 may mediate this effect via IGF2. In our experimental setting we can obviously overcome the inhibitory effect of IGFBP7 by administration of recombinant IGF2 but not IGF1 when used in the same concentration.

We now discuss this in greater detail on page 9, lines 9-14.

Referee 1, point 13:

He/she says: *"It should be excluded that treatments have effect on locomotor function"*

We now provide novel data showing that none of the treatments affect locomotor function (See novel supplemental figure 8 and decribe this in the revised manuscript (page 9, line 14).

Referee 1, point 14:

He/she says: *"It is puzzling that survival of new neurons is decreased after new fear learning. If new leaning critically involves survival of newly generated neurons, why should they decrease?"*

We appreciate this comment. Indeed numerous data suggested that various types of memories required hippocampal neurogenesis. However, depending on the experimental paradigm it was also found that exposure of mice to fear conditioing could transiently impair neurogenesis (e.g. see Pham et al., 2005 Neuroscience, 130). We now discuss this issue in greater detail on page 11, lines 7-10.

Referee 1, point 15:

He/she says: *"It is stated that IGF-I receptor is the main receptor through which IGF-II exerts its biological functions. The statement is incorrect. IGF-II exerts its functions via both IGF-II receptors (toward which it has higher affinity) as well as IGF-I receptors."*

We appreciate this insightful comment and apologize for any misunderstanding. We were referring to the fact that the majority of the literature shows that the major role of IGF2 receptor is to clear IGF2 and to attenuate its signaling. We now rephrase this point on page 12, lines 14-17.

We also cite a recent manuscript showing that blocking IGF2 receptor in the hippocampus impairs memory formation (Chen et al., Nature 2011). We used the same strategy to block IGF2 receptor during fear extinction and found that IGF2 receptor is not required for fear extinction. This data is now provided as a novel supplemental figure 11.

Referee 1, point 16:

The referee states: *"Concerning neurogenesis, the authors refer to literature that is distant from the subject of their study. Out of the five articles that they discussed as motivating them to look at IGF-II and neurogenesis, only one actually showed any role of IGF-II, as the others all referred to IGF-I or insulin. Indeed, in that one article (Leventhal, 1995), the authors show that IGF-II promotes cell growth of a neuroblastoma cell line. The literature should be carefully revised and correctly cited."*

The cited literature refered to the role of IGF signaling in general since less is known on the role of IGF2 when compared to the availabe data on IGF1. We now cite additional literature and describe this issue in even greater detail. See page 10, line 2.

Referee 1 mentions two addition points.

-In the abstract the authors state that: "This type of memory function (extinction) is of great clinical importance because the extinction of fear memory is an essential mechanism to treat anxiety disorders". Although extinction is indeed employed in clinical practice, this is an overstatement. It is used to treat phobias and OCD. However, it is not "an essential mechanisms to treat anxiety disorders" in general. I suggest changing the text.

We changed the abstract accordingly.

- The manuscript needs editing

The manuscript has been prove readen by a native speaker.

Referee #2 (Remarks to the Author):

This referee states that our experiments are "well designed and effective" and asks some specific qustions.

Referee 2, point 1:

He/she feels that *"One main concern of the paper is the variability of the control animals during extinction trials. Particularly in the microcannulae implantation experiments, the vehicle-treated controls in all of the experiments vary quite a bit. For example, the control animals in fig 4A appear to extinct the fear memory much faster than control animals in fig 4B. By day 5 of extinction trials,*

fig 4A animals are at roughly 12% freezing whereas fig 4B animals are at roughly 33%. This is also observed between experiments. In fig 7 the control animals appear to extinct the fear memory very fast compared to the other control animals of fig 3, fig 4, and fig 6. Assuming that the fear extinction protocols are the same between experiments, this observation should be explained. Most importantly, the authors should state whether the experimenters were blind to the groups while conducting the behavioral tests. "

We thank this referee for this insightful comment. All experiments were conducted by researchers blind to the treatment. In addition, we would like to point out that the employed extinction paradigm consistently leads to significant reduction of learned fear and has been successfully used in numerous laboratories - including ours - to unravel the molecular mechanisms of fear extinction (Marsicano et al., 2000, Nature 418; Kamprath et al., 2004, Learning & Memory, 11; Myers & Davis, 2007, Mol Psychiatry. 2; Sananbenesi et al., 2007, Nature Neuroscience, 10). As such, while independent experiments might show slightly different dynamics, every experiment is always compared to a control group that has been treated at the same time under the same conditions. Thus, the dynamics of fear extinction should only be compared amongst the groups of a given experiment.

It is important to note that mice are likely to show variation in response to behavior paradigms due housing conditions, handling etc. (Crabbe et al., 1999, Science, 284; Wahlsten et al., 2003, J. Neurobiol, 54; Lewejohann et al., 2006, Genes Brain Behav., 5). Although we try to control housing, handling etc. as much as possbible many factors may affect the behavior of a given group of mice and it has to be reiterated that our experiments have not been conducted at the same time but througout the time course of 3 years.

For example in a recent work we described the function of a hippocampal Rac1-Cdk5-Pak1 signaling cascade during fear extinction (Sananbenesi et al., Nature Neuroscience, 2007). In this manuscript the dynamics of fear extinction among the vehicle treated groups in figures 1 and 3 are also slightly different. We describd this issue within the methods and material section of the manuscript (Sananbenesi et al., 2007 Nature Neuroscience, 10, see page 1018 under METHODS/Animals). Importantly, independent of small variations among individual experiments our data demonstrated that Cdk5 regulates fear extinction, a findings that has been confirmed (although the dynamics of fear extinction were different) by others using Cdk5 knock out mice of a different strain background in a different laboratory (Hawsali et al, Nature Neuroscience, 2007, 10). Simlar variations in the dynamics of fear extinction amongst individual experiments can be foudn througout the literature; e.g. see Huk et al. Learn & Memory (2009) and compare freezing levels of the control groups throughout experiments.

We now adress this issue more specifically in the material and methods section. See page 17, line 16-19 from bottom in the revised manuscript.

Referee 2, point 2:

He/She says that: *"The second main concern is with the last experiment where the authors look at the involvement of 17-19 day old neurons in fear extinction. On pages 10 and 11, the authors discuss cfos expression of these neurons after fear extinction but there is no data to support this claim aside from one picture (fig. 5H). Quantification with cfos expression in these 17-19 day old neurons needs to be included. The discussion section also speculates about the involvement of these immature neurons in fear extinction and the authors should include data to support this statement. One experiment could be to follow the same paradigm as fig 5D except sacrifice the animals immediately after ELF , stain for cfos and look at co-localization of cfos and BrdU. This would provide evidence that the 17-19 day old neurons are activated during extinction."*

The a im of the described experiment was to show that 17-19 day old neurons are able to respond to an environmental stimulus. However, we agree with this referee that quantification cFOS/BrdU postive cells would be a meaingful experiment. Since our gene-expression analysis data indicates that cFos gene expression is induced upon E1 but not upon ELF exposure, we quantified the number of 17-19 day old cFOS/BrdU positive cells in mice upon E1 exposure. In line with the interpretation of our data we find that the number of cFOS/BrdU positive cells is enhanced upon E1 exposure when compared to the FC-group suggesting that new born neurons are activiated upon extinction

training. This data is now presented as a novel panel of Figure 5 (Figure 5I) and described within the text of the revised mansucript, see page 12, lines 2-4.

Referee 2 raises some minor concerns.

Referee2, Minor point 1:

He/she says: *"In the manuscript, the term ELF is defined by "the extinction trial on which the freezing behavior was significantly reduced" but it is confusing (fig 1A) why E3 or E4 are not ELF. Both E3 and E4 seem to be significantly reduced from the original freezing percentage. The term needs to be more clearly stated"*

We apologize for any misunderstanding. We now descibe this issue in greater detail and point to the fact that the term ELF could be suitable to define extinction trial that are suitable to perfom molecular analysis. However, we agree that in a pure behavioral experiment the term ELF makes less sense and we changed the revised masnucript accordingly. See page 5 lines 14-16 of the revised manuscript.

Referee2, Minor point 2:

He/she remarks that: *"In fig 2B, it is interesting to note the mRNA upregulation and downregulation of Igf2 and Igfbp7 at E1 and ELF but it would be helpful to include more time points in between. At which extinction trial does the upregulation of Igf2 stop? At what extinction trial does the downregulation of Igfbp7 start?"*

This is very good suggestion. However, taking into account that we are investigating a complex biological system in vivo we reasoned that the most straightforward approch to gain insight about the molecular mechansims would be to compare to clearly distinguishable time points, in this case E1 vs. ELF. In future research we are planning to investigate the precises mechanisms by which the expression of Igf2 and Igfbp7 is regulated during fear extinction. We discuss this issue within the revised version of our mansucript, see page 14, line 5 from bottom.

Referee2, Minor point 3:

He/she states: *"In fig 2E, the IGF2 protein level at ELF appears to be below control levels. Is this significant? If so, please explain."*

Quantification of the immunoblot data showed that IGF2 levels do not signifincantly differ upon ELF exposure (See Figure 2E in the original and revised mansucript).

Referee 2, Minor point 4:

This referee points out that: *"For the BrdU survival experiments, the number of surviving BrdU/Calbindin cells in control animals for figs 6 and 7 are almost twice as high as surviving BrdU/Calbindin cells in controls of fig 5C-5E. The difference between the experiments is that animals in figs 6 and 7 received intrahippocampal injections whereas animals in fig 5 did not. Given that the BrdU survival paradigms were the same for each experiment, are the intrahippocampal surgeries somehow increasing BrdU cell survival in control animals?"*

We thank this referee for this comment. As depicted in the experimental design in Fig 5C-E "control" in this case stands for animals that received BrdU injection and were sacrificed 42 days later without any behavioral manipulation.
Such a group does not exist in the experiments depicted in Figs 6 & 7. Here the "vehicle-group" is a group of mice that received BrdU injection and was subjected to fear extinction training 14 days later. During extinction training this group received vehicle injection while the experimental groups received IGF2 or IBFBP7 injection.
Although the experiments depicted in Figs 6 & 7 and Fig 5 should not be compared directly (one group received intrahippocampal injection and one not), the "vehicle-group" in Figs. 6 & 7 would be most similar to the FC/E1-ELF-groups in Fig. 5D. Please note that the number of surviving cells is similar amongst these groups. This data suggests that the surgery and injection procedure by

intself does not severly affect neuronal surival.
We discuss this issue now in greater detail on page 12, lines 1-4 from bottom of the revised manuscript.

Referee 2, Minor point 5:

He/she says: *"On page 11, the authors state... "Our finding that fear extinction can be manipulated by intrahippocampal injection of recombinant IGF1, IGF2ab or IGFBP7 (see Fig. 3&4) shows that IGF2 and IGFBP7 mediate this effecting a paracrine manner." The data from these experiments do not exclude the possibility that IGF2 and IGFBP7 are acting in an autocrine manner."*

We now add this point in the revised version of our manuscript. See page 12, lines 6-8

Referee 2, Minor point 6:

He/she point out that: *"On page 9, the authors state... "Adult neurogenesis is a complex process that consists of two main phases." It would be more appropriate to say 3 main phases: proliferation, differentiation into neurons, and survival of newborn neurons."*

We thank this referee for the insightful comment and rewrote the sentences as suggested. See page 10 line 8 of the revised mansucript.

Referee 2, Minor point 7:

He/she requests: *"Include at what age the animals were tested."*

All animals were tested at 3 month of age. We now specifically state this in the revised version of the manuscript. Page 18, line 1.

Referee 2, Minor point 8:

He/she requests:. *"Include confocal images of BrdU/calretinin staining"*

We now include this data within novel supplemental Figure 10 of the revised mansucript.

Referee 2, Minor point 9:

This referee says *" There are a number of typos and grammatical errors within the text and figures."*

We apologize and now carfully checked the revised version of this mansucript for typos.

2nd Editorial Decision   18 July 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees to review the revised manuscript and I have now received the comments.

While referee #1 still express concerns with the paper, referee #2 appreciates the carried out revisions and supports publication here. Having looked closely at the remaining concerns raised by referee #1, I find that they can be addressed with appropriate text changes: Point #1, you have already adressed this issue in the text and in the previous point-by-point response and so there is no need to do anything further regarding this point. Point #2 and 4 please clarify/respond to this with appropriate text changes either in the manuscript file or in the point-by-point response. Point #3, as far as I can see you do use an IGF1 receptor antagonist in Fig S11 and so this point should resolved. Please also respond to the minor concerns raised.

Once we receive the revised manuscript we will proceed with its acceptance for publication here.

Best wishes

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This resubmission by Agis-Balboa et al. has addressed only partially the issues raised in the previous reviews. Unfortunately there are still major flaws with the study.

1- The validation of the array analysis with qPCR is not in agreement with the arrays' data. It remains unclear whether the array experiments and analyses are technically sound. Although qPCR may indeed pick up additional changes, if the qPCR data are in disagreement with the arrays' data the validation is not confirmed. Although any screening may reveal a partial list of genes, from which one can follow specific candidates, the results need to be validated.

2- One critical issue is that IGF2 and IGFBP7 have no known functional links. In this study, they are found differentially regulated in two very different conditions, one is after the first extinction trial and the other is after the 5th extinction trial 4 days later. Thus, the fact that they are part of the IGF family seems to be the only known commonality. The manuscript emphasizes an association that yet needs to be proven, and the data showing their regulation at different time points and after different behavioral experiences is not supporting any functional link. Hence there is no IGF2/IGFBP7 pathway (stated in the manuscript), as it still needs to be demonstrated and the effects of IGF2 and IGFBP7 on extinction could be totally distinct and affect distinct pathways. The fact that IGF2 rescues the blocking effects of IGFBP7 does not prove that they target the same pathway. IGF2 may be independently activating the system targeting a downstream mechanism or different mechanisms that are able to overcome the deficit.

3- The IGF2-mediated effect on extinction was not characterized. The authors suggest that it occurs via IGF1 receptors (page 13) but no experiments are included to support this hypothesis. Blocking IGF1 receptors can test this hypothesis.

4- It's curious that IGF1R inhibitor blocks extinction, however IGF1 does not enhance it and does not rescue the IGFBP7-mediated impairment. Does the IGF2-function blocking antibody used in the study also recognize and bind IGF1?

Minor issues

1- In Figure 2, it is unclear from the text or the figure legend what the controls were. Are these the mice that were exposed to the conditioning context without foot shock or mice that were sacrificed at the same time points.

2- The language used is at times unclear. For example, in the abstract it is stated ""we show that inhibition of contextual memory initiates..." to refer to extinction.
This language is too general and does not inform what the "inhibition" refers to. The manuscript would benefit from editing.

3- Numerous labeling mistakes are found in the main text. The authors write Figure 1D when they mean 2D, they write 2E when they mean 2E&2F, or when they meant IGF2R when they write IGF2 etc. There were numerous font changes in the references throughout the text. Some Figures are not labeled.

4- Page 6, the reference Chen et al. 2011b referred to IGF-1 upregulation in the hippocampus is not in the Reference list. Furthermore, Chen et al. 2011 Nature is reported twice in the Reference list.

Referee #2 (Remarks to the Author):

The authors have addressed the previous concerns and made some good revisions to the manuscript.
I feel that it is good enough to be published in EMBO.

2nd Revision - authors' response   21 July 2011

We are very thankful that the referees now feel that our manuscript would be suitable for publication
in EMBO Journal.

While referee 2 says that we "have addressed the previous concerns and made some good revisions
to the manuscript", referee 1 mentions some points which have however already been addressed in
the previous revision and thus required only minor changes to the text.

As suggested please find the point-to-point rebuttal with respect to point 2, 3 & 4 and the minor
issues pointed out by referee 1.

We hope that this revised version of our manuscript will be suitable for publication in EMBO J.

The entire text excluding references includes 54,700 character (including spaces), 7 figures and
supplemental material including 12 supplemental figures and 1 supplemental table.

Referee 1, point 2:
*"2- One critical issue is that IGF2 and IGFBP7 have no known functional links. In this study, they
are found differentially regulated in two very different conditions, one is after the first extinction
trial and the other is after the 5th extinction trial 4 days later. Thus, the fact that they are part of the
IGF family seems to be the only known commonality. The manuscript emphasizes an association
that yet needs to be proven, and the data showing their regulation at different time points and after
different behavioral experiences is not supporting any functional link. Hence there is no
IGF2/IGFBP7 pathway (stated in the manuscript), as it still needs to be demonstrated and the
effects of IGF2 and IGFBP7 on extinction could be totally distinct and affect distinct pathways. The
fact that IGF2 rescues the blocking effects of IGFBP7 does not prove that they target the same
pathway. IGF2 may be independently activating the system targeting a downstream mechanism or
different mechanisms that are able to overcome the deficit."*

As pointed out in the previous revision of our manuscript up-regulation of IGF2 and down-
regulation of IGFBP7 would eventually both increase IGF2 signaling. Since fear extinction is a
dynamic process that occurs throughout subsequent days of re-exposure we propose that the
hippocampal circuitry achieves prolonged elevation of IGF2 signaling via the initial up-regulation of
IGF2 and eventually induce longer-lasting changes that involve down-regulation of IGFBP7. As
stated in the previous version of our manuscript it would be interesting to study the precise
molecular mechanisms by which Igf2 and Igfbp7 genes are regulated during fear extinction and we
have already started to address this issue. However, we feel that to unravel the precise mechanisms
by which Igf2 and Igfbp7 are regulated during fear extinction would certainly be beyond the scope
of the current manuscript.
Moreover, we clearly state in our manuscript that IGFBP7 may also affect fear extinction via
mechanisms that do not involve IGF2 signaling. The most likely candidate would be IGF1.
However, in the previous revision of our manuscript we showed that IGF1 does not affect fear
extinction and cannot rescue the effect of IGFBP7.
We now discuss this issue in even greater detail to avoid any possible confusion.
See page 9, lines 15-17 from bottom.

Referee 1, point 3:

"3- The IGF2-mediated effect on extinction was not characterized. The authors suggest that it

occurs via IGF1 receptors (page 13) but no experiments are included to support this hypothesis. Blocking IGF1 receptors can test this hypothesis."

Referee 1 may have overlooked that we had already provided the requested experiment within novel supplemental figure 11 in the previously revised manuscript.

Referee 1, point 3:

"4- It's curious that IGF1R inhibitor blocks extinction, however IGF1 does not enhance it and does not rescue the IGFBP7-mediated impairment. Does the IGF2-function blocking antibody used in the study also recognize and bind IGF1?"

As pointed out in the previous version of our manuscript the IGF2 blocking antibody was selected for its ability to neutralize specifically the bioactivity IGF2. Even if one would assume the very unlikely possibility that the IGF2 blocking antibody would affect the function of IGF1 rather than IGF2, we provide multiple lines of evidence that IGF2 and not IGF1 affects fear extinction and fear-extinction mediated neuronal survival. As described in our manuscript it is well-established in the literature that IGF2 can mediate its biological function via IGF1-receptor. Thus it appears that under physiological conditions of fear extinction, the action of IGF1 is tightly controlled, probably by the binding to highly expressed IGFBP other than IGFBP7.

We now address this issue in even greater detail. See page 15, lines 10-13 of the revised manuscript.

Minor issues

"1- In Figure 2, it is unclear from the text or the figure legend what the controls were. Are these the mice that were exposed to the conditioning context without foot shock or mice that were sacrificed at the same time points."

As already pointed out in the previous revision (See response to referee1 point 9&10) we had addressed this point but now describe this in even greater detail greater detail on page 5, line 8.

"2- The language used is at times unclear. For example, in the abstract it is stated "we show that inhibition of contextual memory initiates..." to refer to extinction.
This language is too general and does not inform what the "inhibition" refers to. The manuscript would benefit from editing."

We now checked the manuscript carefully and avoid the phrase "inhibition of contextual fear memory".

3- Numerous labeling mistakes are found in the main text. The authors write Figure 1D when they mean 2D, they write 2E when they mean 2E&2F, or when they meant IGF2R when they write IGF2 etc. There were numerous font changes in the references throughout the text. Some Figures are not labeled.

We apologize for any editing mistakes. This has now been corrected.

4- Page 6, the reference Chen et al. 2011b referred to IGF-1 upregulation in the hippocampus is not in the Reference list. Furthermore, Chen et al. 2011 Nature is reported twice in the Reference list.

We have now corrected the list of references