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Steroid-induced microRNA *let-7* acts as a spatio-temporal code for neuronal cell fate in the developing Drosophila brain

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(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 10 June 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees appreciate the findings reported, but significant concerns are also raised regarding many of the findings reported. The referees indicate that extensive additional work would be needed to consider publication here. Should you be willing and able to address the concerns in full then we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the raised 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, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, but given that extensive work is needed I can offer to extend the revision time to 6 months. If you would like an extension just let me know.

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

Yours sincerely

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Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This study demonstrates a cell-autonomous requirement of let-7 miRNA for proper axon guidance of the pupal-born a/b neurons in the Drosophila mushroom bodies. It further establishes a model, linking ecdysone hormone through let-7 and abrupt (negatively regulated by let-7) to up-regulation of FasII, for temporal regulation of MB neuronal morphogenesis. However, the involvements of ecdysone and abrupt in MB morphogenesis were poorly characterized, leaving their interactions with let-7 in the specification of various MB neurons unresolved. In addition, systematic efforts should be taken to better assess cell fate changes versus morphogenetic defects.

First, let-7 MBN clones should be thoroughly characterized to reveal their clone sizes and neuron type compositions (which is possible with diverse subtype-specific MB drivers), to critically examine the implicated role of let-7 in MB temporal fate specification. In addition, it is unclear why let-7 MBN clones induced at L3 show less severe a/b abnormalities than clones generated earlier or later (Table S6), given that let-7 is required cell-autonomously in individual a/b neurons.

Second, ecdysone is required before the production of a/b neurons for a/b lobe development, as suggested from stage-specific inhibition of ecdysone production. This argues for an undocumented role for ecdysone signaling in MB temporal fate transition, contradictory to earlier findings with USP clones or EcR mutants. It is therefore important to scrutinize the phenotypes in further detail by determining MB neuron type compositions and their birth dates, to substantiate its role in MB temporal fate change from a'/b' to a/b. One should also examine where ecdysone acts to govern MB a/b lobe development.

Third, one could not really determine if lack-of-ecdysone has elicited a similar spectrum of a/b lobe defects as loss-of-let-7 without detailed phenotypic analysis or single-cell labeling.

Fourth, one should try to rescue the lack-of-ecdysone a/b lobe defects with targeted expression of transgenic let-7 to directly examine their possible linear actions in the regulation of MB a/b lobe development.

Fifth, as shown in Figure S6, ab MBN clones induced at L3 or earlier acquired complex phenotypes with abnormal and rather thin a/b projections, paradoxical to the argument that ab is dispensable to MB a/b development. Subtype-specific drivers are again needed to better assess neuron type compositions in ab MBN clones.

Sixth, it is not clear from Table S6 what really happened to those ab mutant single-cell clones generated at L3. There was even no information about how many ab single-cell versus MBN clones were obtained at L3.

Seventh, the ab MBN clone induced at L1 (Figure 6E) showed ectopic FasII in cell bodies of an apparently very small clone, arguing much more complex roles of ab in MB neurogenesis which were not mentioned or addressed at all. In addition, how could one know for sure these residual neurons are early-born neurons without birthdating with BrdU, given that NB clones may contain neurons born through the lineage development? Given the complexity of loss-of-function clones, one should also examine the suppression of FasII expression by ectopic ab using various a/b-specific MB drivers.

Referee #2

This manuscript explores the role of the miRNA let7 and one of its target, abrupt, in the formation of the Drosophila mushroom body neurons. The data are generally convincing and add up to an

interesting, novel and important story. Suggestions:
1.A semantic issue: the cluster of MB neurons and neuroblast is not called the calyx; the calyx is the neuropile domain that contains the dendrites of the mushroom body neurons. The cell body cluster has no specific name attached to it ("MB cell body cluster")
2.A text and diagram that details the anatomical differences between the lbes, and the succession in which the 3 neuron classes are produced (like the diagram in Fig.2) should come at the very beginning; already the Intro should explain these details, so that the reader is well equipeed with foreknowledge when diving into the data.
3.The in situ of let7, shown in supplementary figure S1, is disappointingly faint. Only looking at the photographs, I would not even be convinced it is real. Are there no better data using fluorescent probes? It also would be nice to show the time course of expression, for which now we always rely on the driver line
4.when the term "let 7 loss of function" is used: does this refer to a homozygous mutation in the gene, that results in the MB phenotype shown? The reader should have that info without referring to the Materials section.
5.My main problem concerns aspects of the phenotype and, related to that, the interpretation of let7 function.
-in Fig.1H, L (the "mutant") it looks as if only the b lobe is much thinner; the a lobe looks OK. This would imply a branching defect: normally, an a/b neuron, or a'/b' neuron, has a branch in both corresponding lobes; in the mutant, one of these branches is (presumably) absent in the majority of the a/b neurons. This would better be characterized as a differentiation defect: the "specification" is OK, given that the other lobe (a) is normal in size. This confusion is tangible throughout several parts of the text. Fore example Fig.4G: overexpression of abrupt in a/b neurons results in the absence of the a, but not the b lobe! These are branches of the same neuron; on is lost. The authors should re-think the wording of their interpretation, and its implications.
-At the same time, the existing literature on this kind of MB defects should be introduced from the beginning: I believe that published mutations in many genes, including fasII, result in such branch defects; there is also, if I remember correctly, a literature on separable behavioral functions of the a vs b lobe.
-Is the loss of one vs the other branches consistent? It looks as if in the let7 mutant in panel 1 H, mostly the b lobe is affected; in 1 F, it is the a lobe (or both lobes?).
6.clones: a nice result. Question: how can one interpret a'/b' neurons resulting from inductions in the pupa? I thought only a/b neurons are generated during this phase? One related question/suggestion: did the authors convince themselves thoroughly that only heat pulses induced the clones? I believe from proactical experience that it is very rare that one have that sort of control, and no leakiness. Were never any clones recovered in controls without heat pulse?

Referee #3

The authors describe a function for the Drosophila let-7 miRNA in temporal patterning of neuronal subtypes in identified neuroblast lineages of the mushroom body (MB). Using a let-7-complex Gal4 line, the authors show specific MB expression which is upregulated following the pupal ecdysone peak. Ecd temperature shifts imply that ecdysone signaling is directly or indirectly required for alpha/beta neurons and that it upregulates let-7 in MB lineages. They also demonstrate a specific let-7 requirement for the specification or morphogenesis of late alpha/beta but not earlier alpha'/beta' neurons. This is associated with a reduced olfactory learning index in the adult fly. In the second part of the paper, the authors dissect the molecular targets that mediate the effects of let-7 on MB lobe development. They show that the expression of Let-7-c-Gal4 is complementary to that of Abrupt (Ab), and that let-7 overexpession represses Ab. Overexpressing Ab also affects alpha/beta neuron development, while autonomous loss of Ab restricts neurons to the late alpha/beta fate. Loss of Ab also rescues the alpha/beta phenotype of the let 7 complex mutant. This argues that Ab is an important target of Let7 for the specification of MB lineages. Finally, the authors correlate the extent of alpha/beta morphogenesis observed in let-7 or Ab manipulations with levels of the cell adhesion molecule FasII, leading to a linear model for MB neurogenesis that culminates surprisingly in differential adhesion regulating chronological neurogenesis.

The let-7 miRNA has already been studied in Drosophila in some detail. However, this manuscript describes for the first time to my knowledge, evidence for a temporal patterning function in the context of identified neuroblast lineages. It therefore potentially adds a valuable new component to

the temporal patterning model in MBs. Nevertheless, in my opinion, the data (as they currently stand) fall short of providing watertight evidence for the model in Fig.7, thus leaving alternative possibilities open. There are also some cases of data over interpretation. All this uncertainty is revealed in the Fig. 7 model, which is little more than a summary and lacks any kind of real genetic wiring diagram. A revised manuscript would need to address thoroughly all of the criticisms below. Where additional experiments are requested, these are only intended to test or to lend support to the authors existing conclusions and not to explore new ones.

MAJOR POINTS

1. The evidence that FasII mediates any aspect of let-7 function relevant to the temporal switch between alpha'/beta' to alpha/beta neurons is not strong enough. Moreover, there appears to me to be over interpretation on p.13: "These data allow us to conclude that alpha/beta neuron specification depends upon precise expression levels of the cell adhesion protein FasII that is spatially and temporally modulated by the ecdysone-induced miRNA let-7 and its target, the transcription factor Ab". Breaking this down into two issues:
a) The evidence that let-7 regulates FasII in a cell autonomous manner should be strengthened. Yes, the observed reductions in FasII mRNA (Fig.6A) and FasII antibody staining will of course correlate with the overall numbers of FasII-expressing neurons. However, in Fig. 1H it appears that the alpha and even the few beta neurons that remain in let-7 mutant brains are still able to express FasII. This suggests that the effect may therefore be very indirect. It is possible that single cell resolution analysis of FasII expression in let-7 MARCM clones might help here.
b) Is the FasII phenotype really a downstream subset of the let-7 phenotype? More phenotypic analysis is required to demonstrate that there are common features of these two phenotypes in the alpha'/beta' to alpha/beta switch and to support the linear model in Fig7 (where differential adhesion regulates chronological neurogenesis) and in the p.13 statement cited above. Given that Let-7c-Gal4; UAS-FasII-RNAi didn't generate a statistically significant alpha/beta lobe phenotype (although it did affect the midline structure), it is not clear that the current evidence supports the authors conclusions about the role of adhesion in mediating the effects of Let-7 or Ab. It is possible that additional carefully controlled epistasis tests with FasII and let-7 etc. might be insightful here in distinguishing how let-7, Ab, and FasII are genetically wired up. Regarding the role of Ab, I am also confused as to how this links with let-7 as let-7-C-Gal4; UAS-ab appears to primarily affect alpha neurons (Fig.4G) whereas let-7 deletion appears to primarily affect beta neurons (Fig 1H).

2. Is the primary function of let-7 in MB lineages really temporal patterning? The let-7 deletion phenotype shown in Fig.1G.H and quantified in table S1 shows a much more dramatic effect on beta morphology/volume than on alpha. If the primary role of let-7 really is in temporal patterning of the alpha/beta neuronal cohort, then why are both neuronal classes not equally disrupted? The authors need to explain this clearly as, otherwise, it appears possible that let-7 might act primarily in some neuronal differentiation process that is downstream of the temporal switching common to both alpha and beta classes.

A related issue here is to clarify the fate of the let-7 mutant cells ectopically expressing Abrupt (which presumably would have made alpha/beta neurons in the wild type). i.e are the excess Ab+ cells incorporated into the alpha'/beta' lobe or do they differentiate into some other fate/die. The former would strengthen the conclusion that the primary role of let-7 is in temporal patterning, the latter may point to some other role.

3. Let-7-C-GAL4 is used as a proxy for let-7 expression but we have no idea how well or not this recapitulates the endogenous let-7 miRNA pattern. The problem here is that the LNA in situs in Fig. S1E,F do not provide convincing evidence of MB-specific expression. To validate let-7-C-GAL4, we need to see direct comparisons of the let-7 miRNA LNA in situ patterns in control versus let-7 deletion mutants.

4. To strengthen the link with ecdysone in the model in fig.7, allowing a genetic wiring diagram to be drawn, an additional element is needed. Namely evidence as to whether or not ecdysone acts cell autonomously in MB lineages-the ecd temperature shift experiments do not distinguish. It is possible that the use of available UAS dominant-negative and/or RNAi lines for Ecdysone Receptor would help to resolve this important issue.

MINOR POINTS

5. The fig.6A FasII mRNA levels graph really needs an abrupt single mutant histogram bar to allow proper interpretation of the partial restoration of FasII levels.

6. Figure 5b and Table S6. Is the 80:20 to 90:10 change in control versus delta let-7 statistically significant? Surely the experiment was done more than once and we need to see standard deviations and p values to judge this issue, which has important implications for whether the let-7 phenotype is or is not indicative of a true temporal switch involving an increase in alpha'/beta' at the expense of alpha/beta.

7. There may well be published links between JAK/STAT signaling and Abrupt in other contexts but there appears to be no direct experimental evidence for it in the context of temporal neuronal patterning in MB lineages. As the JAK/STAT/Abrupt link might be context dependent, either tone it down from the abstract and discussion or strengthen it by providing direct experimental evidence.

8. It is highly unlikely that all the let-7-negative or Ab-negative cells are GMCs (as stated on p.10 & Fig. 3 legend) as these are very short-lived and so there are only a few adjacent to each neuroblast. At least some of the negative cells are likely to be immature neurons.

9. The discussion is rather long and rambling and would benefit from shortening and refocusing.

1st Revision - authors' response 25 September 2012

Point-by-Point Response

Referee #1

This study demonstrates a cell-autonomous requirement of let-7 miRNA for proper axon guidance of the pupal-born a/b neurons in the Drosophila mushroom bodies. It further establishes a model, linking ecdysone hormone through let-7 and abrupt (negatively regulated by let-7) to up-regulation of FasII, for temporal regulation of MB neuronal morphogenesis. However, the involvements of ecdysone and abrupt in MB morphogenesis were poorly characterized, leaving their interactions with let-7 in the specification of various MB neurons unresolved. In addition, systematic efforts should be taken to better assess cell fate changes versus morphogenetic defects.

First, let-7 MBN clones should be thoroughly characterized to reveal their clone sizes and neuron type compositions (which is possible with diverse subtypespecific MB drivers), to critically examine the implicated role of let-7 in MB temporal fate specification. In addition, it is unclear why let-7 MBN clones induced at L3 show less severe a/b abnormalities than clones generated earlier or later (Table S6), given that let-7 is required cell-autonomously in individual a/b neurons.

Now we thoroughly characterized let-7 MBN clones depending on their size and time of the clone induction. We have analyzed more clonal brains to increase sample size and calculated the significance between single and double-cell induced clones versus MBN-derived clones. This confusion between L3 and Pgenerated let-7 clones was in part due to low numbers and the fact that MBNderived clones were also taken into account. We now considered only single/double cell clones generated at the specific developmental stage, and added a scheme explaining the outcome of clonal induction at different stages (L1, L3 and pupa) in different cell populations (MBN and GMC/neuron), which will help to illustrate the processes and results to the reader. let-7 single/double cell clones induced at L1 and L3, like parental MARCM clones, generate properly specified gamma and α/β neurons, while 68% of let-7 mutant neurons induced at pupal stage generate MB neurons that project instead of α/β into α'/β' lobe,

confirming that let-7 is required for the α'/β' to α/β neuron identity switch. In addition, around 70% of MBs containing MBN-derived let-7 clones generated at L1 and L3 had underdeveloped α/β lobes. The new data is added to Figures and Tables (Figure 1K, 5D,F, S2A, Supplementary Table VIII). To evaluate the identity of let-7 mutant MB neurons we labeled them in let-7 loss of function mutant brains with the temporal and spatial α/β specific let-7 Gal4 driver expressing membrane CD8GFP (Figure 1L) and found that in the absence of let-7 miRNA these neurons migrate together with Trio expressing α'/β' neurons. We now also used diverse subtype-specific MB drivers when possible (due to the complexity of genotypes) and established the cell autonomous role for ecdysone signaling, Ab, and FasII in α'/β' versus α/β neurons using these drivers.

Second, ecdysone is required before the production of a/b neurons for a/b lobe development, as suggested from stage-specific inhibition of ecdysone production. This argues for an undocumented role for ecdysone signaling in MB temporal fate transition, contradictory to earlier findings with USP clones or EcR mutants. It is therefore important to scrutinize the phenotypes in further detail by determining MB neuron type compositions and their birth dates, to substantiate its role in MB temporal fate change from a'/b' to a/b. One should also examine where ecdysone acts to govern MB a/b lobe development.

As suggested by the reviewer we now studied in a greater detail the role of ecdysone signaling in the governing MB α/β lobe development. For this we used multiple ecdysone pathway mutants: ecd1ts, the dominant negative ecdysone receptor hs EcRDN and co-receptor hs uspDN, EcR RNAi driven by temporal let-7 Gal4 and α/β specific c739 Gal4 drivers, and hs EcR.A and hs EcR.B1. We introduced ecdysone pathway mutations specifically at the time of α/β birth (12 h APF), which resulted in the "slim α/β lobe" phenotype (Figure 2F-K, Supplementary Tables IV-V). We also shortened the heat-shock treatment for the ecd1ts mutant to one day at the pupal stage in order to narrow down the exact time when ecdysone signaling is required for α/β transition, as well as to eliminate the wide error margin in the α/β lobe morphological phenotypes that was previously observed after a two-day treatment (Supplementary Table IV). The new data show that ecdysone signaling is required at 12-24 h APF for proper α/β neurogenesis. This shortened period was used to induce ecdysone signaling deficit in hs EcRDN, hs uspDN, hs EcR.A and hs EcR.B1 (Figure2H, Supplementary Table V). The usage of subtype-specific MB drivers with EcR RNAi allowed us to conclude that ecdysone signaling cell autonomously acts for α/β neuron cell fate specification (Figure2H,J, Supplementary Table V). We were also able to rescue the ecdysone signaling α/β MB lobe phenotype by overexpression of let-7, confirming that ecdysone signaling regulates α'/β' to α/β cell fate change via this miRNA (Figure2I,L, Supplementary Table IV).
The role of ecdysone signaling in α/β lobe development has not been studied before, previously it has been shown that usp and EcR.B1 are required for gamma lobe remodeling (Cell-Autonomous Requirement of the USP/EcR-B Ecdysone Receptor for Mushroom Body Neuronal Remodeling in Drosophila, Lee at al., Neuron, 2000).
We are very grateful to you for suggesting these additional experiments, since the new obtained data made the whole point about the role of ecdysone signaling stronger. The results are included in the new panels in Fig 2, Tables VI and V and the text.

Third, one could not really determine if lack-of-ecdysone has elicited a similar spectrum of α/β lobe defects as loss-of-let-7 without detailed phenotypic analysis or single-cell labeling.

To address this critique we down-regulated the ecdysone receptor EcR using temporally regulated let-7 Gal4 driver and α/β specific c739 Gal4 (let-7 Gal4/EcR RNAi and c739 Gal4/EcR RNAi) and observed mutant defects similar to let-7 and other components of ecdysone pathway. We also performed anti-EcR antibody

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staining and found that EcR is expressed in let-7C expressing cells. Analysis of transheterozygous genetic interactions between let-7 and EcR and usp (let-7/EcRQ50, let-7/EcRM554fs, let-7/usp4) were examined. These epistatic interactions resulted in α/β MB lobes mimicking the let-7 and ecdysone mutant phenotypes. The new data now are included in Figure2H, Table V.
Fourth, one should try to rescue the lack-of-ecdysone a/b lobe defects with targeted expression of transgenic let-7 to directly examine their possible linear actions in the regulation of MB a/b lobe development.

As suggested, we performed the genetic rescue experiment of the lack of ecdysone α/β lobe defects with forced expression of transgenic let-7 in dominantnegative EcR and usp mutants (hsGal4-uspLBD/UAS-let7, hsGal4-EcRLBD/UASlet7) and found that overexpression of let-7 induced in the period of α/β lobe formation can rescue α/β defects caused by the ecdysone-signaling deficit at the pupal stage (Figure 2I,L, Table IV).

Fifth, as shown in Figure S6, ab MBN clones induced at L3 or earlier acquired complex phenotypes with abnormal and rather thin a/b projections, paradoxical to the argument that ab is dispensable to MB a/b development. Subtype-specific drivers are again needed to better assess neuron type compositions in ab MBN clones.

This discrepancy is seen only when large MBN-derived ab clones are analyzed and this phenotype can be explained due to the requirement of properly formed earlier born lobes for the guidance of the later lobes (Shin and DiAntonio, 2011). Therefore, now we focused on single and/or double cell clones and confirmed that elimination of Ab is required for proper identity switch from α'/β' to α/β (Figure 5CF, Supplementary Figure 6A-E and Table VIII). We also used temporal let-7 Gal4; UAS GFP, α'/β' specific c305aGal4, α/β specific c739Gal4 and heat-shock inducible act<CD2<Gal4 drivers to asses neuronal cell identity of ab overexpressing neurons (Figure 4F-I, Supplementary Figure 6F-H, Table X).

Sixth, it is not clear from Table S6 what really happened to those ab mutant single-cell clones generated at L3. There was even no information about how many ab single-cell versus MBN clones were obtained at L3.

Now we analyzed two additional independent sets of MARCM clonal analysis experiments and more thoroughly re-analyzed all previously found phenotypes (total 4-5 independent sets for each genotype and hs stage). The statistical analysis on the number of single-cell versus MBN-derived clones obtained during each stage of clonal analysis and each analyzed genotype is now added in Table VIII. There was no significant difference between genotypes in the frequency of MBN and single-cell clone induction. Due to the complexity of MBN-derived phenotypes, we now included data on only single-/double cell clones (Figure 5DF, Table VIII).

Seventh, the ab MBN clone induced at L1 (Figure 6E) showed ectopic FasII in cell bodies of an apparently very small clone, arguing much more complex roles of ab in MB neurogenesis which were not mentioned or addressed at all. In addition, how could one know for sure these residual neurons are early-born neurons without birthdating with BrdU, given that NB clones may contain neurons born through the lineage development? Given the complexity of loss-of-function clones, one should also examine the suppression of FasII expression by ectopic ab using various a/b-specific MB drivers.

We should not have tried to compare single cell and MBN-derived clones. Now we replaced the figures to compare appropriate types of clones. Single cell derived parental and ab clones induced at different stages are shown in Figure 6H-M. We carefully staged flies before exposing to heat shock treatment following descriptions in http://www.devbio.net/node/112 and

http://www.devbio.net/node/45 to ensure the birthdating of clonal cells. Furthermore, as you suggested, we induced ab clones via heat shocking L1 larvae and fed L3 stage animals EdU to prove that ab clonal cells born after L3 contain FasII, unlike non-clonal α'/β' neurons. These data are now included in the figure S6I and the text. Also, as suggested, we examined the suppression of FasII expression by overexpression of ab using let-7 Gal4 and c739 Gal4 α/β MBsubtype specific drivers (Figure 4F-G and Figure S6G-H).

Referee #2

This manuscript explores the role of the miRNA let7 and one of its target, abrupt, in the formation of the Drosophila mushroom body neurons. The data are generally convincing and add up to an interesting, novel and important story.

Suggestions:

1. A semantic issue: the cluster of MB neurons and neuroblast is not called the calyx; the calyx is the neuropile domain that contains the dendrites of the mushroom body neurons. The cell body cluster has no specific name attached to it ("MB cell body cluster")
Thank you very much for pointing out this mistake, it has been corrected now.

2. A text and diagram that details the anatomical differences between the lbes, and the succession in which the 3 neuron classes are produced (like the diagram in Figure2) should come at the very beginning; already the Intro should explain these details, so that the reader is well equipeed with foreknowledge when diving into the data.
Thank you for this helpful suggestion, the scheme is now included in the Figure 1A, which definitely helps to introduce information necessary for understanding the data right from the beginning.

3. The in situ of let7, shown in supplementary figure S1, is disappointingly faint. Only looking at the photographs, I would not even be convinced it is real. Are there no better data using fluorescent probes? It also would be nice to show the time course of expression, for which now we always rely on the driver line
We are grateful for the suggestion to perform fluorescent LNA in situ that finally allowed us to analyze the chronological let-7 expression in the developing brain. The new data are added in Figure 2C. In the Figure S1E-F we also inserted images of let-7 expression pattern in adult brains of WT flies and let-7 mutants.

4. when the term "let 7 loss of function" is used: does this refer to a homozygous mutation in the gene, that results in the MB phenotype shown? The reader should have that info without referring to the Materials section.
We changed it in the text sections.

5. My main problem concerns aspects of the phenotype and, related to that, the interpretation of let7 function.

-in Figure1H, L (the "mutant") it looks as if only the b lobe is much thinner; the a lobe looks OK. This would imply a branching defect: normally, an a/b neuron, or a'/b' neuron, has a branch in both corresponding lobes; in the mutant, one of these branches is (presumably) absent in the majority of the a/b neurons. This would better be characterized as a differentiation defect: the "specification" is OK, given that the other lobe (a) is normal in size. This confusion is tangible throughout several parts of the text. Fore example Figure4G: overexpression of abrupt in a/b neurons results in the absence of the a, but not the b lobe! These are branches of the same neuron; on is lost. The authors should re-think the wording of their interpretation, and its implications.
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introduced from the beginning: I believe that published mutations in many genes, including fasII, result in such branch defects; there is also, if I remember correctly, a literature on separable behavioral functions of the a vs b lobe.
-Is the loss of one vs the other branches consistent? It looks as if in the let7 mutant in panel 1 H, mostly the b lobe is affected; in 1 F, it is the a lobe (or both lobes?).

Firstly, you brought up a very interesting and valid point. To address the first issue, we carefully re-analyzed all let-7 loss of function brains and found that the differences in the frequencies of slimmer-alpha versus slimmer-beta are not significant. We changed the panels in Figure 1G and 1K and now they show both alpha and beta lobes reduced in size, which better describes let-7 phenotype. However, as you pointed out, overexpression of Ab using let-7Gal4 driver does affect primarily the alpha lobe. This suggests that apart from the regulation of cell identity, transcription factor Ab has additional targets that regulate neuron differentiation. This hypothesis is now added to the text. However, we found that the majority of GFP positive cells that also overexpress Ab (let7CGal4/UASCD8GFP marker, UAS-Ab) do not fail to branch, instead they express less of FasII and co-localize with Trio-expressing α'/β' neurons, indicating the cell identity defect.

6. clones: a nice result. Question: how can one interpret a'/b' neurons resulting from inductions in the pupa? I thought only a/b neurons are generated during this phase? One related question/suggestion: did the authors convince themselves thoroughly that only heat pulses induced the clones? I believe from proactical experience that it is very rare that one have that sort of control, and no leakiness. Were never any clones recovered in controls without heat pulse?

As suggested by the reviewer, we now analyzed the leakiness of heat-shock promoter and found that without heat pulse, MARCM clones can be generated with the frequency of 3.20+1.68 clonal cells per brain. However, only 0.10+0.18 clones per MB cell cluster, which is not significantly different from the null. After hs induction every brain had at least one or more single/double-cell MB clone. These data are now included in the legend of the Sup. Table VIII. This additional control confirmed that we really are analyzing mutant clones induced at certain stages and not spontaneously mutated neurons. When we induce let-7 clones during the α'/β' to α/β identity switch, due to loss of let-7, Ab is not downregulated, which results in inability to turn on expression of Fas II and, probably, many other downstream targets of the putative transcription factor Ab. These Ab positive let-7 mutant neurons cluster together with Fas II negative and Trio positive α'/β' neurons, which implies that they remain as α'/β' neurons and their identity was not switched. We also provide new data showing that misregulation of just one cell adhesion molecule, Fas II is enough to change MB neuron differentiation (Figure 6O-R).

Referee #3

The authors describe a function for the Drosophila let-7 miRNA in temporal patterning of neuronal subtypes in identified neuroblast lineages of the mushroom body (MB). Using a let-7-complex Gal4 line, the authors show specific MB expression which is upregulated following the pupal ecdysone peak. Ecd temperature shifts imply that ecdysone signaling is directly or indirectly required for alpha/beta neurons and that it upregulates let-7 in MB lineages. They also demonstrate a specific let-7 requirement for the specification or morphogenesis of late alpha/beta but not earlier alpha'/beta' neurons. This is associated with a reduced olfactory learning index in the adult fly. In the second part of the paper, the authors dissect the molecular targets that mediate the effects of let-7 on MB lobe development. They show that the expression of Let-7-c-Gal4 is complementary to that of Abrupt (Ab), and that let-7 overexpression represses Ab.

Overexpressing Ab also affects alpha/beta neuron
development, while autonomous loss of Ab restricts neurons to the late alpha/beta fate. Loss of Ab also rescues the alpha/beta phenotype of the let 7 complex mutant. This argues that Ab is an important target of Let7 for the specification of MB lineages. Finally, the authors correlate the extent of alpha/beta morphogenesis observed in let-7 or Ab manipulations with levels of the cell adhesion molecule FasII, leading to a linear model for MB neurogenesis that culminates surprisingly in differential adhesion regulating chronological neurogenesis.

The let-7 miRNA has already been studied in Drosophila in some detail. However, this manuscript describes for the first time to my knowledge, evidence for a temporal patterning function in the context of identified neuroblast lineages. It therefore potentially adds a valuable new component to the temporal patterning model in MBs. Nevertheless, in my opinion, the data (as they currently stand) fall short of providing watertight evidence for the model in Figure7, thus leaving alternative possibilities open. There are also some cases of data over interpretation. All this uncertainty is revealed in the Figure 7 model, which is little more than a summary and lacks any kind of real genetic wiring diagram. A revised manuscript would need to address thoroughly all of the criticisms below. Where additional experiments are requested, these are only intended to test or to lend support to the authors existing conclusions and not to explore new ones.

MAJOR POINTS

1. The evidence that FasII mediates any aspect of let-7 function relevant to the temporal switch between alpha'/beta' to alpha/beta neurons is not strong enough. Moreover, there appears to me to be over interpretation on p.13: "These data allow us to conclude that alpha/beta neuron specification depends upon precise expression levels of the cell adhesion protein FasII that is spatially and temporally modulated by the ecdysone-induced miRNA let-7 and its target, the transcription factor Ab". Breaking this down into two issues:
a) The evidence that let-7 regulates FasII in a cell autonomous manner should be strengthened. Yes, the observed reductions in FasII mRNA (Figure6A) and FasII antibody staining will of course correlate with the overall numbers of FasIIexpressing neurons. However, in Figure 1H it appears that the alpha and even the few beta neurons that remain in let-7 mutant brains are still able to express FasII. This suggests that the effect may therefore be very indirect. It is possible that single cell resolution analysis of FasII expression in let-7 MARCM clones might help here.

To prove that let-7 regulates FasII in a cell autonomous manner, we overexpressed let-7 in α'/β' neurons and found higher levels of FasII in a single cell showing that let-7 regulates FasII cell autonomously, which confirms our previous statement that let-7 acts upstream of FasII in MB neurogenesis. Unfortunately, we found it impossible to detect downregulation of FasII in MB cell body cluster, since FasII is seen only in axonal part of MB neurons in WT animals. It is really hard to get a good resolution to evaluate FasII levels in a single cell clonal axon surrounded by hundreds of other FasII expressing axons. Also it is known that miRNAs normally do not switch gene expression "on-off", they are used as an additional layer of regulation of precision of gene expression, therefore deletion of let-7 miRNA affects FasII and Ab expression only to some extend, these factors are certainly regulated via additional regulators. Now this statement is added to the text in the "Ab but not Apt is a miRNA let-7 target in MB neurons" section.

b) Is the FasII phenotype really a downstream subset of the let-7 phenotype? More phenotypic analysis is required to demonstrate that there are common features of these two phenotypes in the alpha'/beta' to alpha/beta switch and to support the linear model in Fig7 (where differential adhesion regulates

chronological neurogenesis) and in the p.13 statement cited above. Given that Let-7c-Gal4; UAS-FasII-RNAi didn't generate a statistically significant alpha/beta lobe phenotype (although it did affect the midline structure), it is not clear that the current evidence supports the authors conclusions about the role of adhesion in mediating the effects of Let-7 or Ab. It is possible that additional carefully controlled epistasis tests with FasII and let-7 etc. might be insightful here in distinguishing how let-7, Ab, and FasII are genetically wired up. Regarding the role of Ab, I am also confused as to how this links with let-7 as let-7-C-Gal4; UASab appears to primarily affect alpha neurons (Figure4G) whereas let-7 deletion appears to primarily affect beta neurons (Fig 1H).

Thank you for pointing to us the inaccuracy in the statement on p.13, what we say now is : "These data allow us to conclude that α'/β' neuron differentiation depends on repression of the cell adhesion protein Fas II by the let-7 target Ab. Nonetheless, a reduction of just one homophilic cell adhesion molecule, Fas II using the chronologically regulated let-7 did not fully phenocopy the severity of let-7 and ecd1ts MB defects, showing that additional factors are involved in the processes of MB neurogenesis. In the process of the α'/β' to α/β neuronal identity switch, the spatio-temporal steroid-induced miRNA let-7 targets the transcription factor Ab that promotes Fas II expression."
Also, as suggested by the reviewer, we performed a series of additional experiments to provide further evidence on the role of FasII in differential neurogenesis. First of all, qPCR data show that let-7 LOF and overexpression of Ab (UAS-ab/let7Gal4 and UAS-ab/c739Gal4) result in lower levels of FasII in the brain (Figure 6A and new data in Table IX). On the cellular level let-7 overexpression (UAS let-7/c309Gal4 - in all MB lobes and UAS let-7/actGal4 – in single cell clones) and Ab LOF (MBN derived clones and single cell clones) results in higher levels of FasII (Figure 6B-D, H-M and SupFigure S6I). Next, we overexpressed FasII with alpha prime/beta prime lobe specific driver (c305a-Gal4) and observed changed cell differentiation of Trio positive cells, which now are located where alpha/beta lobe neurons were (Figure 6O-P). We also generated actGal4-UAS-FasII MBN derived clones induced at L2 and observed that FasII overexpressing cells fail to project into alpha prime/beta prime, but extended into alpha/beta lobes (Figure 6Q-R). Additionally, we tested a genetic interaction between FasII and let-7 and observed the "slim α/β lobe" phenotype (Figure 6E, Table 5). As suggested by the reviewer we now changed our model (Figure 7).
For the alpha versus beta lobe phenotype, please refer to the answer to Referee 2, Remark 5.

2. Is the primary function of let-7 in MB lineages really temporal patterning? The let-7 deletion phenotype shown in Figure1G.H and quantified in table S1 shows a much more dramatic effect on beta morphology/volume than on alpha. If the primary role of let-7 really is in temporal patterning of the alpha/beta neuronal cohort, then why are both neuronal classes not equally disrupted? The authors need to explain this clearly as, otherwise, it appears possible that let-7 might act primarily in some neuronal differentiation process that is downstream of the temporal switching common to both alpha and beta classes.

Please refer to the answer to Referee 2, Remark 5.

A related issue here is to clarify the fate of the let-7 mutant cells ectopically expressing Abrupt (which presumably would have made alpha/beta neurons in the wild type). i.e are the excess Ab+ cells incorporated into the alpha'/beta' lobe or do they differentiate into some other fate/die. The former would strengthen the conclusion that the primary role of let-7 is in temporal patterning, the latter may point to some other role.

Based on the size of obtained let-7 MARCM clones, their frequency and the Caspase3 staining we conclude that loss of the microRNA let-7 does not affect the survival of the neuron. The cell fate change of the clonal cell was quantified

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from the analysis of clones co-stained with alpha/beta lobe marker FasII and alpha'/beta' lobe marker Trio. These data are included in the text.

3. Let-7-C-GAL4 is used as a proxy for let-7 expression but we have no idea how well or not this recapitulates the endogenous let-7 miRNA pattern. The problem here is that the LNA in situs in Figure S1E,F do not provide convincing evidence of MB-specific expression. To validate let-7-C-GAL4, we need to see direct comparisons of the let-7 miRNA LNA in situ patterns in control versus let-7 deletion mutants.

As suggested by the reviewer we now redid let-7 LNA in situ experiments in L3, prepupal, pupal,and pharate brains (Figure 2C) and in WT versus let-7 mutant adult brains (Figure S1E). Please also refer to the reply to Referee 2, Remark 3

4. To strengthen the link with ecdysone in the model in Figure7, allowing a genetic wiring diagram to be drawn, an additional element is needed. Namely evidence as to whether or not ecdysone acts cell autonomously in MB lineages-the ecd temperature shift experiments do not distinguish. It is possible that the use of available UAS dominant-negative and/or RNAi lines for Ecdysone Receptor would help to resolve this important issue.

We are grateful for the suggested experiments, new results are included in Figure 2D-L, Tables IV and V and the text. Please also refer to the reply to Referee 1, Remarks 1-4.

MINOR POINTS

5. The Figure6A FasII mRNA levels graph really needs an abrupt single mutant histogram bar to allow proper interpretation of the partial restoration of FasII levels.

Now we tested FasII mRNA levels in Ab hypomorphic mutants and found increased FasII mRNA levels in the heads of these mutants. Introduction of Ab mutation into the let-7 mutant background still could only partially rescue FasII levels in let-7 mutant brains suggesting that components other than let-7 and Ab are involved in FasII regulation.

6. Figure 5b and Table S6. Is the 80:20 to 90:10 change in control versus delta let-7 statistically significant? Surely the experiment was done more than once and we need to see standard deviations and p values to judge this issue, which has important implications for whether the let-7 phenotype is or is not indicative of a true temporal switch involving an increase in alpha'/beta' at the expense of alpha/beta.

Please, see responses to Referee 1, remarks 1, 5 and 6 and Referee 2, remark 6. The data are included in the new Table VIII and Figure 5D,F.
Statistics between MBN-derived versus single/double cell clones were calculated using a two-tailed Student's t-test and statistics for the frequencies between cell identities of single/double MARCM clonal neurons were calculated using two-way tables and chi-squared test.

7. There may well be published links between JAK/STAT signaling and Abrupt in other contexts but there appears to be no direct experimental evidence for it in the context of temporal neuronal patterning in MB lineages. As the JAK/STAT/Abrupt link might be context dependent, either tone it down from the abstract and discussion or strengthen it by providing direct experimental evidence.

We agree with the reviewer that more experiments are required to prove the link between JAK/STAT signaling and Ab in our system and since this is not the subject of this paper, we agree that the speculation on JAK/STAT involvement

should be removed from the Intro and the text. However, we believe that JAK/STAT will be important in future research, thus it is left in the discussion.

8. It is highly unlikely that all the let-7-negative or Ab-negative cells are GMCs (as stated on p.10 & Figure 3 legend) as these are very short-lived and so there are only a few adjacent to each neuroblast. At least some of the negative cells are likely to be immature neurons.

We are thankful for this important remark. Now we corrected our mistake and stated that these Ab negative cells are GMCs and immature neurons. This statement is corrected in the text and in the figure 3 legends.

9. The discussion is rather long and rambling and would benefit from shortening and refocusing.

We tried to contract the discussion and to omit the non-vital to the substance details, however we wanted to leave the reader to be able to speculate on the idea on their own about the evolution of the complexity of the brain and possible role of miRNA in the process.

Additional Correspondence 15 October 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by referees # 1 and 2. As you can see below, both referees appreciate the introduced changes and support publication here. Referee #1 suggests an additional experiment namely to look at let-7 expression in USP mutant clones. As you do show downregulation of let-7 miRNA expression in an ecdysoneless temperature sensitive mutant I find this good enough at this stage. Therefore no further experiments are needed. Referee #2 suggests a minor text change in the abstract. I think it sounds reasonable, if you do too then go ahead and send me a modified text file by email and we will upload it for you. Once we get this last minor issue taken care then we will accept the paper for publication here.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The revised manuscript addressed satisfactorily most of my previous concerns. However, the evidence for the cell-autonomous requirement of EcR/USP for upregulation of let-7 in MB a/b neurons remains indirect with use of transgenic RNAi or dominant-negative transgenes. The authors should directly check let-7 expression in USP mutant NB clones to validate this key message of the paper.

Referee #2:
The authors have made a great effort in revising the manuscript. They addressed all suggestions/criticisms with changes in the text, as well as additional experiments and figures. I noted that in the abstract (a highly visible part of the paper), there is a sentence that should be fixed: "....the signaling pathway controls the expression levels of the cell adhesion molecule Fasciclin II in differentiating neurons that ultimately influences their differentiation". I assume I understand the sentence, but the tow "differentiating" is confusing. Better: "controls ....of Fasciclin II in DEVELOPING neurons that ultimately influences their differentiation.

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