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Neuropilin-1 expression in GnRH neurons regulates prepubertal weight gain and sexual attraction

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1st Editorial Decision 1st Apr 2020

Dear Dr. Prevot,

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the two reports on your study.

As you can see from the comments below, the referees appreciate the analysis and find it suitable for consideration here. They raise a number of different concerns that I would like to ask you to address in a revised version. As you can see from the comments below the manuscript needs to be streamlined to make it more accessible.

I think it would be helpful to discuss the revisions further either by phone or video conferencing. I am also aware that with the current situation that it is difficult to discuss a timeline for the revisions and don't worry about the revision time at this stage.

I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the major 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:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing further with you the revisions.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes

made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 30th Jun 2020.

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Referee #1:

This paper reports interesting findings after deleting Neuropilin-1 in GnRH neurons, including excess GnRH neurons in the brain due to their enhanced survival and migration. In female mice, these alterations result in early prepubertal weight gain, premature attraction to male odors and precocious puberty. The findings are novel and interesting, though as written often hard to follow (see below), especially for a general audience.

Major

1) Figures are confusing and too much presented that is not necessary - often have same data repeated in different format (graph vs histogram). See below for specifics.

2) Few actual numbers (data) are ever given in text or figure legends. This makes it difficult to compare between groups -at least percent changes can be included in text so reader can see if magnitude of change is similar or different.

3) Most cre mice lines express cre in transient populations during development - a control here would be to show no changes in another Nrp1 expressing cell type both developmentally (also see below) and postnatal. For postnatal it would strengthen data to examine the arcuate since cell types here known to influence energy balance/weight and project to GnRH cells. Paper by van der Klaauw et al (cell, 2019) show that knock down of Nrp1 in arcuate alter POMC fiber outgrowth to

PVN and VMH which could alter weight gain. Does POMC change to GnRH cells? No change in fibers would strengthen overall conclusion - directly related to GnRH neurons and not changes in other metabolic circuits (inputs to GnRH cells) and would help address sentences in manuscript -
Page 10: 'Altogether, these data suggest that Nrp1 signaling does not influence the intrinsic excitability of GnRH neurons, and that differences in spontaneous activity may therefore be the result of their altered connectivity.'
Page 12: 'potentially alters their integration into the hypothalamic network controlling their activity after birth.'

Abstract:
1) using a system of guidance cues, including the Semaphorins and their receptors, the Neuropilins and Plexins (as written sounds like only ones)
aberrantly to the accessory olfactory bulb - always a few here in normal mice thus not really aberrantly
2) and accelerates the maturation of their activity - how are authors defining - should delete
3) Our findings suggest that rather than being influenced by peripheral energy state - experiments suggested to show no changes in arcuate?

Introduction
Page 4:
1) They also form the scaffold along which GnRH neurons migrate into the brain - should be olfactory axons otherwise reads as if Sema and Nrp1 form scaffold
2) Nrp1 expression in GnRH neurons is required to control the size of the GnRH neuronal population generated during embryogenesis and their proper migration into the brain - all the right places - just wrong numbers? This concept needs to be revised/clarified.
3) abnormal migration of excess neurons into the accessory olfactory bulb - delete abnormal- definitely excess - a few cells are found there under normal conditions (certainly documented in rats - Witkin and Silverman, 1983). Really first part of this sentence seems redundant with previous sentence.

Results:
Pg 5
1) (hereafter termed "mutant") and Nrp1loxP/loxP (hereafter termed "control") mice - but in fact not used throughout. Correct throughout manuscript
2)GFP - 'to better visualize and isolate these neurons' Unclear when GFP antibody was used if at all for staining? Please clarify and if not staining put GFP in figures.
3) showed a decrease in Nrp1 mRNA levels - percent decrease? This was associated with a decrease in PlexinA1 mRNA levels - percent decrease?

Page 6:
1) 'While the total number of GnRH neurons was equivalent in Gnrh::cre; Nrp1loxP/loxP and Nrp1loxP/loxP embryos at E12.5, it was significantly higher in mutant embryos at E14.5 ((22)=2.56, p=0.02, n=5 to 6) and E18.5 (t(22)=2.17, p=0.04, n=4 to 5) (Figure 2A)' - by how much? Actual numbers here or percent increase is needed.
2) It is unclear whether GnRH staining or GFP alone or enhanced by staining was used for data in fig. 2 since 2B indicates GnRH on figure? 2F should be labeled postnatal.
3) For adult counts were both males and females used? Must indicate and important for final hypothesis, i.e. did cell number also increase in males? In methods there is a reference number so no way to decipher procedure - appears only females used for adult counts? If true, then males

would be important to examine since no change in their sexual maturation detected. But one assumes embryos were not sexed so those represent male/female group.
4) 'At E14.5, these excess cells were located both in the olfactory bulbs ($t(36)=2.20$, $p=0.03$, n=5 to 6) and in the VFB ($t(36)=2.39$, $p=0.02$, n=5 to 6) (Figure 2B and 2C) suggesting that a higher proportion of cells had already migrated further towards their destinations at this embryonic stage in mutants than in control littermates (Figure 2C).' Remove total number since presented in 2A, then reader could actually see increase/area. But again, numbers in the text (other than statistics) are required.
5) 'GnRH neurons in mutant mice clearly accumulated in the accessory olfactory bulb (AOB).' The video of the AOB does not show anything more than figure 2D and a higher mag inset is needed in 2D to see cell bodies in mutant.

Page 7:
1) The GnRH cre specific control - show GnRHcre/lac and GnRH staining at E14.5 in control mouse - % overlap - if close no problem, if more GnRH crelacz cells interpretation might need to change.
2) 'This therefore suggests that Nrp1 signaling in differentiated GnRH neurons controls their own survival.' How does one explain more labeled cells E14.5 in VFB but in adult more rostral?

Page 8:
1) 'Together, these in vivo results indicate that the size of the GnRH neuronal population is regulated by apoptosis at the post-mitotic stage, and that Nrp1 signaling in newborn GnRH neurons may be involved in this process.' Could be that more get in and they survive due to 'CNS' factor? Or leaving junction area they do not see factor that causes apoptosis? Do you get same cell reduction with antibody treatment in explants?
2) 'Together with the in vivo findings on the aberrant migration of GnRH neurons that lack Nrp1, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons." - reversed order or how data is presented would be best to revise with survival first and migration second.

Page 9:
1) 'the ectopic accumulation of GnRH neurons in the AOB' - delete ectopic
2) 'However, at P20, although control juvenile females showed a preference for female bedding, 4 out of 8 mutant females presented marked preference for male odors ($t(8)=5.64$, $p=0.0005$, n=4 to 6; Figure 4A). - Why are only 4 animals plotted (figure 4A) when 8 tested? How does one account for differences between these 4 mice? What was the weight of these mice?
3) Firing frequency - Dulka & Moenter, 2017 only reported change in female mice - unclear what sex was used in present study for recording? The number of animals recorded from, sex of animals and location of cells needs to be indicated. Only 50% of the female mice showed preference in sniffing - did only 50% show reduced activity?. Also weight of mice recorded from would be helpful.

Page 10/11: Organize figure 5 (see below) to go with text, as written reader is jumping all over figure.
Was WAT measured in males? Important for final hypothesis.

Discussion:
1) 'GnRH release at the median eminence has indeed been shown to be dependent on action potentials (Glanowska & Moenter, 2015)' - if puberty initiation by kisspeptin - this was shown to be action independent at ME. In addition, this study used male mice.
2)'Our results suggest that ectopic GnRH release in the AOB may itself be responsible for these changes in sexual receptivity during postnatal development' - these may still target ME any

evidence of projections of these cells in mutant mice?

Figures: General Note: it is very difficult to see black dots in gray bars or dark purple - colors should be changed and many times information presented in figure could be put in text and de-congest figures.

1D: Dots representing data are nice to see but using black asterisk for significance on these graphs is not. Perhaps red would work
1E: must be different regions since mutant heavy fibers whereas control primarily cells. Similar regions should be taken so reader can compare and when redone should contain inset with a higher magnification. Also add arrow to show reader where fibers are. This figure does not show - no alteration of the olfactory sensory or terminal tracts was detected in Gnrh::cre; Nrp1loxP/loxP embryos at any age (Figure 1E)

Figure 3:
A) Graph of BrdU should be right of BrdU labeling micrograph and not underneath.
C) One cannot see anything in low mag. Numbers (actual numbers, with stats) can be presented in text.
Can present schematic of explant with graph E (since also shows increase in cell number).
F-I) Give data for scratch assay in text (with actual numbers and stats) and just display H/I

Figure 5: Needs organization revisions - remove and indicate in text (with actual numbers and stats) measurements that did not change - at least 5A, B, E, F and H - giving room and organization for those of interest.

Minor:
1) A few references appear as numbers in text, but reference format not numbered. This makes reviewing more difficult and shows not well proofed.

Referee #2:

General summary: In this report the authors examine the effect of knockout of Nrp1 expression specifically in GnRH neurons in a transgenic mouse model. The study results posit an interesting hypothesis about the role of NRP1 signaling in GnRH neurons resulting in accelerated and aberrant migration and distribution of embryonic GnRH neurons, earlier postnatal adiposity changes that the authors suggest may underlie precocious puberty and changes in sexual behavior in female mice. Overall, the manuscript is well-written, and the author's conclusions are in line the study results. The significant strengths of this study include the use of complementary in vitro molecular and electrophysiologic approaches and in vivo studies.

I have a few specific comments about some aspects of this nice paper.
1. The authors allude to the critical role for MKRN3 expression in central precocious puberty. Did the authors examine MKNR3 expression in postnatal stages? It has been previously reported that reduction of MKNR3 expression in the hypothalamus may signal initiation of puberty (Abreu et al N Engl J Med 368: 2467-75). Does this hold true in this transgenic model?
2. Given the link between SEMA3A signaling and obesity is also emerging, did the transgenic mice

show any differences in food intake, energy expenditure, metabolic markers such as leptin, effects on glucose homeostasis? If so, it would be interesting to speculate any direct role for GnRH activation on metabolic changes.
3. The gender discordance of the phenotypes is interesting, and this merits discussion about what factors may underlie this discordance.
4. Fig 2B - The text suggests a significantly higher GnRH neurons in olfactory bulbs, but the figure does not indicate any markers (*) of statistical significance. Please correct.

Point-by-point response to the Referees:

We thank the Referees and the Editor for their careful review and appreciation of our manuscript and their insightful comments, which have been of tremendous help to us in improving the manuscript.

All changes to the text are highlighted in yellow.

Referee #1:

This paper reports interesting findings after deleting Neuropilin-1 in GnRH neurons, including excess GnRH neurons in the brain due to their enhanced survival and migration. In female mice, these alterations result in early prepubertal weight gain, premature attraction to male odors and precocious puberty. The findings are novel and interesting, though as written often hard to follow (see below), especially for a general audience.

Major

1) Figures are confusing and too much presented that is not necessary - often have same data repeated in different format (graph vs histogram). See below for specifics.

We apologize for the lack of clarity of our first set of figures. We now have changed and reorganized the figures to comply with the referee's recommendations, as further detailed below.

2) Few actual numbers (data) are ever given in text or figure legends. This makes it difficult to compare between groups -at least percent changes can be included in text so reader can see if magnitude of change is similar or different.

We have now added actual data and information in the text to enable the reader to visualize the magnitude of the change.

3) Most cre mice lines express cre in transient populations during development - a control here would be to show no changes in another Nrp1 expressing cell type both developmentally (also see below) and postnatal.

The transient expression of cre in some cell populations across the lifespan, including in the germline, is a key point that has now been clarified in the methods as follows: "Importantly, unexpected germline recombination has recently been reported to occur in distinct cre driver lines (Luo, Ambrozkiewicz et al., 2020). Because recombination in the *Gnrh::cre* mouse line can occur in some oocytes (Hoffmann, Larder et al., 2019), only male *Gnrh*::*Cre* mice are used to generate bigenic mice (i.e., *Gnrh::Cre*$^{(het)}$; *Nrp1*$^{loxp/loxp}$ males are crossed with *Gnrh::Cre*$^{(wt)}$;*Nrp1*$^{loxp/loxp}$ females to generate about 50% mutants and 50% controls in the same litter)."

We are convinced as to the selectivity of the invalidation of Nrp1 expression and its restriction to GnRH neurons in the hypothalamus of our mouse model because of a built-in negative control. To be precise, immunofluorescence analyses clearly show that Nrp1 expression is unaltered in the olfactory/vomeronasal fibers that GnRH neurons use as a scaffold for their migration between nose and brain in *Gnrh::Cre*; *Nrp1*$^{loxp/loxp}$ mice when compared to *Nrp1*$^{loxp/loxp}$ control littermates during embryogenesis (Figure 1E). In addition, one of our previous studies shows that in adult *Gnrh::Cre*; *Nrp1*$^{loxp/loxp}$ mice, Nrp1 expression is absent in GnRH-immunoreactive nerve terminals, but not in other neuroendocrine axons residing in the median eminence (Giacobini et al., 2014 Plos Biology, cited in the text).

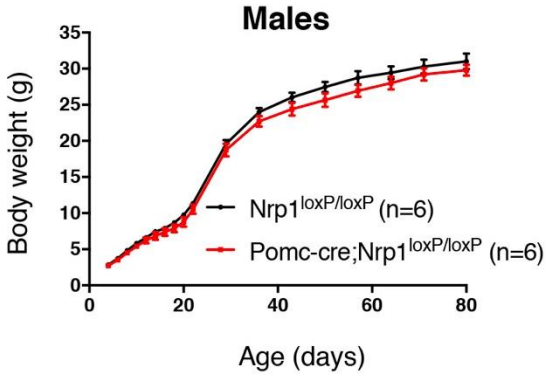
For postnatal it would strengthen data to examine the arcuate since cell types here known to influence energy balance/weight and project to GnRH cells. Paper by van der Klaauw et al (cell, 2019) show that

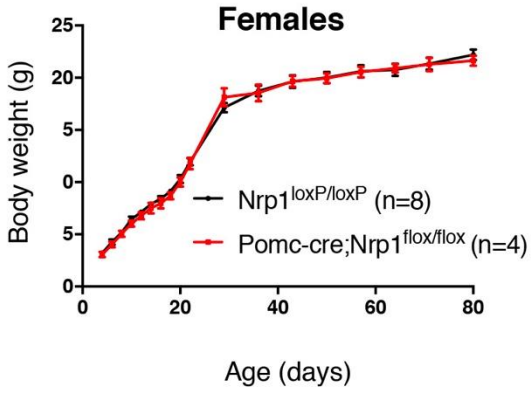
knock down of Nrp1 in arcuate alter POMC fiber outgrowth to PVN and VMH which could alter weight gain. Does POMC change to GnRH cells?

No change in fibers would strengthen overall conclusion - directly related to GnRH neurons and not changes in other metabolic circuits (inputs to GnRH cells) and would help address sentences in manuscript –

Page 10: 'Altogether, these data suggest that Nrp1 signaling does not influence the intrinsic excitability of GnRH neurons, and that differences in spontaneous activity may therefore be the result of their altered connectivity.'
Page 12: 'potentially alters their integration into the hypothalamic network controlling their activity after birth.'

Indeed, Van der Klaauw et al. (Cell, 2019) have shown the importance of Neuropilins and their ligands in the development of hypothalamic circuits involved in energy homeostasis. In particular, knocking out Neuropilin-2 (Nrp2) in POMC neurons disrupts their projections and alters weight gain. However, preliminary data from our laboratory suggest that knocking out Nrp1 in POMC neurons does not have any marked effect on postnatal growth (see the two graphs below).





By disrupting Nrp1 in GnRH-expressing cells, we do not expect to alter POMC projections because we do not suppose that local Sema3A gradients are altered, only the way GnRH neurons sense and respond to these gradients. However, as mentioned by the referee, our electrophysiological data suggest, surprisingly, that the decreased firing of GnRH neurons in mutant juvenile animals could be due to changes in the connectivity of GnRH neurons rather than to changes in their intrinsic electrical properties. The main excitatory inputs to GnRH neurons being GABAergic, and a subpopulation of POMC neurons being known to express GABA (Hentges et al. 2004, J.Neurosci), it

would indeed be interesting to investigate POMC neurons, among others. It particular, it would be intriguing to determine whether the ability of GnRH neurons to attract POMC afferents is altered when Nrp1 is knocked out, and to identify the molecular mechanism underlying this process, but this would require a whole new line of investigation that is beyond the scope of the present study.

Abstract:
1) using a system of guidance cues, including the Semaphorins and their receptors, the Neuropilins and Plexins (as written sounds like only ones)

This sentence has been changed to make it clearer that there are other molecules involved.

aberrantly to the accessory olfactory bulb - always a few here in normal mice thus not really aberrantly

By "aberrantly", we were referring to their numbers, not their location. We have modified the entire sentence to make this clearer.

2) and accelerates the maturation of their activity - how are authors defining - should delete

The activity of GnRH neurons was measured by their frequency of firing, which we found to be decreased to a juvenile pattern at an earlier time point in mice lacking Nrp1 in GnRH neurons. We believe that this is an important change reflecting the connectivity of these neurons, quite apart from the effect of Nrp1 deletion on GnRH neuron numbers and position, and that it contributes to the reproductive phenotype of our mutant mice. As such, we believe that the presence of this phrase or something similar in the abstract is justified. In modifying the sentence to respond to the previous comment, we have also modified this phrase.

3) Our findings suggest that rather than being influenced by peripheral energy state - experiments suggested to show no changes in arcuate?

We apologize if we have misunderstood the question, but is the referee asking us whether we have tested for changes in the arcuate nucleus? While this is possible, for the simple reason that, as discussed in several places in the text, having an abnormal number and distribution of GnRH neurons would necessarily alter the neuroglial networks that they are part of, we do not see the point of these experiments, as the primary change here is in and only in GnRH neurons, not peripheral tissues that signal energy stores and that might signal through the arcuate. Other changes, if they were to take place, would be driven by this change in GnRH neurons themselves. The prevalent hypothesis that peripheral energy status determines the timing of puberty, rather than this being a central process, is therefore without merit, which is the only point we are trying to make here.

Introduction
Page 4:
1) They also form the scaffold along which GnRH neurons migrate into the brain - should be olfactory axons otherwise reads as if Sema and Nrp1 form scaffold

This sentence has been changed, and now reads “Olfactory axons expressing Nrp1 also form the scaffold along which GnRH neurons migrate…”

2) Nrp1 expression in GnRH neurons is required to control the size of the GnRH neuronal population generated during embryogenesis and their proper migration into the brain - all the right places - just wrong numbers? This concept needs to be revised/clarified.

Indeed, this concept has been extensively detailed in the results and discussion sections. Here in the introduction, we have opted to keep things simple so as not to anticipate the results and their

significance. However, we have replaced "their proper migration into the brain" by "their migration to their proper destinations in the brain in the correct proportions" for clarity.

3) abnormal migration of excess neurons into the accessory olfactory bulb - delete abnormal- definitely excess - a few cells are found there under normal conditions (certainly documented in rats - Witkin and Silverman, 1983). Really first part of this sentence seems redundant with previous sentence.

We agree with the referee that "abnormal migration" is a bit ambiguous, but in our humble opinion, the word "abnormal" itself is necessary since our observations differ from those in WT mice. To resolve this issue, the sentence has now been changed to read ‘We also show that the knockout of Nrp1 specifically in GnRH neurons results in the accumulation of an abnormally high number of neurons in the accessory olfactory bulb (AOB)”.

We now refer to the work of Witkin and Silverman, 1983 in the discussion, page 17.

Results:
Pg 5
1) (hereafter termed "mutant") and Nrp1loxP/loxP (hereafter termed "control") mice - but in fact not used throughout. Correct throughout manuscript

We now use “mutant” and “control” consistently throughout the manuscript from page 5 onwards.

2) GFP - 'to better visualize and isolate these neurons' Unclear when GFP antibody was used if at all for staining? Please clarify and if not staining put GFP in figures.

We do apologize for that. We have now clarified throughout the text when GnRH neurons identified by the expression of GFP were used. In fact, the triple transgenic animals (with GFP-identified neurons) were used only for cell-sorting (Figure 1) and electrophysiological recordings (Figure 4) and did not require the use of a GFP antibody. All counts were carried out using immunohistofluorescence for GnRH only. This has now also been clarified in the main text and in the legends.

3) showed a decrease in Nrp1 mRNA levels - percent decrease? This was associated with a decrease in PlexinA1 mRNA levels - percent decrease?

The percent decrease in transcript expression is now mentioned in the text for both genes.

Page 6:
1) 'While the total number of GnRH neurons was equivalent in Gnrh::cre; Nrp1loxP/loxP and Nrp1loxP/loxP embryos at E12.5, it was significantly higher in mutant embryos at E14.5 ((22)=2.56, p=0.02, n=5 to 6) and E18.5 (t(22)=2.17, p=0.04, n=4 to 5) (Figure 2A)' - by how much? Actual numbers here or percent increase is needed.

The percent change in the total number of GnRH neurons as well as the mean+/- SEM are now mentioned in the text

2) It is unclear whether GnRH staining or GFP alone or enhanced by staining was used for data in fig. 2 since 2B indicates GnRH on figure? 2F should be labeled postnatal.

All labeling corresponds to GnRH immunohistofluorescence alone. This has now been clarified in the text and the legend. The word “Adults” has been added to Figure 2F.

3) For adult counts were both males and females used? Must indicate and important for final hypothesis, i.e. did cell number also increase in males? In methods there is a reference number so no way to

decipher procedure - appears only females used for adult counts? If true, then males would be important to examine since no change in their sexual maturation detected. But one assumes embryos were not sexed so those represent male/female group.

Cell counts are now mentioned whenever possible in the text. Indeed, embryos were not sexed in our study. Both males and females were used for electrophysiological recordings. Male data points have now been highlighted in blue in the figure and the statistics for each sex are provided in the main text, page 10. The quantification of GnRH neurons in transparentized tissue was only done in females. The data on GnRH neuron quantification based on immunohistofluorescence in slices were initially shown for females only, although quantification was done for both sexes. However, in response to the referee's comment, we have now added the data obtained in males, which show the same trend as in females but are not significant on their own due to the low number of animals analyzed (n=4). Male data points have now been added to the graph, increasing the total number of analyzed brains to 13 per group; these are highlighted in blue in the new Figure S1.

We sincerely apologize for the missed references in the methods in our previous version of the manuscript. References are now properly indicated in the methods.

4) 'At E14.5, these excess cells were located both in the olfactory bulbs (t(36)=2.20, p=0.03, n=5 to 6) and in the VFB (t(36)=2.39, p=0.02, n=5 to 6) (Figure 2B and 2C) suggesting that a higher proportion of cells had already migrated further towards their destinations at this embryonic stage in mutants than in control littermates (Figure 2C).' Remove total number since presented in 2A, then reader could actually see increase/area. But again, numbers in the text (other than statistics) are required.

The total numbers of GnRH neurons at E14.5 has been removed from Figure 2C, and the percent increase and numbers are now provided in the text, page 6.

5) 'GnRH neurons in mutant mice clearly accumulated in the accessory olfactory bulb (AOB).' The video of the AOB does not show anything more than figure 2D and a higher mag inset is needed in 2D to see cell bodies in mutant.

We believe that being able to actually see how GnRH neurons are distributed in the olfactory bulb in 3D over several millimeters is a valuable addition to the manuscript. We feel that it would be of interest to keep these movies, but will leave the decision up to the Editor.

Page 7:
1) The GnRH cre specific control - show GnRHcre/lac and GnRH staining at E14.5 in control mouse - % overlap - if close no problem, if more GnRH crelacz cells interpretation might need to change.

Hoffman and colleagues (2019, Neuroendocrinology, see answer to major comment #3) have shown that, between the two existing mouse lines at the present time, the mouse cre line from the Dulac laboratory, which is the one we are using, is the one with the lowest number of off-targets during both embryogenesis and adulthood. Furthermore, they could identify using a LacZ reporter mouse line that cre is expressed in about 96% of the GnRH neurons in the brain. In any case, here we are not using any reporter gene to quantify the number of GnRH neurons, but GnRH immunoreactivity, which would not occur in putative off-target cells.

2) 'This therefore suggests that Nrp1 signaling in differentiated GnRH neurons controls their own survival.' How does one explain more labeled cells E14.5 in VFB but in adult more rostral?

The ventral forebrain (VFB) at E14.5 is the presumptive territory that will give rise to the anterior hypothalamus later on during development. At E14.5, we also quantified the number of cells present in the olfactory bulb, where the total number of GnRH neurons also tended to be higher in mutants but did not reach statistical significance. However, the p value (p=0.07) is now reported in Figure 2C and cited in the text, page 6, to reflect this. One could speculate that the increased migration of

GnRH neurons from the nose to the AOB occurs at a later phase of development in mutants, i.e., between E14.5 and E18.5, during which GnRH neurons are known to continue to migrate.

Page 8:
1) 'Together, these in vivo results indicate that the size of the GnRH neuronal population is regulated by apoptosis at the post-mitotic stage, and that Nrp1 signaling in newborn GnRH neurons may be involved in this process.' Could be that more get in and they survive due to 'CNS' factor? Or leaving junction area they do not see factor that causes apoptosis? Do you get same cell reduction with antibody treatment in explants?

This is a very interesting point. One could speculate that the regulation of the survival of GnRH neurons would depend on their location. In that case, Nrp1 could be involved in controlling only GnRH neuronal migration, but not directly their survival, with the environment in the nose and in the brain being different in terms of the concentration of chemo-attractant and repellent molecules, including Sema3A. However, our nasal explant experiments showing an increased number of total GnRH neurons migrating in mutants argues against this hypothesis and suggests that knocking down Nrp1 expression in GnRH neurons does indeed increase their survival.

Nasal explants were isolated from the brain and cultured at 11.5, when recombination has not yet occurred or is in the process of occurring (i.e. when GnRH starts to be expressed). Unfortunately, the quantity of explants obtained from littermates being low, we were not able to treat control explants with the neutralizing Nrp1Ab. However, nasal injections of Nrp1Ab carried out in embryos *in vivo* induced an increase in GnRH neuron number, but these supernumerary cells were found to be equally distributed in the nose and the brain (the distribution of these neurons is not illustrated in the paper).

In addition, experiments using Nrp1Ab on nasal explants *ex vivo* would perhaps have not been conclusive, because one could expect that the vomeronasal projections used by GnRH neurons to migrate outside the explant would have been impaired under these conditions since they require Sema3A-Nrp1 signaling to be established and maintained (Hanchate *et al.* 2012, PLoS Genet).

2) 'Together with the in vivo findings on the aberrant migration of GnRH neurons that lack Nrp1, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons." - reversed order or how data is presented would be best to revise with survival first and migration second.

We understand this comment by the referee, but we feel that reversing the order of Figures 2 and 3 would be a bit awkward since Figure 3 is actually meant to provide mechanistic insights into the findings illustrated in Figure 2, as mentioned at the beginning of the section, page 7 “To understand the origin of these supernumerary GnRH neurons in mutant embryos and adult mice, …”.

Page 9:
1) 'the ectopic accumulation of GnRH neurons in the AOB' - delete ectopic
2) 'However, at P20, although control juvenile females showed a preference for female bedding, 4 out of 8 mutant females presented marked preference for male odors (t(8)=5.64, p=0.0005, n=4 to 6; Figure 4A). - Why are only 4 animals plotted (figure 4A) when 8 tested? How does one account for differences between these 4 mice? What was the weight of these mice?

We have replaced "ectopic" by "aberrant" since neuron numbers are altered. We now represent all mutant animals in Figure 4A. Unfortunately, body weight was not recorded for these 4 animals at the time of experiment.

3) Firing frequency - Dulka & Moenter, 2017 only reported change in female mice - unclear what sex was

used in present study for recording? The number of animals recorded from, sex of animals and location of cells needs to be indicated. Only 50% of the female mice showed preference in sniffing - did only 50% show reduced activity?. Also weight of mice recorded from would be helpful.

Both males and females were used for electrophysiological recordings. This is now specified both in the text, page 10, and in figure 4D, where data from males have been highlighted in blue. New statistical analyses that are now reported in the text actually show that GnRH neurons behave in exactly the same way during postnatal development in male and female littermates of both genotypes. All recorded GnRH neurons were located in the hypothalamic preoptic area at the level of the OVLT (this detail has been added to the methods, page 22), but no actual segregation was noticed with respect to the firing behavior of GnRH neurons between mutant female mice. Unfortunately, the weight of these animals was not recorded at the time of the experiment.

Page 10/11: Organize figure 5 (see below) to go with text, as written reader is jumping all over figure.

The text and Figure 5 have been rearranged accordingly.

Was WAT measured in males? Important for final hypothesis.

The WAT was unfortunately not measured in males.

Discussion:
1) 'GnRH release at the median eminence has indeed been shown to be dependent on action potentials (Glanowska & Moenter, 2015)' - if puberty initiation by kisspeptin - this was shown to be action independent at ME. In addition, this study used male mice.

We respectfully disagree with the reviewer on this point. Indeed, puberty being the acquisition of the ability to reproduce and carry litters, i.e., the first ovulation in females, this phenomenon is thought to be controlled, at least in mice, by the activation of the kisspeptin neurons in the AVPV, which may control surge release by acting on GnRH cell bodies. When kisspeptin is added to median eminence explants, it indeed induces the release of GnRH (from GnRH nerve terminals, which likely express the kisspeptin receptor), but this appears to be rather artificial as in mice, in contrast to monkeys or sheep, there are no kisspeptin fibers in the median eminence. In mice, the action of kisspeptin in the tuberal region of the hypothalamus is more likely to occur at the level of the ARH, where kisspeptin neurons interact with GnRH “dendrons” to control the pulsatile release of LH, but probably not the preovulatory surge of LH.

The timing of puberty is more ambiguous in males since there are no noninvasive indicators of mature spermatozoa production, unlike ovulation, which can be deduced by monitoring the estrous cycle. However, in the current work, we did use both male and female mice, and we observe changes in activity in electrophysiological recordings from GnRH neurons at 2 weeks of age, when kisspeptin is not yet expressed, at least in the preoptic region. We have modified the text, page 10, to clarify the use of both sexes.

2)'Our results suggest that ectopic GnRH release in the AOB may itself be responsible for these changes in sexual receptivity during postnatal development' - these may still target ME any evidence of projections of these cells in mutant mice?

Experiments conducted in the AOB of voles (Dluzen, Ramirez et al., 1981) clearly suggest that AOB GnRH neurons could project to the median eminence. Unpublished results from our laboratory using retrograde viral approaches indeed show that some GnRH neurons in the olfactory bulb project to the median eminence. However, we have not conducted these experiments in our mutant mice.

Figures: General Note: it is very difficult to see black dots in gray bars or dark purple - colors should be changed and many times information presented in figure could be put in text and de-congest figures.

1D: Dots representing data are nice to see but using black asterisk for significance on these graphs is not. Perhaps red would work

Thank you for pointing this out. We have now changed the visualization of the dots and transferred some data (mainly from Figure 5) to the text.

1E: must be different regions since mutant heavy fibers whereas control primarily cells. Similar regions should be taken so reader can compare and when redone should contain inset with a higher magnification. Also add arrow to show reader where fibers are. This figure does not show - no alteration of the olfactory sensory or terminal tracts was detected in Gnrh::cre; Nrp1loxP/loxP embryos at any age (Figure 1E)

The images in the initial Figure 1E were too contrasting and not properly oriented, which made them difficult to interpret. We do apologize for that. The new Figure 1E now shows the same images with the correct orientation at the nose-brain junction, and olfactory sensory and terminal tracts can now be properly visualized and are indicated by arrowheads in both conditions.

Figure 3:
A) Graph of BrdU should be right of BrdU labeling micrograph and not underneath.

Figure organization has been changed accordingly.

C) One cannot see anything in low mag. Numbers (actual numbers, with stats) can be presented in text.

Can present schematic of explant with graph E (since also shows increase in cell number).

The figure has been changed. We now show a better image of a representative explant. As mentioned previously, some data have been added to the text.

F-I) Give data for scratch assay in text (with actual numbers and stats) and just display H/I

This figure has been removed and the results reported in the text.

Figure 5: Needs organization revisions - remove and indicate in text (with actual numbers and stats) measurements that did not change - at least 5A, B, E, F and H - giving room and organization for those of interest.

The figure has been simplified and many results are now included in the main text (pages 11 and 12).

Minor:
1) A few references appear as numbers in text, but reference format not numbered. This makes reviewing more difficult and shows not well proofed.

We are sorry about this again. The problem has now been fixed.

Referee #2:

General summary: In this report the authors examine the effect of knockout of Nrp1 expression specifically in GnRH neurons in a transgenic mouse model. The study results posit an interesting hypothesis about the role of NRP1 signaling in GnRH neurons resulting in accelerated and aberrant

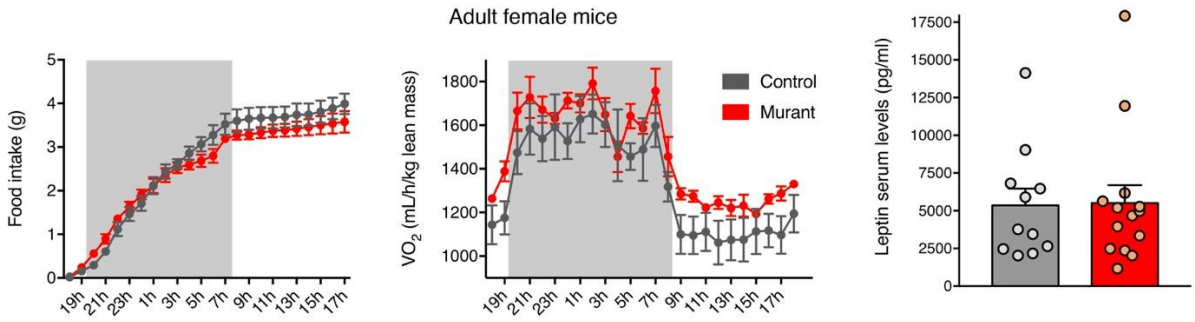
migration and distribution of embryonic GnRH neurons, earlier postnatal adiposity changes that the authors suggest may underlie precocious puberty and changes in sexual behavior in female mice. Overall, the manuscript is well-written, and the author's conclusions are in line the study results. The significant strengths of this study include the use of complementary in vitro molecular and electrophysiologic approaches and in vivo studies.

I have a few specific comments about some aspects of this nice paper.
1. The authors allude to the critical role for MKRN3 expression in central precocious puberty. Did the authors examine MKNR3 expression in postnatal stages? It has been previously reported that reduction of MKNR3 expression in the hypothalamus may signal initiation of puberty (Abreu et al N Engl J Med 368: 2467-75). Does this hold true in this transgenic model?

We thank the reviewer for raising this very interesting point. Indeed, we had addressed this in the original manuscript, but have now fleshed it out in the discussion, pages 15-16, to read "To date, the most frequently mutated gene in pedigrees with central precocious puberty is *MKRN3* (Abreu, Dauber et al., 2013), whose early hypothalamic repression causes precocious puberty in female rats (Heras, Sangiao-Alvarellos et al., 2019)…". We do not have any actual data concerning this yet, as we did not perform any transcriptomic analyses in the tuberal region of the hypothalamus, but it is definitively an intriguing point to address in the future.

2. Given the link between SEMA3A signaling and obesity is also emerging, did the transgenic mice show any differences in food intake, energy expenditure, metabolic markers such as leptin, effects on glucose homeostasis? If so, it would be interesting to speculate any direct role for GnRH activation on metabolic changes.

Data to support such a role would indeed be very interesting. However, we have been unable to collect extensive metabolic data in the cohort of animals under scrutiny, since juvenile animals have proven to be very sensitive to stress. Indeed, we did try to put them in metabolic cages shortly after weaning, but the animals simply stopped eating (both controls and mutants). In adults, our preliminary results using metabolic cages show that overall food intake and energy expenditure are unchanged between the controls and mutants (n=3 to 4 per group), and that leptin levels are also similar (see the three graphs below). One could speculate that the percent increase in body weight is not sufficient to trigger any change in leptin levels. We have not explored glucose homeostasis in these mice. In our opinion, these preliminary results do not justify the inclusion of such a speculation in the discussion at present.



3. The gender discordance of the phenotypes is interesting, and this merits discussion about what factors may underlie this discordance.

We had some reservations about discussing actual sexual dimorphism in our paper since our study has some limitations due to the fact that puberty onset is much more difficult to pinpoint in males than in females. Indeed, as mentioned in our reply to Referee 1 above, while the first ovulation in

females can be spotted by the daily inspection of vaginal smears following vaginal opening, identifying the onset of puberty in males would require killing the animals at different pre- and peri-pubertal ages to detect the presence of the first spermatozoa in the vasa deferentia of both genotypes. Precocious puberty in male mice is very rare, due to the fact that the duration of spermatozoid maturation, which starts at P12, i.e. at minipuberty, is almost incompressible (about 6 weeks), and has only been found, to our knowledge, in animals expressing activatory mutations of the LH receptor, which are also known to cause precocious puberty in boys (see Hess & Renato de Franca, 2008 and McGee & Narayan, 2013 now cited in the text). However, one cannot rule out the possibility that male mice in which Nrp1 expression is selectively knocked out in GnRH neurons undergo precocious puberty, as females do. This is all the more plausible as our results suggest that both migratory defects and changes in postnatal neuronal activity occur in male as well as in female mutants, even though the initiation of sexual maturation (i.e. vaginal opening and balanopreputial separation) is seemingly normal in mutants of both sexes.

We have attempted to present a nuanced discussion of this issue in the revised discussion, pages 14-15.

Concerning sexual dimorphism in terms of metabolism, this is a distinct possibility since it is well known that in females, puberty onset is preceded by an increase in fat mass, while in males it is preceded by an increase in lean mass. This latter fact is now mentioned in the discussion, page 15.

4. Fig 2B - The text suggests a significantly higher GnRH neurons in olfactory bulbs, but the figure does not indicate any markers (*) of statistical significance. Please correct.

This has been fixed. The number of GnRH neurons in the OB of mutant embryos tends to be higher than in controls but the difference is not significant.

1st Revision - Editorial Decision 25th May 2020

Dear Vincent,

Thank you for submitting you revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #1. The referee appreciates the introduced changes and is overall supportive of publication here.

Regarding the remaining points raised as the referee indicates if you have no further data to address the raised issues please ensure that you discuss limitations and caveats of the remaining points.

When you re-submit your revised manuscript will you please take care of the following issues as well.

- we need a COI as well as an Autor Contribution section DATA NOT SHOWN: N/A

- There is a figure call out missing to Figure 4E

- each movie needs to zipped with its legend

- Fig S2 is missing scale bar

- Please relabel Figure S1-S4 as EV fgures and correct call oput in MS text. Please see also author guidelines.

- We also require a Data Availability Section - if there is no data deposited in external databases please state: This study includes no data deposited in external repositories

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels).

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days.

Let me know if we need to discuss anything further - happy to do so.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Aug 2020.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The revised version of this manuscript is greatly improved. However, several revisions are indicated. If such revisions are not feasible, then the authors should be less assertive with their results and increase alternatives explanations in the discussion. For example - Unfortunately we did not correlate behavior (sniffing) with cell count that might have given additional insight...is better than not addressing issue. Other examples given below.

1) Most cre mice lines express cre in transient populations during development. I apologize to the authors for not specifically stating my concern. I agree that the majority of GnRH cells co-expressed the GnRHcre-reporter line used in this paper. However, the comment was directed at other cells during development that transiently express GnRH, which could have NrP1 excised as well. Skynner et al., 1999, reported a transient population of GnRH cells within the forebrain (ganglionic eminence,

subpallium, septal region) that are expressed during embryonic development and gone by P16. The Allen brain atlas indicates Nrp1 expression in a similar region at E13.5. and Tamamaki et al., JCN, 2002 indicated Nrp1 in ganglionic eminence at E16 in rat. This information does not alter the data obtained but may alter the strength of the interpretation unless the authors can show that these transient GnRH cells do not express Nrp1. Thus, the statement "In contrast, littermates with a GnRH-neuron-specific Nrp1 knockout did not show any detectable Nrp1 immunolabeling in GnRH neurons, but displayed Nrp1 in GnRH-negative fibers, such as the vomeronasal/terminal nerves that support GnRH neurons in their migration (Figure 1E). These observations confirm the genetic deletion of Nrp1 in GnRH neurons in mutant mice. - page 5 - is true - but it would also be important to be able to say 'and transient GnRH cells during development do not express Nrp1'. Otherwise one must mention in discussion these transiently expressing cells, which might contribute to the phenotype.

2) Page 6 : At E14.5, these excess cells were located in the ventral forebrain where their number was found to be increased by 41% ...(Figure 2B and 2C) suggesting that a higher proportion of cells had already migrated further towards their destinations at this embryonic stage in mutants than in control littermates.
Values look like two populations and since females show odor change, one should go back and sex genotype (Sry) from cryo or processed sections - protocols for this are available. This would be for animals in figure 2C and 3D. If data cannot be obtained, should at least discuss possibility.

3) were measurements of bulbs taken? mutant bulbs look smaller in 2D

4) Page 7 : "The distribution of GnRH neurons in the forebrain/hypothalamus of adult males (n=4) did not appear to differ markedly from that in females (Figure S1A). Together these results show that alterations of GnRH neuronal number and migration during embryonic development in mutant mice have clear repercussions on their distribution in the postnatal brain.
Distribution may not really be changing - if proportion via increased total number remains constant, i.e. should check to see if distribution changed or just proportional numbers due to increase in cell number - ovlt cell number/total for each genotype - different or the same ? Using numbers from paper E14.5 control has 32% in ventral forebrain and mutant has 36% - not sure these values would be significantly different. Thus, distribution may be appropriate for extra cells. This needs to be examined at E14.5, E18.5 and adult (and reported) before one can say distribution changing.

5) Page 8: Together, these in vivo results indicate that the size of the GnRH neuronal population is regulated by apoptosis at the post-mitotic stage, and that Nrp1 signaling in newborn GnRH neurons may be involved in this process.
The authors should describe where the GnRH cells were located that they counted after AB injections (in figure 3D), ie in reference to figure 2C - in response to first reviewer comments the authors indicate that 'However, nasal injections of Nrp1Ab carried out in embryos in vivo induced an increase in GnRH neuron number, but these supernumerary cells were found to be equally distributed in the nose and the brain (the distribution of these neurons is not illustrated in the paper). - This should be included in revised manuscript and addressed why change in migration did not occur.

Consistent with our in vivo results, the total number of GnRH neurons was roughly twice as high in nasal placode explants from mutant embryos compared to their controls after 8 days of culture was this total = both on and off in periphery or just off?

6) page 9: Together with our in vivo findings on the aberrant migration of GnRH neurons that lack

Nrp1, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons.
In keeping with last sentence of this paragragh, transwell should come before explants -transwell migration, explants cell survival and migration. Revise last sentence to read: Together with our in vivo findings, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons. - you also show increase in cell number in vivo so why highlight only in vivo migration?

7) Page 11 - (two-way repeated measure ANOVA, current step $F(7,133)=865.80$, $p<0.0001$, genotype $F(1,19)=0.09$, $p=0.76$, interaction $F(7,133)=0.54$, $p=0.80$, Figure 4D).
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'does not influence the intrinsic excitability of GnRH neurons, and that differences in spontaneous activity may therefore be the result of their altered connectivity.
Delete their

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May not be acquisition of adult-like pattern since only one time point. Could be response from day 1 - unknown - early age required to make this statement - otherwise should discuss that did not record earlier - maybe cells were always active in the KO.

Point-by-point response to the Referee:

We thank Reviewer 1 and the Editor for their careful review and appreciation of our revised manuscript.

We have taken care of all the issues raised by the editor and the publisher. Our answers to Reviewer 1 are as follows (please note that all changes to the text are highlighted in yellow).

Referee #1:

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The transient expression of cre in some cell populations across the lifespan is a key point that was clarified in the revised version of the manuscript by adding a paragraph in the methods. Indeed, the *cre* driver line we used, which was generated by Catherine Dulac in 2005 and has been extensively characterized, has been shown by Hoffmann and colleagues to have very little ectopic expression of *cre*, including during embryogenesis, in contrast to the GnRH promoters used to drive other reporter genes in previous studies, such as the one by Skynner et al. in 1999. However, we agree that this was not addressed in the main text. We now mention in the Results section, page 6: “These observations, together with the fact that in the specific *Gnrh::cre* mouse model we used, the expression of the transgene is very limited in ectopic neuronal populations during embryogenesis (Hoffmann, Larder et al., 2019), confirm the genetic deletion of Nrp1 in GnRH neurons in mutant mice. In addition, Nrp1 expression remains intact in the non-GnRH hypophysiotropic systems of mutant mice (Giacobini, Parkash et al., 2014), indicating that any neuroendocrine actions of Nrp1 deletion are mediated by GnRH neurons exclusively.”

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Values look like two populations and since females show odor change, one should go back and sex genotype (Sry) from cryo or processed sections - protocols for this are available. This would be for animals in figure 2C and 3D. If data cannot be obtained, should at least discuss possibility.

The data illustrated in Figure EV1 show that selectively knocking out *Nrp1* in GnRH neurons alters GnRH neuronal distribution in a similar manner in the hypothalamus of postnatal male and female mice. It is thus highly unlikely that GnRH neuronal migration is differentially altered in male and female mutant embryos, or that any such differences are linked to putative defects to behavior, since behavioral changes occur during postnatal life (i.e., when GnRH neuronal migration has ended).

However, whether juvenile male mutants exhibit alterations in social odor perception similar to those in mutant females was not explored in the present study. We take the Reviewer's point, and this limitation is now mentioned in the Discussion, page 19, where it reads “Whether similar changes in the perception of social odors also occur in juvenile male mutants remains to be explored.”.

3) were measurements of bulbs taken? mutant bulbs look smaller in 2D

We do apologize for the former Figure 2D, which indeed gives the erroneous impression that mutant bulbs are smaller (due to the angle of the picture taken from the 3D view). The new Figure 2D now shows two representative pictures of a frontal section of the bulb in a control and a mutant.

4) Page 7 : "The distribution of GnRH neurons in the forebrain/hypothalamus of adult males (n=4) did not appear to differ markedly from that in females (Figure S1A). Together these results show that alterations of GnRH neuronal number and migration during embryonic development in mutant mice have clear repercussions on their distribution in the postnatal brain.
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We thank the reviewer for this discussion. Unfortunately, the number of neurons in the nose in adulthood is almost impossible to test because of calcification. However, we have an ensemble of known facts and current results that support our conclusions. Firstly, as indicated in Figure EV2, the birth of new GnRH neurons is complete by E11.5, but their migration into the brain starts soon after this stage and is ongoing at E14.5. Considering that at E12.5, the total number of GnRH neurons is comparable between the two genotypes but that at E14.5, this number remains comparable in the nose despite there being a significantly higher number of GnRH neurons in the ventral forebrain (Figure 2A-C), the trend is clearly towards an increase in the ratio of brain neurons to nose neurons, i.e. a change in distribution. Secondly, a proportional change in neuronal numbers would be reflected in all areas, and might even be expected to be higher in more proximal regions such as the OB while migration is still ongoing. However, the opposite is true, as seen in Figure 2B and 2C, with the increase in the number of GnRH neurons becoming both more pronounced and more significant along the migratory path. Even though the final percentage of neurons may appear similar, this is both more difficult to measure statistically, and less informative than the trend towards increased cell numbers in the hypothalamus that we observe. We have tried to express this more clearly in the Results section, page 7, showing that while the number of GnRH neurons in the nose does not differ between mutants and controls at E14.5, it is dramatically increased in the brain of mutants, thus suggesting a marked change in their distribution: “At E14.5, while the total number of neurons in the nose did not change (two-way ANOVA, anatomical region, $F_{(1,9)}$=309.3, p<0.001, genotype $F_{(1,9)}$=12.7, p=0.006, interaction $F_{(1,9)}$=7.5, p=0.02; Fisher’s LSD multiple-comparison test, Control 325±36 vs. mutant 368±45, $t_{(18)}$=0.63; p=0.78, n=6 and 5), the number of neurons reaching the brain markedly increased in mutant embryos at the same developmental stage (Control 1027±53 vs. mutant 1327±56, $t_{(18)}$=4.46; p<0.001, n=6 and 5).”

5) Page 8: Together, these in vivo results indicate that the size of the GnRH neuronal population is regulated by apoptosis at the post-mitotic stage, and that Nrp1 signaling in newborn GnRH neurons may be involved in this process.
The authors should describe where the GnRH cells were located that they counted after AB injections (in figure 3D), ie in reference to figure 2C - in response to first reviewer comments the authors indicate that 'However, nasal injections of Nrp1Ab carried out in embryos in vivo induced an increase in GnRH neuron number, but these supernumerary cells were found to be equally distributed in the nose and the brain (the distribution of these neurons

is not illustrated in the paper). - This should be included in revised manuscript and addressed why change in migration did not occur.

With regard to the Nrp1Ab experiments, as suggested by the Reviewer, we feel that it would be better to remain cautious in our interpretation. Accordingly, on page 8, it now reads “Of note, the treatment of E11.5 embryos with Nrp1 neutralizing antibodies is likely to disrupt the vomeronasal/terminal nerve projections used by GnRH neurons to migrate from the nose to the brain (Hanchate et al., 2012), in addition to GnRH neurons themselves; because of this confounding factor, we do not report the distribution of GnRH neurons at E14.5 in the group of experiments represented in Figure 3I.”

Consistent with our in vivo results, the total number of GnRH neurons was roughly twice as high in nasal placode explants from mutant embryos compared to their controls after 8 days of culture was this total = both on and off in periphery or just off?

Only the neurons migrating outside the explant were counted. This has now been clarified in the legend of the new Figure 3D.

6) page 9: Together with our in vivo findings on the aberrant migration of GnRH neurons that lack Nrp1, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons.

In keeping with last sentence of this paragragh, transwell should come before explants -transwell migration, explants cell survival and migration. Revise last sentence to read: Together with our in vivo findings, these in vitro results suggest that, in addition to acting as the receptor for a guidance cue and thus regulating cell migration, Nrp1 regulates the survival of newly born GnRH neurons. - you also show increase in cell number in vivo so why highlight only in vivo migration?

Thank you for this suggestion. We have changed the order of the panels in Figure 3 and amended the text accordingly.

7) Page 11 - (two-way repeated measure ANOVA, current step $F(7,133)=865.80$, $p<0.0001$, genotype $F(1,19)=0.09$, $p=0.76$, interaction $F(7,133)=0.54$, $p=0.80$, Figure 4D).

I believe this should be 4E.

'does not influence the intrinsic excitability of GnRH neurons, and that differences in spontaneous activity may therefore be the result of their altered connectivity.

Delete their

We thank the reviewer for his/her careful assessment of our manuscript and apologize for the errors. We have edited the text accordingly.

8) Page 14: In the present study, the lack of Nrp1 signaling also appears to affect the neurophysiology of postmigratory GnRH neurons, as seen by the early acquisition of a mature firing pattern by GnRH neurons during postnatal development.
May not be acquisition of adult-like pattern since only one time point. Could be response from day 1 - unknown - early age required to make this statement - otherwise should discuss that did not record earlier - maybe cells were always active in the KO.

Contrary to what one might think, it has been shown previously by Moenter and others that GnRH neurons fire at a high frequency shortly after birth and that the maturation of their firing pattern translates into a decrease rather than an increase in frequency. Here we observe that this decrease occurs earlier in mutants than in controls (Figure 4C and 4D).

2nd Revision - Editorial Decision 13th Jul 2020

Dear Vincent,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Manuscript Number: EMBOJ-2020-104633R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to pre-determine sample size. Sample sizes in this study were estimated based on previous studies using similar mouse models and literature documentation of similar experiments. We have tried to use the lowest number of animals to achieve statistical significance, without compromising possible outcomes. All sample sizes are provided in the texte and Figures.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Statment included in the Statistics paragraph of Methods section
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples of animals were excluded based on outlier analysis (GraphPad Prism software, ROUT, Q=1%)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All experiments were done in a strictly double-blind manner.
For animal studies, include a statement about randomization even if no randomization was used.	Selection of mice was based solely on genotype and sex. All other factors wer randomized
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes. All experiments and analysis was done in double-blind manner
4.b. For animal studies, include a statement about blinding even if no blinding was done	The measurements were all performed by an experimenter blind to the genotype
5. For every figure, are statistical tests justified as appropriate?	Staistical tests used for every figure are justified in the text
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All analyses were performed using Prism 7 (GraphPad Software) and assessed for normality (Shapiro-Wilk test) and variance, when appropriate.

Is there an estimate of variation within each group of data?	Variance is estimated as part of the statistical analysis wherever necessary
Is the variance similar between the groups that are being statistically compared?	Not all variances were similar. If alternative statistical tests that are not affected by dissimilar variance was available, it has been used for that analysis

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We added the details of antibody used in the Method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The GN11 mouse cell line (Zakaria et al., 1996) was used and was tested negative for micoplasms.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All relevant details are present in the Methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	As stated in the Methods all animal studies were performed with the approval of the Institutional Ethics Committees for the Care and Use of Experimental Animals of the University of Lille and the French Ministry of National Education, Higher Education and Research (APAFIS#2617-2015110517317420 v5 and APAFIS#13387-2017122712209790 v9), and under the guidelines defined by the European Union Council Directive of September 22, 2010 (2010/63/EU).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We comply with he ARRIVE guidlines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Statement included in the Methods section
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A