

Tmem117 in AVP neurons regulates the counterregulatory response to hypoglycemia

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study the authors convincingly demonstrate that the hypothalamic protein Tmem117 protects against elevated ROS and cell death in AVP neurons, and that knocking out Tmem117 in these neurons leads to cell death. However, the major thrust of this paper was to demonstrate that Tmem117, in AVP neurons plays a role in hypoglycemia counter regulation (per the title). The data supporting this hypothesis are much less convincing.

****Major comments****

There are 2 major findings of this study that are problematic (#1 and 2 below). Further experiments are needed to support their conclusions.

1. In figure 4 and throughout the text they refer to AVP neurons as being "directly" inhibited by glucose but they do not show this. They do show membrane depolarization in response to decreased glucose from 2.5 to 0.1 mM; however this could easily be an indirect effect. To demonstrate that they directly sense glucose, it is necessary to block presynaptic input (generally done with the sodium channel blocker tetrodotoxin). In addition, the example recordings are not very good. The neurons seem to just decrease action potential frequency throughout the recording, despite the membrane depolarization. In the methods section, they state that cell resistance was measured but they do not report it. Looking at the traces, it appears that resistance does not change in response to decreased glucose which further supports a presynaptic action. In order to characterize these neurons as directly glucose sensing, they must use a blocker of presynaptic activity such as tetrodotoxin and the resistance values should be reported. They should also choose better representative traces.

2. The relationship between the AVP/Tmem117 knockout and glucagon secretion is also not very convincing - especially in terms of the data showing that the effect seen at 1-week reverses by 3 weeks post knockdown in males when the neurons have died. First, the effect is very slight to begin with. Second, and more of a concern, the over secretion is still evident at the later time point, it just did not reach significance. This could have been due to the difference in sample size. Sample size in the early timepoint is 15-16 and in the late time point is reported as 8-9 (almost half the size), and in fact when I try to count data points, it really looks like the WT sample size is only 5. To make the conclusion that the effect disappears at the later time it is necessary to increase sample size so that the studies are equivalent. This is very important because these data are what is supporting the hypothesis that Tmem117 in AVP neurons regulates (inhibits) glucagon secretion. It is also concerning that the increased glucagon secretion in the knockout did not translate into differences in the depth of hypoglycemia raising concerns about physiological relevance.

3. It is also curious that the females in proestrus were similar to males but that females in other stages showed no effect. If, as mentioned in the discussion, estradiol was involved one would expect that males would be more similar to females in stages with

low estradiol. The sex difference should be discussed more fully since it does seem to be one of the strong conclusions in the study

4. The discussion needs to be adjusted in line with the findings.

****Minor comments:****

The BXD mice should be defined and the reason for their use explained

The abbreviation cQTL needs to be defined at first use in the text of the results

2. Significance:

Significance (Required)

The observation that Tmem117 is protective against ROS and cell death is interesting. However, the other conclusions are not supported by the data which decreases the overall significance of the study

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The present study led by Gaspari, et al shows a new role for Tmem117 as a major regulator in the CRR. The authors used a variety of techniques to demonstrate that Tmem117 inactivation modulates hypoglycemia-induced responses by regulating AVP action in the hypothalamus. I found the rationale of the study interesting, the results seem to support the author's claims and conclusions seem to be proper. However there are some minor issues that needs to addressed:

1. Page 5, line 111: Fig1 shows Tmem117 expression in the hypothalamus. Tmem117 expression in AVP neurons in the PVN and SON are highlighted, however, AVP is not only expressed in these areas in the hypothalamus and in extra-hypothalamic areas. Was Tmem117 also expressed in AVP neurons beyond these areas?
2. Page 10, line 219: Fig2 shows CRR responses after conditional Tmem117 ko in AVP neurons. A sex-dependent difference was observed in the increased CRR after Tmem117 inactivation. A similar pattern between males and females during pro-estrus in co-peptin and glucagon was observed. Was AVP expression and AVP neuron response to hypoglycemia also analyzed in both sexes, and in the case of females, were the results analyzed during the pro-estrus phase?
3. Many of the experiments performed after Fig 3 (glucose responsiveness of AVP neurons, ER stress and ROS production, Ca²⁺ imaging) are mainly analyzed in the SON, but not the PVN. What is the rationale for analyzing only the SON in some experiments? Analyzing the PVN is of relevance due to its known role in CRR triggering.
4. Page 16, line 346: Again here, only BIP expression in AVP neurons is shown in the SON. I would be interested in looking at expression in AVP neurons in the PVN. Same goes for activation of these neurons (measured by Ca²⁺) after insulin administration.
5. What would be the role of Tmem117 in CRR triggering after hypoglycemia? Would its expression change during hypoglycemic conditions versus normoglycemia? Would that be different in models of pathology, like diabetes? Speculation of its physiological role in the discussion might be of help to visualize its role beyond inactivation.

2. Significance:

Significance (Required)

The study of Gaspari, et al proposes a new player in the hypoglycemia-induced CRR, specially affecting AVP modulation of CRR. The results and conclusions are of relevance to the field, as the mechanisms mediating hypoglycemia-induced CRR and how these are affected during pathology are not completely understood. The study is relevant in the metabolic field and for a neuroendocrinology public.

Field of expertise: Neuroendocrinology, hypoglycemia, diabetes.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

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Yes

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Glucagon secretion counters hypoglycaemia, and this is in part regulated by the CNS.

The authors investigated the role of hypothalamic Tmem117, which was previously identified in a genetic screen as a regulator of the counter-regulatory response to hypoglycaemia. They show that Tmem117 is expressed in vasopressin neurons and that inactivation of Tmem117 in AVP neurons increases hypoglycemia-induced vasopressin secretion leading to higher glucagon secretion. They show this is oestrus cycle phase-dependent.

The manuscript is generally well written and the results of good quality. The findings are important.

I have some comments:

****Major:****

1. The in vivo calcium data is not convincing (e.g. Figure 5j). Why is the activity not similar to that normally seen from populations of neurons (and in particular AVP neurons) in vivo - bursting/spikes. This is just a background increase in calcium... not an increase in activity?

2. Why would this response be oestrus cycle dependent? This is given very little thought in the manuscript.

****Minor:****

1. How does the regulation make sense in the context of AVPs normal role in whole body physiology?
2. What do the authors think about the role of AVP in islets, some authors suggest it actually has the opposite effect.
3. Why have the authors left the recent study by Kim, Rorsman and colleagues et al. 2022 (eLife) to the discussion? This manuscript is an important piece in this field and should be introduced in the introduction.
4. The intro should also mention that islets also can intrinsically regulate glucagon release.
5. Has *tmem117* shown up in any human screens of hypoglycaemia risk? Do *tmem117* variants show in any osmoregulation or insipidus risk?
6. Please clarify what N= is? What is a statistical unit in each case in the graphs. It is not clear when it is a mouse, cell or experiment. This should be justified in each case.

2. Significance:

Significance (Required)

This is important, but some of the in vivo calcium data is weak. The manuscript is also riddled with self-citations and doesn't really put the results into a broader context. In addition, the finding is in a sense piggybacking off a recent revelation in the field (AVP neuron importance) using a technique that is available to the authors (*Tmem117* manipulation) rather than trying to address the hypothesis/question with THE BEST approach to answer the question. The question feels like: "how can we use our mouse model to probe this AVP neuron hypothesis ourselves". Strange. The finding that the results are only relevant during a particular phase of the oestrus cycle feels a bit random and lacks adequate explanation. I still like the manuscript very much, the experiments are very technically challenging, the results are very interest, the data (mostly) of excellent quality, and the relevance to the field highly important, but I feel there is still room for improvement (unlikely more experiments, definitely some thinking and rewriting).

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Yes

Full Revision



Manuscript number: RC-2022-01806

Corresponding author(s): Bernard, Thorens

1. General Statements

We thank the reviewers for their critical comments. We have addressed these comments as detailed below.

2. Point-by-point description of the revisions

Reviewer #1

Major comments:

1. In figure 4 and throughout the text they refer to AVP neurons as being "directly" inhibited by glucose but they do not show this. They do show membrane depolarization in response to decreased glucose from 2.5 to 0.1 mM; however this could easily be an indirect effect. To demonstrate that they directly sense glucose, it is necessary to block presynaptic input (generally done with the sodium channel blocker tetrodotoxin). In addition, the example recordings are not very good. The neurons seem to just decrease action potential frequency throughout the recording, despite the membrane depolarization. In the methods section, they state that cell resistance was measured but they do not report it. Looking at the traces, it appears that resistance does not change in response to decreased glucose which further supports a presynaptic action. In order to characterize these neurons as directly glucose sensing, they must use a blocker of presynaptic activity such as tetrodotoxin and the resistance values should be reported. They should also choose better representative traces.

Answer: By "directly inhibited" we do not refer to a cell-autonomous effect but to a direct glucose responsiveness. Our cFOS experiments (Fig 3) and preexisting *in vivo* fiber photometry data (Kim et al., PMID: 34787082; Mandelblat-Cerf et al., PMID: 27989461) reported activation of AVP neurons in conditions of hypoglycemia. We further wanted to verify that the activation was triggered directly by the decreased glucose levels and not by accompanying systemic signals (for example insulin levels, stress response, etc). Furthermore, we aimed at identifying if inactivation of *Tmem117* in AVP neurons affects their glucose responsiveness (at the circuit level). Therefore, we decided to take into account the synaptic inputs. Of course, this approach provides information at the circuit level (that we consider essential for CRR characterization) but is also accompanied with highly variable firing patterns. We focused our analysis on the membrane potential, as an indicator of cell excitability state that based on our previous experience was proven to be consistently affected in glucose sensing neurons regardless of cell-type, inputs or topology (Strembitska et al., PMID: 36180454; Kessler et al., PMID: 34622169; Quenneville et al., PMID: 32839348; Quenneville et al., PMID: 32839348; Labouèbe et al., PMID: 29218531; Steinbusch et al., PMID: 27422385; Labouèbe et al., PMID: 27322418; Lamy et al., PMID: 24606905). We used

membrane resistance as an indicator of our patch-clamp quality. As specified in the Materials and Methods section (pages 33, lines 702-704), neurons with an access resistance $>25 \text{ M}\Omega$ or changing by $>20\%$ during the recording were excluded from the analysis.

We understand that the term “directly affected” has created some confusion since it could be interpreted as cell-autonomous effect. Therefore, we have exchanged the term as follows:

- Results section (page 14, line 281): “To determine whether AVP neurons were directly sensitive to hypoglycemia and whether Tmem117 would modify this sensitivity” was converted to “To determine whether AVP neurons were sensitive to the decreased glucose availability and whether Tmem117 would modify this sensitivity”
- Discussion section (page 27, lines 548-553): “Thus, AVP neurons can directly respond to hypoglycemia. Interestingly, a recent study reported that hypoglycemia can also activate AVP neurons indirectly, through afferent connections arising from GI neurons of the basolateral medulla (Kim et al., 2021). Thus, AVP neurons are part of a brainstem-hypothalamus neuronal circuit where hypoglycemia can be sensed by neurons located at multiple sites to activate the secretion of AVP leading to increased secretion of GCG.” was converted to “Thus, AVP neurons can respond to decreased glucose levels. Interestingly, a recent study reported that hypoglycemia can also activate AVP neurons through afferent connections arising from GI neurons of the basolateral medulla (Kim et al., 2021). Thus, AVP neurons are part of a brainstem-hypothalamus neuronal circuit where hypoglycemia can be sensed by neurons located at multiple sites to activate the secretion of AVP leading to increased secretion of GCG.”

Regarding the membrane resistance measurements, 6 out of 7 *AVP^{TM117KO}* GI cells and 6 out of 9 *AVP^{TM117WT}* GI cells show a decrease in conditions of low glucose suggesting a cell autonomous effect, at least in a subpopulation of AVP GI neurons. We realized, based on the reviewer’s comment, that not including these data can lead to misinterpretation of the results, therefore we included the membrane resistance measurements in figure 4 and added the corresponding explanation in the Results section (page 15, lines 295-298).

Regarding the representative traces, those included in the figure clearly show the increase in membrane potential that was consistent in all our GI cells. Choosing a representative trace for firing patterns is unrealistic given the high variability observed without the use of synaptic blockers.

2. The relationship between the AVP/Tmem117 knockout and glucagon secretion is also not very convincing - especially in terms of the data showing that the effect seen at 1-week reverses by 3 weeks post knockdown in males when the neurons have died. First, the effect is very slight to begin with. Second, and more of a concern, the over secretion is still evident at the later time point, it just did not reach significance. This could have been due to the difference in sample size. Sample size in the early timepoint is 15-16 and in the late time point is reported as 8-9 (almost half the size), and in fact when I try to count data points, it really looks like the WT sample size is only 5. To make the conclusion that the effect disappears at the later time it is necessary to

increase sample size so that the studies are equivalent. This is very important because these data are what is supporting the hypothesis that *Tmem117* in AVP neurons regulates (inhibits) glucagon secretion. It is also concerning that the increased glucagon secretion in the knockout did not translate into differences in the depth of hypoglycemia raising concerns about physiological relevance.

Answer: The sample size required based on our power calculations for these traits (plasma glucagon and copeptin) in male mice is 8 animals per group. Figure 2 consists of data merged from two individual experiments, both of which showed a significant effect. Figure 6 is the 3rd replication of our phenotypic observations with the addition of a later timepoint measurement. The number of animals included is 9 *AVP*^{TM117WT} and 8 *AVP*^{TM117KO}. Only for copeptin levels (panel F), as stated in the figure legend, the n is 8 mice per group, since for one of the *AVP*^{TM117WT} mice the baseline levels of copeptin were undetectable by ELISA. Counting the datapoints in a 2D graph can cause misinterpretation since similar values can be depicted as overlapping points. We had decided to present in Fig 6 only the later timepoint that corresponds to the IF results reporting AVP cell death. We now realize based on the reviewer's comment that this can cause misinterpretation of the phenotypic outcome, therefore we have exchanged graphs E-G (containing 2 timepoints) for the corresponding graphs that contain all 3 timepoints. We believe that with this addition the concern will be addressed since with the same sample size there is a significant increase in glucagon and copeptin secretion in *AVP*^{TM117KO} mice at the early timepoint that is no longer evident at the later timepoint.

Regarding the concern of effect size, we acknowledge the fact that we are reporting a small effect. But the CRR is controlled by a highly complex network of hypoglycemia activated neurons, which are located in different brain regions and act in an integrated manner to induce glucagon secretion through autonomic control, the HPA axis, and the secretion of AVP (reviewed in Steinbush et al., PMID: 26163755; Thorens, PMID: 34989155). Therefore, in this highly redundant and robust system, suppressing only one hypoglycemia sensing node, cannot be expected to have major consequence on the glucagon secretion. Nevertheless, we provide solid evidence that inactivation of *Tmem117* in AVP neurons leads to a slight upregulation of AVP secretion that is accompanied by a subsequent increase in the glucose-stimulating hormone glucagon. To further highlight this observation, we added in figure 6 the correlation analysis between glucagon and copeptin levels of each tested sample that is reporting a significant positive correlation in both genotypes.

Regarding the concern for physiological relevance, we would like to clarify that in this model we have not followed the reestablishment of normoglycemia over time. The timepoint of blood collection and glycemic measurement was selected based on our previous experience. We have performed multiple times full insulin tolerance tests in various mouse models, and we selected here (and in other papers: Picard et al., PMID: 35339728; Strembitska et al., PMID: 36180454) the one-hour time point because this is always the nadir of the glycemic curves, where CRR has the strongest effect in stimulating the secretion of CRR hormones. Glycemia at this time point depends on the insulin sensitivity of the animal. Thus, finding similar glycemia at 60 minutes after insulin injection indicates similar insulin sensitivity in *AVP*^{TM117WT} and *AVP*^{TM117KO} mice, further

supporting that the increased glucagon secretion is directly correlated to the increased AVP secretion.

3. It is also curious that the females in proestrus were similar to males but that females in other stages showed no effect. If, as mentioned in the discussion, estradiol was involved one would expect that males would be more similar to females in stages with low estradiol. The sex difference should be discussed more fully since it does seem to be one of the strong conclusions in the study

Answer: We acknowledge that the discussion of the sex-specific phenotype was minimal in the initial version of our manuscript. We consider this finding very important for the general understanding of sex-differences in CRR, therefore we have included it in our main figures. But given that our observations remain in the phenotypic level our intention was to keep the discussion minimal and not add a lot of speculations that would not be supported by experimental data. We have performed all our further mechanistic analysis only in male mice and therefore we are not able to comment about this sex specific effect with evidence on the underlying mechanism. Of course, we have our literature-based explanation/hypothesis for the observed phenotype and given that this point was raised by all 3 reviewers, we have expanded this section of the discussion (pages 26, lines 520-542).

4. The discussion needs to be adjusted in line with the findings.

Answer: Several adjustments both in the Results and Discussion sessions have been incorporated based on the reviewers' comments. Details on the adjustments for each point raised can be found in the corresponding answer.

Minor comments:

The BXD mice should be defined and the reason for their use explained

Answer: We did not include further information in the first place because all the details and rationale for the use of BXD mice were included in the original publication of this genetic screen (Picard et al., PMID: 35339728). We have now added further details on the origin of the BXD mouse lines accompanied with the corresponding reference (page 4, lines 83-85).

The abbreviation cQTL needs to be defined at first use in the text of the results

Answer: We added the abbreviation (page 5, line 102).

Reviewer #2

Minor comments:

1. Page 5, line 111: Fig1 shows Tmem117 expression in the hypothalamus. Tmem117 expression in AVP neurons in the PVN and SON are highlighted, however, AVP is not only expressed in these areas in the hypothalamus and in extra-hypothalamic areas. Was Tmem117 also expressed in AVP neurons beyond these areas?

Answer: Based on our immunofluorescent detection Tmem117 is only observed in AVP neurons of the PVN and SON. This pattern (Fig 1A), the percentage of AVP-Tmem117 co-positive cells in these two areas (Fig 1H, I) and the presence of strong positive staining in the neurohypophysis (Fig 1B) points towards specific expression in magnocellular neurosecretory AVP cells. We do not observe staining in other AVP positive neurons. An example can be found in Fig 1A where Tmem117 staining is localized at the PVN and SON, but not at the Suprachiasmatic nucleus.

2. Page 10, line 219: Fig2 shows CRR responses after conditional Tmem117 ko in AVP neurons. A sex-dependent difference was observed in the increased CRR after Tmem117 inactivation. A similar pattern between males and females during pro-estrus in copeptin and glucagon was observed. Was AVP expression and AVP neuron response to hypoglycemia also analyzed in both sexes, and in the case of females, were the results analyzed during the pro-estrus phase?

Answer: Unfortunately, a detailed analysis of the mechanistic underpins of the estrus phase-dependent phenotype in female mice would exceed the spectrum of this initial characterization. We do believe that this finding is of great importance for the general understanding of sex-differences in CRR, therefore we have included it in our main figures. But our observations remain at the phenotypic level. We have performed all our further mechanistic analysis only in male mice. Of course, we have our literature-based explanation/hypothesis for the observed phenotype and given that this point was raised by all 3 reviewers, we have expanded this section of the discussion (pages 26, lines 520-542).

3. Many of the experiments performed after Fig 3 (glucose responsiveness of AVP neurons, ER stress and ROS production, Ca²⁺ imaging) are mainly analyzed in the SON, but not the PVN. What is the rationale for analyzing only the SON in some experiments? Analyzing the PVN is of relevance due to its known role in CRR triggering.

Answer: The reason for focusing our single-cell level analysis only in SON is its homogeneous population of AVP-Tmem117 double positive cells (Fig 1I). In the PVN about 10% of AVP neurons are negative for Tmem117 (Fig 1H). Therefore, we would be unable to distinguish between the cells with *Tmem117* inactivation and those that did not express it at the first place. We agree with the reviewer that PVN is of great relevance for CRR induction, and this is why for all our *in vivo* phenotyping experiments we aimed at targeting both areas. Our *AVP^{TM117KO}* mouse model is generated by injection of the AAV6-AVP-icre virus in the posterior pituitary and is targeting both regions (Fig S2E). This model was used for all our phenotypic analyses (Fig 2, 6) and for the fiber photometry recordings (Fig 5H-M). The Ca²⁺ signal we obtain is a collective output of AVP magnocellular terminals in the posterior pituitary deriving from both PVN and SON. But when it comes to the analysis of responses at the single-cell level, not having the ability to identify the subpopulation of Tmem117 negative cells could have a big impact on the results and their interpretation.

4. Page 16, line 346: Again here, only BIP expression in AVP neurons is shown in the SON. I would be interested in looking at expression in AVP neurons in the PVN. Same goes for activation of these neurons (measured by Ca²⁺) after insulin administration.

Answer: As mentioned above, we did not quantify these single-cell events in AVP neurons of the PVN due to the inability of effectively distinguishing between cells with *Tmem117* inactivation and those that do not express *Tmem117*. But based on our supporting data (activation of PVN AVP cells in Fig 3 and decreased number of AVP PVN cells upon *Tmem117* inactivation in Fig 6) we believe that the effect of *Tmem117* inactivation is comparable between the *Tmem117*-positive AVP cells of both areas.

5. What would be the role of *Tmem117* in CRR triggering after hypoglycemia? Would its expression change during hypoglycemic conditions versus normoglycemia? Would that be different in models of pathology, like diabetes? Speculation of its physiological role in the discussion might be of help to visualize its role beyond inactivation.

Answer: We focused our discussion strictly on the acquired results, but we agree with the reviewer than including speculations regarding the physiological role of *Tmem117* would enhance the communication of our results. We have incorporated some extra points focusing on the possible role of *Tmem117* expression in physiological conditions and pathological states in the Discussion section (pages 27-28, lines 562-571). Furthermore, we have included some extra data from microdissected PVN and SON tissue reporting downregulation of *Tmem117* in the SON as a physiological response to insulin-induced hypoglycemia (Fig 3G-I).

Reviewer #3

Major comments:

1) The in vivo calcium data is not convincing (e.g. Figure 5j). Why is the activity not similar to that normally seen from populations of neurons (and in particular AVP neurons) in vivo - bursting/spikes. This is just a background increase in calcium... not an increase in activity?

Answer: Our aim with the fiber photometry experiments was not to detect the activity of AVP neurons but to evaluate the Ca^{2+} fluctuations in AVP neuronal terminals, directly at the site of AVP exocytosis. The activity of AVP neurons in conditions of hypoglycemia has been previously detected with similar techniques at the level of cell bodies in the SON (Kim et al., PMID: 34787082; Mandelblat-Cerf et al., PMID: 27989461) and was further supported by our cFOS and ephys experiments (Fig 3, 4). These experiments showed that inactivation of *Tmem117* did not affect the activation of AVP neurons in conditions of hypoglycemia. But our phenotypic characterization revealed higher secretion of AVP (Fig 2) and our *in vitro* (Fig S3) and *in vivo* (Fig 5A-G) data demonstrated increased ER stress and ROS in *AVP^{TM117KO}* cells. Therefore, we wanted to assess if this increased stress is associated with increased Ca^{2+} at the level of neuronal terminals which would enhance AVP exocytosis. The acquired signal is a collective output of AVP magnocellular neurons from PVN and SON and although it is not resembling the pattern observed from cell body recordings, we believe it provides important information for the output of this circuit in conditions of hypoglycemia. Our results do not only report that *Tmem117* inactivation is accompanied by an increase in intracellular Ca^{2+} at the level of nerve terminals/exocytosis, but further demonstrate (for the first time to our knowledge) that the activity reported in conditions of hypoglycemia is linked, as expected, to an increase in local Ca^{2+} levels at the posterior pituitary. All the essential controls for normalization and interpretation have been taken into account as mentioned in the

methods section (page 31, lines 655-668). GCaMP7 signal recordings are corrected for artifacts and photobleaching. The isosbestic channel is subtracted to correct for non-GCaMP7 related signal artifacts and then at a second stage (during the DF/F0 normalization) the traces are corrected for photobleaching by the running average algorithm. Furthermore, 60min recordings of mice injected with saline are used as control for determining the increased signal after insulin injection. We have now added a clarification on the origin of the recorded signal in the Results section (page 18, line 368).

2) Why would this response be oestrus cycle dependent? This is given very little thought in the manuscript.

Answer: We acknowledge that the sex-specific phenotype observed was not thoroughly discussed in the initial version of our manuscript. We included the data in a main figure since we consider them as an important piece of information supporting the crucial role of sex hormones in the regulation of CRR. On the other hand, since our observations remain in the phenotypic level, we thought it would be better to keep the discussion on the topic short and not include various literature-based hypothesis that would not be supported by experimental data. We have performed all our further mechanistic analysis only in male mice and therefore we are not able to comment about this sex specific effect with evidence on the underlying mechanism. We have now realized based on the reviewers' comments that further discussion on the topic is needed, therefore we have expanded this section of the Discussion (page 26, lines 520-542).

Minor comments:

1) How does the regulation make sense in the context of AVPs normal role in whole body physiology?

Answer: We focused our discussion strictly on the acquired results, but it has become evident based on the reviewers' comments that a discussion of the broad picture regarding the role of AVP and Tmem117 in physiological and pathological conditions would strengthen the interpretation of our results. We have now incorporated some extra points focusing on the possible role of *Tmem117* expression in physiological conditions and pathological states in the Discussion section (pages 27-28, lines 562-571).

2) What do the authors think about the role of AVP in islets, some authors suggest it actually has the opposite effect.

Answer: The literature on the role of AVP on pancreatic islets shows indeed some controversies. A couple of studies suggest an action on beta cells and insulin secretion without any effect on glucagon secretion. On the other hand, the evidence on the stimulation of glucagon secretion is supported by numerous studies over the years and has been convincingly demonstrated with various *ex vivo* and *in vivo* models. Our study, in line with these findings, verifies the increased AVP secretion in conditions of hypoglycemia and reports a positive correlation between the levels of circulating copeptin and glucagon (Fig 6H).

3) Why have the authors left the recent study by Kim, Rorsman and colleagues et al. 2022 (eLife) to the discussion? This manuscript is an important piece in this field and should be introduced in the introduction.

Answer: We have now added the AVP induced glucagon secretion, accompanied by the supporting references (including the reference mentioned by the reviewer) as integral part of the CRR in our Introduction section (pages 3-4, lines 68-71).

4) The intro should also mention that islets also can intrinsically regulate glucagon release.

Answer: We focused our introduction exclusively to the brain triggered glucagon secretion, but in accordance with the reviewer's comment we have now incorporated the intra-islet regulation and supporting literature in the Introduction (page3, line 55).

5) Has *tmem117* shown up in any human screens of hypoglycaemia risk? Do *tmem117* variants show in any osmoregulation or insipidus risk?

Answer: Not to our knowledge.

6) Please clarify what N= is? What is a statistical unit in each case in the graphs. It is not clear when it is a mouse, cell or experiment. This should be justified in each case.

Answer: We have now justified the corresponding values in all figure legends.

Dear Dr. Thorens,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. Referee 3 still has one more minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few editorial requests will also need to be addressed:

- Please add up to 5 keywords to the ms file.
- Please add a Data Availability Section (DAS) to the end of the M&M section. If you have not deposited any data in public repositories please mention this fact in the DAS.
- The conflict of interest subheading should be corrected to "Disclosure and Competing Interest Statement"
- Please remove the author credits from the ms file. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, if you wish. See also guide to authors <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.
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- FIGURE panels in Figure 3 and 5 are not called out alphabetically; Figure 4J is missing a callout, please correct.
- The order of the ms sections should be: abstract, introduction, results, discussion, materials & methods, data availability section, acknowledgments, disclosure statement and competing interests, references, main figure legends, EV figure legends.
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I would like to suggest some minor changes to the title and abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Tmem117 in vasopressin neurons regulates the counterregulatory response to hypoglycemia

The counterregulatory response to hypoglycemia (CRR), which ensures a sufficient glucose supply to the brain, is an essential survival function. It is orchestrated by incompletely characterized glucose-sensing neurons, which trigger a coordinated autonomous and hormonal response that restores normoglycemia. Here, we investigate the role of hypothalamic Tmem117, identified in a genetic screen as a regulator of CRR. We show that Tmem117 is expressed in vasopressin magnocellular neurons of the hypothalamus. Tmem117 inactivation in these neurons increases hypoglycemia-induced vasopressin secretion leading to higher glucagon secretion in male mice, and this effect is estrus cycle phase-dependent in female mice. Ex vivo electrophysiological analysis, in-situ hybridization and in vivo calcium imaging reveal that Tmem117 inactivation does not affect the glucose-sensing properties of vasopressin neurons but increases ER-stress, ROS production and intracellular calcium levels accompanied by increased AVP production and secretion. Thus, Tmem117 in vasopressin neurons is a physiological regulator of glucagon secretion, which highlights the role of these neurons in the coordinated response to hypoglycemia.

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Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

I do not know embo reports so i cannot comment on whether it is or is not suitable for publication here. I can however confirm that the authors have adequately addressed my comments.

Referee #2:

The authors addressed some of the concerns. Even though the data is interesting, additional mechanistic evidence would be recommended.

Referee #3:

The authors have satisfactorily addressed the majority of my concerns. They have also added the membrane resistance measurements as requested. However, in the text describing figure 4 they indicate that resistance was decreased suggesting a cell autonomous response. Since figure 4 shows no significant difference for membrane resistance in 2.5 and 0.1 mM glucose, this statement is not supported by the data. The statement should be corrected to indicate that while the majority of the cells (6 out of 7 and 6 out of 9) appeared to show a decrease in resistance this did not reach significance.

The authors have addressed all minor editorial requests.

Bernard Thorens
Center for integrative genomics
Department of physiology
Genopode Building
Lausanne, orcid||||| 1015
Switzerland

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](https://doi.org/10.31222/osf.io/9sm4x)). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ^2 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;
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 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and/or clone number - Non-commercial: RRID or citation	Yes	pages 20-21, lines 502-524 & page 22, lines 550-562
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	page 23, lines 565-582
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Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	
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Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository OR supplier name, catalog number, clone number, OR RRID.	Yes	pages 16-17, lines 394-409 (sex is mentioned in the corresponding result section and figure legends)
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Please detail housing and husbandry conditions .	Yes	page 16, lines 390-394
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Include a statement about sample size estimate even if no statistical methods were used.	Yes	sample size and statistical analysis are included in the figure legends
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Include a statement about blinding even if no blinding was done.	Yes	all quantifications were performed by a blinded experimenter and this is mentioned in the materials and methods section (page 22, line 549)
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	sample size and statistical analysis are included in the figure legends
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In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	in the figure legends we have included only sample size. We can add the replication factor
In the figure legends: define whether data describe technical or biological replicates .	Yes	defined in all figure legends

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