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Cold-induced dishabituation in rodents exposed to recurrent hypoglycaemia.

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ABSTRACT

(1) Aims/hypothesis: Recurrent hypoglycaemia (RH) in people with diabetes leads to progressive suppression of counterregulatory hormonal responses to subsequent hypoglycaemia. Recently it has been proposed that the mechanism underpinning this is a form of adaptive memory referred to as habituation. To test this hypothesis, we use two different durations of cold exposure to examine whether rodents exposed to RH exhibit two characteristic features of habituation, namely stimulus generalisation and dishabituation.

(2) Methods: In the first study (Stimulus Generalisation study), hyperinsulinaemic hypoglycaemic (2.8 mmol•L^{-1}) glucose clamps were performed in non-diabetic rodents exposed to prior moderate duration cold (4^{O}C for 3 hours) or control conditions). In the second study (Dishabituation Study), rodents exposed to prior RH or saline injections over 4-weeks underwent a longer duration cold (4^{O}C for 4.5 hours) exposure followed 24 hrs later by a hyperinsulinaemic hypoglycaemic (2.8 mmol•L^{-1}) glucose clamp. Output measures were counterregulatory hormone responses during experimental hypoglycaemia.

(3) Results: Moderate duration cold exposure blunted the adrenaline response (15,266 ± 1,920 vs 7,981 ± 1,258 pmol•L^{-1}, Control vs. Cold, $P < 0.05$) to next day hypoglycaemia in healthy non-diabetic rodents. In contrast, the suppressed adrenaline response (Control 5,912 ± 1,417 vs. RH 1,836 ± 736 pmol•L^{-1}; $P < 0.05$) that is associated with RH was restored following longer duration cold exposure (RH + Cold 4,756 ± 826 pmol•L^{-1}; P = NS vs control).

(4) Conclusions/interpretation: Non-diabetic rodents exhibit two cardinal features of habituation, namely stimulus generalisation and dishabituation. These findings provide further support for the hypothesis that suppressed counterregulatory responses following exposure to recurrent hypoglycaemia in diabetes result from habituation.

Keywords: Counterregulatory responses, Hypoglycaemia, Impaired awareness, Cold, Habituation, Type 1 diabetes

Abbreviations: CRR, Counterregulatory response; IAH, Impaired awareness of hypoglycaemia; RH, Recurrent hypoglycaemia

Research in context:

(1) What is already known about this subject?

- Recurrent hypoglycaemia leads to suppressed counterregulatory responses to subsequent hypoglycaemia, consistent with a form of adaptive memory called habituation.
- In both rodents and people with type 1 diabetes introduction of a single episode of high-intensity exercise as a dishabituating stimulus improves counterregulatory responses to subsequent hypoglycaemia.

(2) What is the key question?

- Is habituation the key pathogenic mechanism underlying the reduced counterregulatory responsiveness demonstrated in rodents and humans with type 1 diabetes following recurrent hypoglycaemia?

(3) What are the new findings?

- We demonstrate that exposing non-diabetic rodents to an alternate physiological stimulus (cold) leads to suppression of counterregulatory responses to subsequent hypoglycaemia through stimulus generalisation; a defined criterion of habituation.
- We demonstrate recovery of counterregulatory responses in recurrently hypoglycaemic non-diabetic rodents exposed to an alternate physiological stimulus (cold) through dishabituation; a further defined criterion of habituation.

(4) How might this impact on clinical practice in the foreseeable future

- If habituation is established as the key pathogenic mechanism underlying the reduced counterregulatory responsiveness demonstrated in rodents and humans with type 1 diabetes following recurrent hypoglycaemia, then habituation theory may also have implications for the clinical management of the related clinical condition, impaired awareness of hypoglycaemia.

INTRODUCTION

In type 1 diabetes, recurrent hypoglycaemia (RH) impairs symptomatic and hormonal counterregulatory responses to subsequent hypoglycaemia [1, 2], culminating in a clinical condition referred to as Impaired Awareness of Hypoglycaemia (IAH). IAH affects approximately 20-25% of all individuals with type 1 diabetes, increasing their risk of severe hypoglycaemia 6-fold and has a well-recognized morbidity burden [3]. The biological process through which RH leads to progressive suppression of counterregulatory hormonal responses (CRR) to subsequent hypoglycaemia remains unknown.

Habituation refers to a reduction in the psychological, behavioural and physiological responses to a stimulus as a result of repeated or prolonged exposure to that stimulus [4]. There are 9 defined criteria of habituation, many of which are also seen in individuals with IAH (for review see [5]). Two of the these criteria are; (i) 'stimulus generalisation', where habituation to one stimulus will also develop in response to other stimuli that are similar to the original stimulus, and (ii) 'dishabituation', where introducing an alternate and usually strong stimulus late in the habituation procedure can cause a temporary restoration of the habituated response [4]. Our group have recently shown in rodents exposed to recurrent hypoglycaemia [6], and humans with type 1 diabetes and IAH [7], that High Intensity Exercise (HIT) as a single alternate dishabituating stimulus successfully increased CRRs during subsequent experimental hypoglycaemia. To further test the Habituation hypothesis in the current study we examine whether an alternate physiological stimulus, cold exposure, can exhibit stimulus generalisation and dishabituation with hypoglycaemia.

METHODS

Animals

Male Sprague Dawley rats (200-250g, Charles River Laboratories) maintained on 12/12-h day/night cycle and provided with food and water ad libitum. Experimental procedures were approved by the University of Dundee Ethical Review Process and performed in accordance with UK Home Office regulations under the auspices of Project License PILPE82C1898.

Experimental Design

Experiment 1 (Stimulus Generalisation).

Experimental procedures were performed over 2 consecutive days. On Day 1, rodents were randomly assigned to either cold (4^{o}C for 3 hours) exposure (Cold; N = 12)) or room temperature for 3 hours in equivalent environment (Control; N = 15). On day 2, a hyperinsulinaemic hypoglycaemic (2.8 mmol•L^{-1}) clamp was performed with sampling of glucose hormones as previously described [8] (Suppl Fig 1A). The primary objective of Experiment 1 was to assess the effect of cold exposure on CRR to subsequent hypoglycaemia.

Experiment 2 (Dishabituation).

After 2 weeks of handling, animals were randomised to receive intraperitoneal insulin-induced (0.5-1 unit kg^{-1} i.p., Novorapid; NovoNordisk Ltd) hypoglycaemia (RH; N = 19) or volume-matched saline injections (Control; N = 19) three times weekly for 4-weeks (Suppl Fig 1B and C). Subsequently, vascular catheters were inserted under general anaesthesia as previously described [8]. Five days post-surgery, a further insulin or saline control injection was administered. On day 6, rodents were then randomised to receive either: 1) cold exposure (4^{o}C for 4.5 hours) or 2) no cold exposure in equivalent environment. The choice of 4.5 hrs cold-exposure duration was based on preliminary studies showing it provided a more robust physiological stimulus. On day 7, animals underwent a similar 90-min hyperinsulinaemic hypoglycaemic (2.8 mmol•L^{-1}) glucose clamp (Suppl Fig 1A). The primary objective of Experiment 2 was to assess the effects of antecedent cold exposure on subsequent CRR to hypoglycaemia in rodents habituated to hypoglycaemia.

Counter-regulatory hormone and metabolite analysis

Blood glucose levels were measured using Biosen (EFK Diagnostics). Hormone levels were assessed as follows: insulin (multiplex ELISA, EMD Millipore Corporation), adrenaline (ELISA; Demeditec Diagnostics GmbH), noradrenaline (ELISA; Demeditec) and BDNF (ELISA; Biosensis Pty Ltd)

Statistical analysis: Data expressed as mean ± SEM. One-way ANOVA or repeated-measures ANOVA followed by Tukey post-hoc correction for multiple comparisons to localize significant effects were used to assess between group differences. P values

less than 0.05 were considered statistically significant. All analyses performed using Statistical Package for Social Sciences (SPSS) Version 25 (IBM SPSS, New York, USA) and GraphPad Prism version 7 (GraphPad Software, La Jolla California, USA).

RESULTS

Experiment 1: Stimulus Generalisation.

During day 2 hyperinsulinaemic hypoglycaemic clamp, steady-state plasma glucose (2.6 ± 0.03 and 2.7 ± 0.1 mmol•L^{-1} for Cold and Control groups respectively) were well matched between the two groups (Fig. 1A). In response to hypoglycaemia, the increase in plasma adrenaline (15,266 ± 1,920 vs 7,981 ± 1,258 pmol•L^{-1}, Control vs. Cold, $P < 0.05$; Fig. 1B), but not plasma noradrenaline (Fig 1C), was significantly attenuated by prior moderate-duration cold exposure. Consistent with suppression of the CRR, exogenous glucose infusion rate was increased after antecedent cold exposure (6.95 ± 1.8 vs 13.4 ± 1.3 mg • kg^{-1} • min^{-1}, $P < 0.05$) (Fig. 1D).

Experiment 2: Dishabituation.

Plasma glucose profiles were matched during the hypoglycaemic clamp ($F = 1.1$, df (2.8, 42.1), P = NS; Fig. 2A). In Control animals, hypoglycaemia induced a pronounced adrenaline response, which, as expected, was markedly blunted following RH (Control 5,912 ± 1,417 vs. RH 1,836 ± 736 pmol•L^{-1}; $P < 0.05$, Fig 2B). In contrast, in RH rodents who had undergone the additional longer duration cold-exposure, adrenaline responses during the clamps study were restored (RH + Cold 4,756 ± 826 pmol•L^{-1}; P = NS vs control). Noradrenaline (Control 1,451 ± 396 vs. RH 900 ± 121 vs. RH + Cold 1,404 ± 197 pmol•L^{-1}; P = NS, Fig 3C) and mean glucose infusion rates [GIR (Control 17.6 ± 2.3 vs. RH 22.0 ± 1.6 vs. RH + Cold 18.7 ± 1.1 mg•kg^{-1}•min^{-1}; P = NS, Fig 3D)] showed similar patterns of change, but did not achieve statistical significance. Control (4-week saline-injected) animals exposed to cold, displayed adrenaline responses that were reduced compared to non-exposed Control animals (3,068 ± 632 pmol•L^{-1}), consistent with stimulus generalisation despite the longer cold exposure.

DISCUSSION

Through demonstration of stimulus generalisation and dishabituation between two physiological stressors, cold and hypoglycaemia, this paper provides further evidence in support of the hypothesis that suppression of counterregulatory responses following recurrent hypoglycaemia results from Habituation. These findings, if supported by additional studies in people with type 1 diabetes and shown to also extend to psychological and behavioural responses to hypoglycaemia, may provide a framework for considering management strategies both for the avoidance of and reversal of impaired awareness of hypoglycaemia.

In the present study, cold was chosen as an alternate physiological stimulus, because previous research had shown an alteration in stress responses to cold exposure in type 1 diabetes suggesting that there might be shared components within each homeostatic response [9]. Cold and hypoglycaemia exhibited stimulus generalisation and dishabituation support this hypothesis. Moreover, because stimulus generalisation is thought to occurs centrally, as opposed to a change in primary sensory afferents [10], our data suggest that habituation to recurrent hypoglycaemia may result from changes in key CNS integrative centres. However, the possibility that adaptations in peripheral organs (e.g. adrenal or peripheral sensors) also contribute to habituation cannot be excluded.

The different duration of cold exposure followed pilot studies showing the noradrenaline response to cold was augmented by 4.5 hrs vs. 3hrs exposure and it was anticipated a stronger stimulus would be required for dishabituation. However, in Experiments 1 and 2 both control groups exhibited stimulus generalisation, so this may not have been required and the difference in outcomes seems to have been determined more by prior habituation to hypoglycaemia than the strength of the alternate stimulus.

In contrast to stimulus generalisation, cold exposure increased CRR in rodents who had been habituated to hypoglycaemia. This is consistent with a previous study using HIT in rodents [6] Both are examples of dishabituation and lend further support to the hypothesis that the adaptation to recurrent hypoglycaemia is through the specialised form of adaptive memory, referred to as habituation.

This study has a number of limitations. Firstly, while dishabituation leads to a restoration of the habituated response, it is possible that (i) this effect is only transient or that (ii) the individual may habituate to the dishabituating stimulus limiting the therapeutic utility of this approach [10]. An on-going clinical study, HIT4HYPOs [11], will directly address this question. Secondly, while we have studied two of the cardinal features of habituation there remain a number of others that need to be tested in order to establish habituation as the mechanism that underpins the development of IAH in humans. Thirdly, a limitation of the rodent model is that it only enables assessment of CRR to hypoglycaemia, whereas people with IAH also demonstrate suppression of symptomatic, psychological and behavioural responses. Despite this it should be recognised that mice, rats and other model systems all respond to recurrent hypoglycaemia in a very similar way to humans [5], and we have recently demonstrated in humans with long-standing type 1 diabetes partial reversal of 3 important facets of IAH, namely hormonal, symptomatic and cognitive performance [7].

In summary, in this paper we have demonstrated in the rodent model that dishabituation with cold exposure leads to, at least temporary, recovery of counterregulatory responses to subsequent hypoglycaemia. Furthermore, we have demonstrated stimulus generalisability between cold and hypoglycaemia, providing further evidence that the reduced responsiveness to hypoglycaemia that follows recurrent exposure develops though habituation. This new understanding, if confirmed by other researchers, may lead to the development of novel approaches to the treatment of IAH.

Acknowledgements: Some of the data from this work were presented as an abstract at the 55th Annual Meeting of the EASD, Barcelona, 2019. The authors would like to thank C. Farrell, C. Forteath, C. Pourreyron and C. Ross, Division of Systems Medicine, University of Dundee, Scotland, for their advice and assistance with clamp studies.

Data availability: Data are available on request from the corresponding author.

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Duality of interest: KV, JB, JRG, ADM, RJM, MLE, BG, UP, BT declare no duality of interest associated with this manuscript.

Contribution statement: KV, JB, JRG contributed to research design, conducted all experiments, acquired and analysed data and drafted the manuscript. ADM and RJM designed the studies, conducted experiments, analysed data and drafted the manuscript. MLE, BG, UP, BT, contributed to the analysis of the data and editing of the manuscript. RJM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

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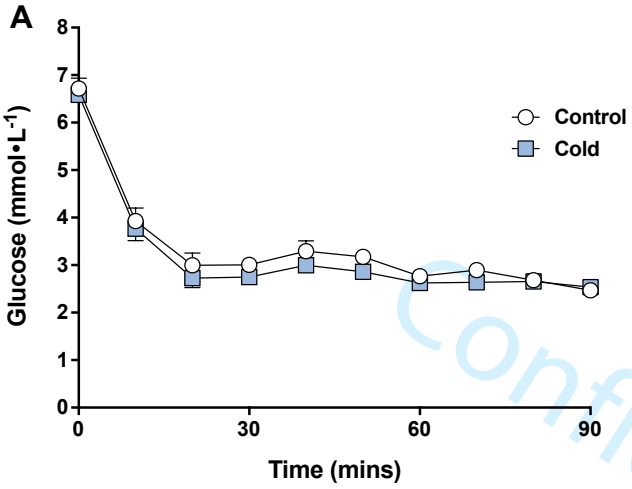
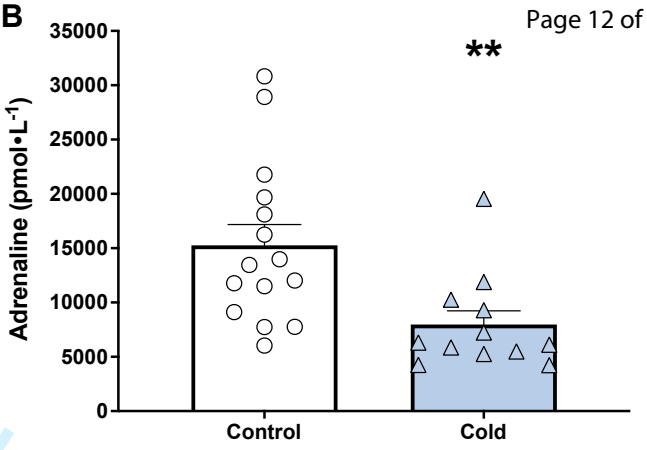
Figures:

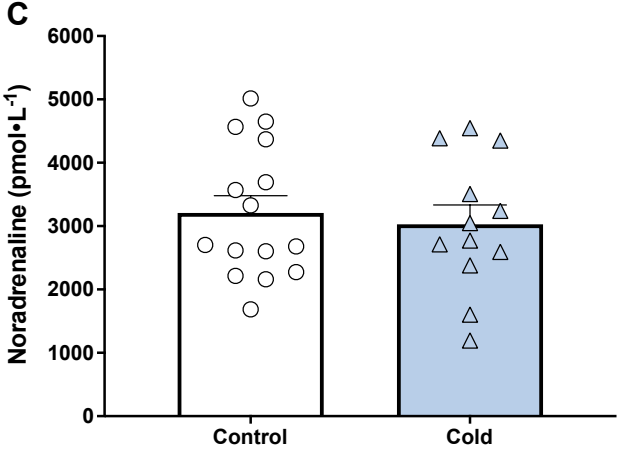
Figure 1 – Stimulus generalisation with antecedent moderate cold exposure (4^{O}C for 3 hours) impairs CRR to subsequent hypoglycaemia. A: Plasma glucose level during hyperinsulinaemic hypoglycaemic clamp study on day 2. Plasma adrenaline (B) and noradrenaline (C) responses to stable hypoglycaemia. Mean exogenous GIR (D) during stable hypoglycaemia (60-90 mins). Control, white circles/bars; Cold, blue squares/bars. Values shown are mean ± SEM. **P < 0.05

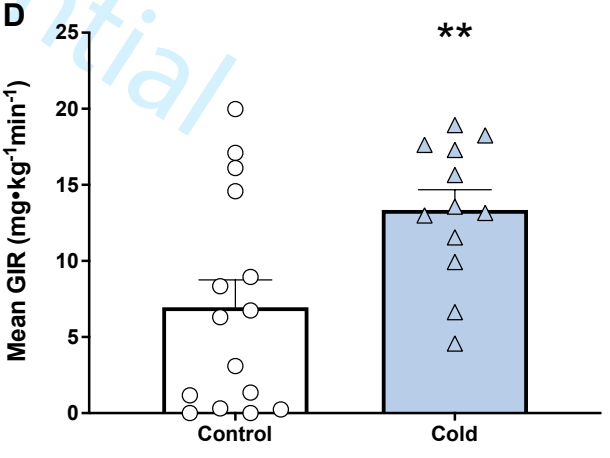
Figure 2 – Dishabituation with acute strong cold exposure (4^{O}C for 4.5 hours) restores defective CRR in RH rodent model to subsequent hypoglycaemia. Plasma glucose level (A) during hyperinsulinaemic hypoglycaemic clamp study. Plasma adrenaline (B) and noradrenaline (C) responses during hypoglycaemia. Mean exogenous GIR (D) during stable hypoglycaemia (60-90 mins). Control, white circles/bars; Control and cold, light blue triangles/bars; RH, grey squares/bars; RH and cold, dark blue diamonds/bars. Values shown are mean ± SEM. **P < 0.05

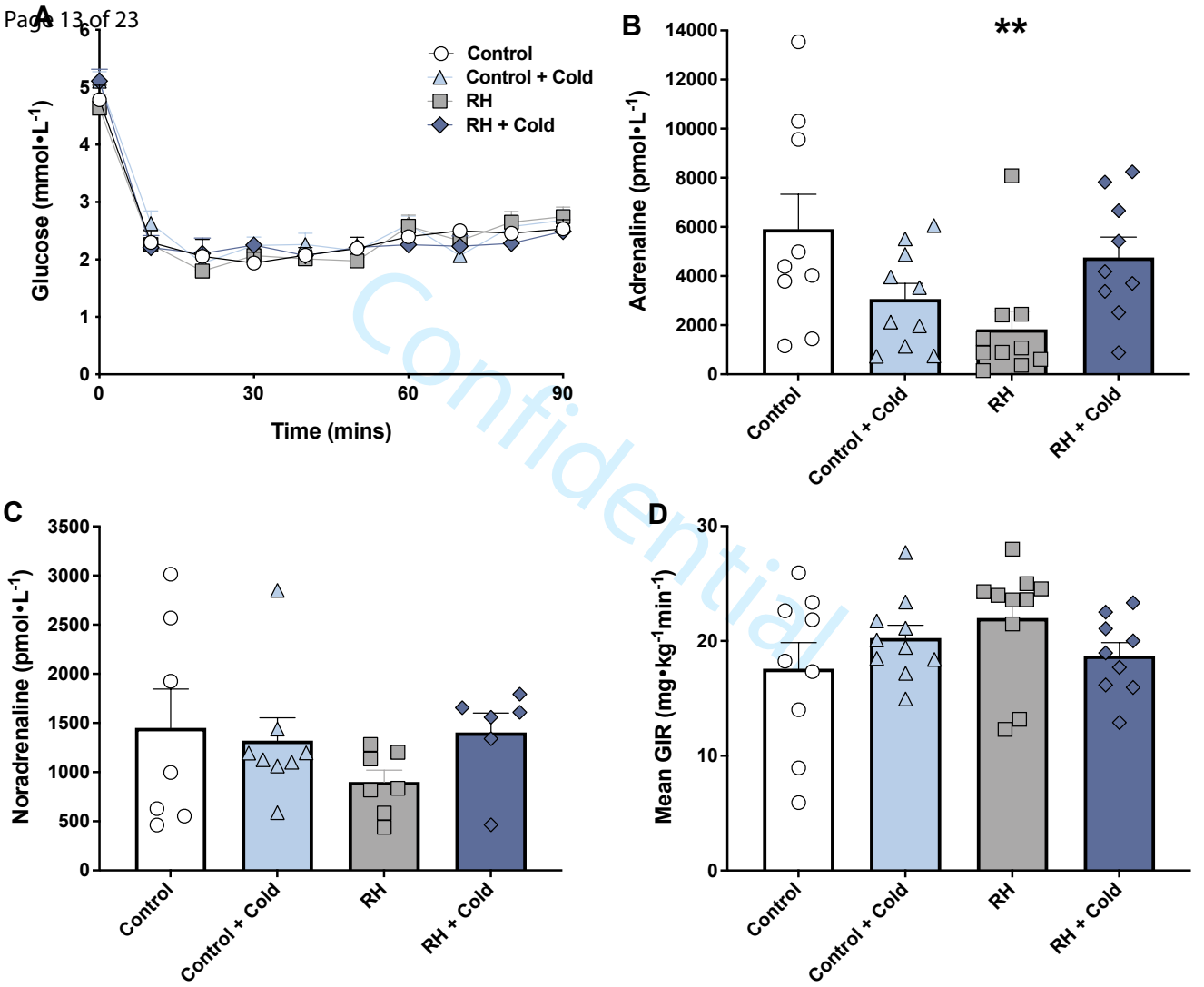
Supplementary Figure 1 – Study design. A: Hyperinsulinaemic hypoglycaemic clamp protocol. Rodents were fasted overnight and vascular catheters were opened and flushed on the morning of the clamp. Animals were allowed to settle for at least 90 mins before a priming dose of insulin (40 mU•kg^{-1}•min^{-1}) was infused. After 10 mins, insulin infusion rate was stepped down to 10 mU•kg^{-1}•min^{-1} and a variable 40% dextrose infusion was adjusted based on bench-side glucose readings every 10 mins. Adrenaline (A) and noradrenaline (N) were sampled at baseline and at 30 min intervals during the clamp. **B:** Study design for Experiment 2. **C:** Insulin dose and blood glucose levels at baseline and during hypoglycaemia over 4 weeks of RH protocol.











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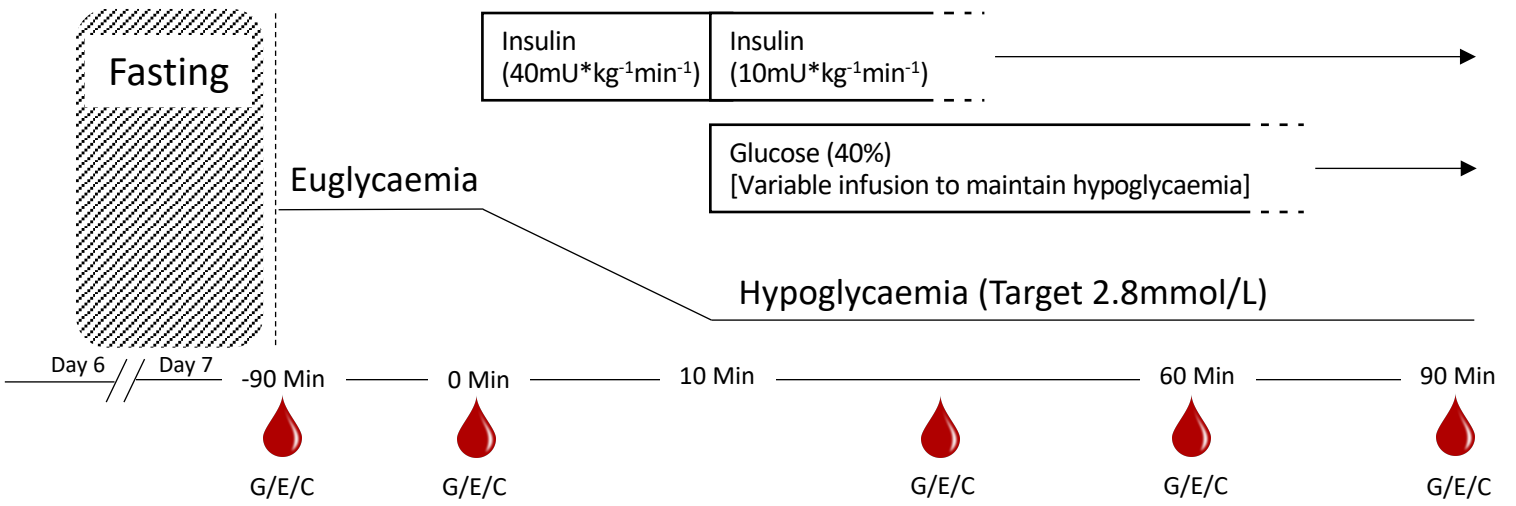
Fasting
Insulin (40mU*kg-1min-1)
Insulin (10mU*kg-1min-1)
Glucose (40%)
[Variable infusion to maintain hypoglycaemia]
Euglycaemia
Hypoglycaemia (Target 2.8mmol/L)
Day 6
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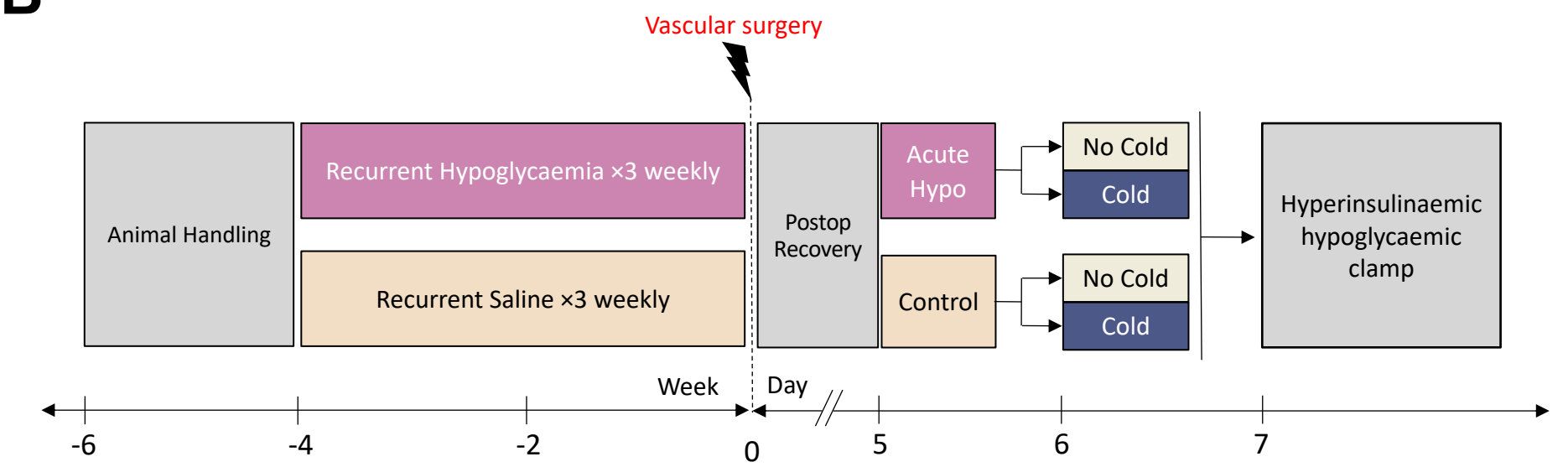
Vascular surgery
Animal Handling
Recurrent Hypoglycaemia ×3 weekly
Recurrent Saline ×3 weekly
Postop Recovery
Acute Hypo
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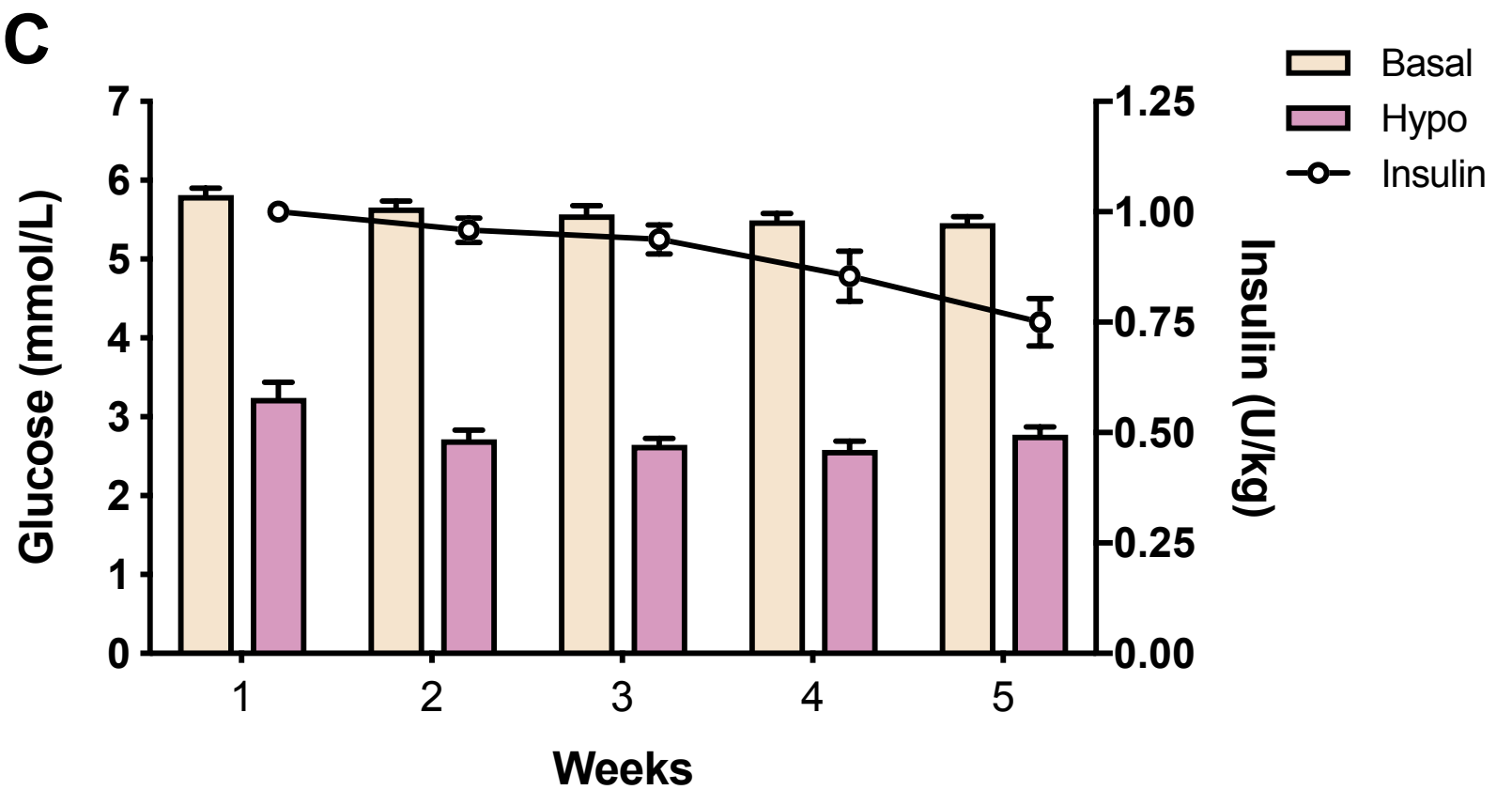
Basal
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Weeks

Dear Sally,

we are very grateful to the reviewers for their consideration of our manuscript and for the positive comments made. We have re-written the manuscript based on these comments and, in response to the Associate Editor's request, re-drafted the paper as a Short Communication. Given that the change to a Short Communication required a major re-write we have not highlighted all these changes in the new submission but have responded to each individual comment below. We hope you will find this acceptable.

Best wishes

Rory

COMMENTS TO THE AUTHOR

Referee #1:

This is a very well-planned study with interesting findings. I have just one comment.

IAH is not a very well-defined condition. Usually, Gold and Clarke scores are used to identify people with IAH. Psychological and physiological factors act together. IAH can be a totally subjective diagnosis, based on an individual's own assessment of their control of their hypoglycaemic episodes. To me, a "rat model of IAH", and "animals developed IAH" sounds a bit off in this regard. I think it would be more appropriate to talk about hypoglycaemia associated autonomic failure (HAAF, as coined by Dagogo-Jack in 1993), as I hardly can imagine that the psychological aspect of IAH could be modelled in rats. This is addressed to some extent in the discussion, but I think it would be preferable to deal with IAH as a condition that is only attributable to humans.

Congratulations on a well performed study.

We thank the reviewer for the positive comments about the paper. The reviewer makes an important point and while the rodent model reflects humans in its response to recurrent hypoglycaemia it is true that it is not possible to address all the individual facets of impaired awareness of hypoglycaemia. We have therefore removed all reference to "rodent models of IAH" and in the discussion referred to the limitations of the rodent model in this respect.

Referee #2:

Comments to the Author
The manuscript by Vickneson et al reports a study testing the idea that the activity-dependent suppression of the counter-regulatory response (CRR) to recurrent hypoglycemia is a form of habituation (that presumably occurs within the nervous system). The reduction in the CRR to repeated episodes of hypoglycemia is a significant clinical problem that affects many type I diabetics. The mechanism(s) underlying this phenomenon are incompletely understood and here the authors extend their previous work showing that the decline in the endocrine component of the CRR in rodents exhibits features of habituation. In particular they examine whether the response shows stimulus generalization (can a related stimulus also suppress the CRR?) and dishabituation (can a novel intense stimulus restore the response?). Using cold exposure as the novel stimulus, the authors conclude that the decrease in epinephrine release following insulin-induced hypoglycemia shows the features expected for a habituated response.

The experiments appear to have been carefully performed and in general the conclusions are supported by the data. I have only a few questions regarding data presentation. My primary concern is that the work is a comparatively small advance on their earlier studies and does not provide any new mechanistic insight into the underlying process.

Major comment

1. Previous work by the same authors have shown that the decline in epinephrine release following recurrent hypoglycemia can be reversed using high intensity exercise as a dishabituating stimulus (McNeilly et al, 2017; Farrell et al, 2020). The present report shows that cold exposure can also reverse habituation of the CRR. I'm not sure that the current ms adds significantly to what is already known on this topic.

We thank the reviewer for the constructive comments. We understand the concern that this may only be a small advance. However, we would argue that this paper advances our understanding in several ways. Firstly, the importance of replication in science. We had previously reported on HIT as a novel stressor in rodents and humans with type 1 diabetes, but mechanisms other than dishabituation may have underpinned these results so to be able to confirm the findings with a different but related physiological stressor provides more robust support for the habituation hypothesis. Secondly, should habituation be established as the model for IAH in humans then we now have two potential therapeutic interventions that can be explored. Thirdly, and importantly, there is confusion in the literature about the impact of exercise on hypoglycaemia. The Reviewer will be aware of the work of Galassetti and Sandoval (e.g. Galassetti et al 2001; Am J Physiol Endocrinol Metab 280(6): E908-917. Sandoval DA et al. 2004; Diabetes 53(7): 1798-1806) showing antecedent exercise suppresses counterregulatory response to subsequent hypoglycaemia whereas we had reported the converse with HIT in subjects with T1D and IAH (and rodents). We took the opportunity in this study on cold to examine the concept of stimulus generalisation , and are able to show that when you present one (cold) of two related stimuli to an untreated rodent (not habituated) you see suppression of the physiological response to the second stimulus (hypoglycaemia). In contrast, in the habituated animal introduction of the second, different, stressor acts to dishabituate and amplify the physiological response to the habituated stimulus. This finding therefore reconciles these disparate findings in the literature, and off course provides the important clinical message that not all exercise modalities have the same effect on hypoglycaemic responses.

Additional comments

1. The discussion section is unfocussed and generic in many places. For example

(i) I don't understand the point the authors' are making about the connection between thermo- and glucoregulation (page 13/14 in the pdf version), beyond the obvious fact that they are homeostatic mechanisms.

We apologise if any confusion has been caused. In redrafting this as a Short Communication this section has been omitted. The point being raised was that apart from both being homeostatic systems, it is recognised that cold- and glucose-sensing share central hypothalamic integrative networks. In addition, it may be of interest for the reviewer to note that as there are glucose-inhibited and glucose-excited neurons, there are also low and high temperature sensitive neurons, which are located both peripherally in the skin and centrally.

(ii) How does their work "support the conceptual framework for glucose sensing proposed by Watts and Donovan" (p. 15)?

Again, we apologise for the lack of clarity and have removed this from the Short Communication. The point we were trying to make was that although Habituation has generally been applied to behavioural research, the classical model for Habituation is the sensory-motor reflex in Aplysia. The premise of Watts and Donovan's paper was that hypoglycaemia counterregulation operated in a similar way to a classical sensory-motor response with low glucose as the internal sensori-stimulus and hormonal counterregulation as the motor response.

(iii) It is noted that large conduction (conductance?) potassium channels play a role in habituation (p. 16). What is relevance of this detail? Please help the reader by explaining the context or implications of your point.

The reviewer raises a valid point. This section has been subsequently removed from the manuscript

(iv) The overall interpretation is very CNS-centric. For example, Herlein et al, 2006 (Endocrinology 147: 2781-8) showed stimulus generalization between hypotension and hypoglycemia. This is the same type of point being made in the current ms however Herlein et al suggested the mechanism could be peripheral. Similarly, Colomer et al, 2008 (J. Neurosci 28: 6616-26) showed long-term cold exposure induced adrenal plasticity and epinephrine release. Maybe a similar effect is also mediated acutely and contributes to the results reported in the present work.

We thank the reviewer for this. Inevitably with word limitations we cannot address all the areas we would like and agree with the reviewer that it seems very likely in an integrated system that adaptations may occur in both central and peripheral aspects of the network.

2. Could the authors speculate why 3 vs 4.5 hrs cold exposure has different effects on the CRR (i.e. the former is sufficient for generalization, but the latter leads to dishabituation)? Ideally the recurrent hypoglycemia protocol should be repeated using the 3 hr cold exposure. This would allow the authors to determine whether the dishabituating effect of cold really requires "higher intensity cold". At present there are too many variables to disentangle this important issue.

The reviewer raises an excellent and very pertinent point. We have modified the manuscript to reflect that fact that we saw stimulus generalisation with both 3 and 4.5 hrs of cold exposure which was unanticipated. In the present study at least, it seems to have been the habituation process itself the was critical to determining the response to Cold

3. The authors find that cold exposure does not alter the systemic levels of epinephrine which seems surprising since this stimulus would be expected to activate a strong sympathetic response (Fig 4). Was epinephrine measured at the end of the period of cold exposure? If so, is it possible that cold rapidly evokes epinephrine release but that the levels have declined by the end of the 3 or 4.5 hr exposure? If cold exposure doesn't increase epinephrine release why does habituation occur?

This is an interesting point of discussion. Cold exposure typically causes much greater elevations in plasma norepinephrine than epinephrine [8]. However, it is possible that we missed a small peak in epinephrine earlier in the cold stimulus. The final point is also interesting, and we would direct the reviewer to criteria #6 of habituation. "The effects of habituation training may proceed beyond the zero or asymptotic response level" - basically if there is a stimulus you can habituate

even if there is no obvious response to that stimulus. An example of this is seen in subjects with IAH who often no longer mount a significant counterregulatory response to levels of glucose between 3-4 mmol/l but do not spontaneously revert because they continue to experience the sensory stimulus (hypoglycaemia).

Figures

1. Could the authors include the data points for individual animals on all the bar graphs so the reader can get a feel for the variability of the data sets?

We agree with the reviewer and the graphs have been modified to show the data points for the individual animals.

2. Fig 1A legend; should this be the study design for experiment 2 (not 1)?

We apologise for the error and it has been rectified. To fit with the new format this figure has been moved to the supplementary section

3. Why are the control values for epinephrine so different in Fig 2B and 3B? Is it because recurrent saline injection suppressed the insulin-induced release of epinephrine?

Unfortunately, we often find marked variation in epinephrine vales between batches of animals even from the same facility. We also believe that the prior saline injections with additional animal handling over 4 weeks contributed to the differences, which is why we included the Control+Cold studies in Experiment 2. That fact that this group also showed stimulus generalisation provides support for the results of Experiment 1 despite the higher adrenaline values.

4. Fig 4; what does “adjusting for baseline as a covariate” involve? Do the mean, unadjusted values for epinephrine vary as a function of cold exposure?

We initially felt that adjusting for baseline would be more statistically robust as compared to one-way ANOVA analysis. Baseline values between animals do vary in any study of this type. In response to the reviewer to include data points for individual animals, we have reverted to the more conventional repeated-measures ANOVA followed by Tukey post-hoc correction for multiple comparisons to localise significant effects and assess between group differences. This did not change our findings

5. Supp Fig 1; what was the n value for this set of experiments? What is “adjusted mean”? What were the values in control animals (i.e. not exposed to cold)?

We have removed this from the resubmitted manuscript

Referee: 3

Comments to the Author

The manuscript by Vickneson and colleagues investigate whether dishabituation can be used to reverse impaired hypoglycemia awareness which the authors propose is an adaptive memory response. They conducted two studies, a “stimulus generalization” study that examined whether the exposure to an alternative stimulus, in this case, cold exposure, can attenuate the response to hypoglycemia in hypoglycemia-naïve rodents and a “dishabituation” study where the authors

examined whether exposure to cold was capable of reversing the "habituated" response to recurring hypoglycemia. The authors found that cold exposure suppressed the counterregulatory response to hypoglycemia and that cold exposure was able to improve the counterregulatory hormone response in recurrently hypoglycemic rats. The authors concluded that "impaired hypoglycemia awareness develops through a habituated process". This is an interesting study that examines a novel hypothesis. The experiments are well conducted. I only have a few comments.

While not mentioned, did the authors measure plasma glucagon responses in their studies? There is typically a reduced glucagon that is observed in the RH rodent models and it would be interesting to see if similar defects occur following cold exposure.

We do agree with the reviewer that a reduced glucagon response is observed with RH in non-diabetic animals. Ordinarily we measure glucagon in our rodent studies. Unfortunately, due to an unanticipated problem encountered in two different glucagon assay kits (Mercodia & EMD Millipore) we were unable to obtain detectable glucagon responses in any of the two experiments presented in this manuscript. We are currently reviewing this area.

Cold exposure typically increases sympathetic nervous system output in rodents, yet in the current study, the authors find it has a detrimental effect on the sympathoadrenal response to hypoglycemia in the stimulus generalization study? How do they reconcile this?

We would argue that the effect of Cold on the hypoglycaemic counterregulatory response is not actually designed to be detrimental. In ordinary circumstances it is an adaptive response presumably to protect the organism from the detrimental effects of repeated stress. However, in type 1 diabetes the presence of high insulin levels following injection combined with loss of alpha-cell glucagon secretion mean that the adaptation paradoxically increases risk of severe hypoglycaemia.

To follow up on the previous question, the one point of confusion here is that in the stimulus generalization studies, it appears that the exposure to one stimulus (cold) impairs the adrenergic response to a different stimulus (hypoglycemia). However, in the dishabituation studies, the opposite seems to occur where the adrenergic response adapts to repeated exposure to the same (hypoglycemia) stimulus, but exposure to a novel (cold) stimulus dishabituates it. By that token, should the response to both cold and hypoglycemia in the stimulus generalization study be the same given they are different stimuli?

The reviewer asks an important question. It is difficult to answer because so much of the literature is based on behavioural rather than physiological responses. All, we think we can say is that we have demonstrated something that is consistent with stimulus generalisation, and that both stimuli involve activation of the sympathetic nervous system, but that the exact nature of that sympathetic response differs between the two stimuli.

Minor:

Consider rewording the first sentence in the concluding paragraph ("…limited in the main to strategies…)

Figure caption 4B should be adrenaline

In the Supplemental Figure caption, the 3°C should read 4°C.

We thank the reviewer for spotting the mistakes and we have edited the manuscript accordingly.

Associate Editor
Comments to the Author:
In this manuscript the Authors show that the activity-dependent suppression of the counter-regulatory response to recurrent hypoglycemia in rodents is a form of habituated response. The study is of potential interest. However, the Authors should better focus on the novelties of their results (that follow previous findings in this area by the same group), and address the other concerns raised by the Reviewers. A new submission should be in the format of a short report.

We thank the associate editor for the constructive comments and have edited the manuscript in the format of a short report.

Diabetologia checklist for preclinical studies

Adapted from the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines Checklist [1, 2] and the NIH Principles and Guidelines for Reporting Preclinical Research [3]

	ITEM	RECOMMENDATION	Page no.
Title	1	Provide as accurate and concise a description of the content of the article as possible.	1
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	2
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. **Animal studies only:** Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	3-4
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	5
METHODS			
Ethics statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), informed consent, and national or institutional guidelines for the care and use of animals, or for the use of human tissue, that cover the research.	4
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals, cells or tissue samples to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals; single cell). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	5
Experimental procedures	7	**Animal studies only** For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	5
	8	**Cell lines only:** Report the source, authentication and mycoplasma contamination status [3].	NA
	9	**Studies involving antibodies:** Report source, characteristics, dilutions and how antibodies were validated [3].	NA

Experimental animals	10	**Animal studies only** a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naive, previous procedures, etc.	4-5
Housing and husbandry	11	**Animal studies only** Provide details of: a. Housing (type of facility e.g. specific pathogen free; type of cage or housing; bedding material; number of cage companions). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	4-5
Sample size	12	a. Specify the total number of animals or samples used in each experiment, and the number of animals or samples in each experimental group. b. Explain how the number of animals or samples was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant, e.g. the number of times a western blot was performed. d. Provide sufficient information on sample collection to allow a clear distinction between independent biological data points and technical replicates.	4
Allocating animals/samples to experimental groups	13	a. Give full details of how animals or samples were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals/samples in the different experimental groups were treated and assessed.	5
Experimental outcomes	14	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	5
Statistical methods	15	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single islet). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach. d. Give details of how data are reported, e.g. mean ± SD, median IQR.	4
RESULTS			
Baseline data	16	**Animal studies only** For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naive) prior to treatment or testing. (This information can often be tabulated).	4
Numbers analysed	17	a. Report the number of animals or samples in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50% [4]). b. If any animals or data were not included in the analysis, explain why.	5
Outcomes and estimation	18	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	6
Adverse events	19	**Animal studies only** a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	6

DISCUSSION			
Interpretation/ scientific implications	20	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal or model, and the imprecision associated with the results [4]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction of the use of animals in research.	7-8
Generalisability/ translation	21	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	7-8
Funding	22	List all funding sources (including grant number) and the role of the funder(s) in the study.	9

- Please note all queries taken or adapted from the ARRIVE guidelines, except where otherwise indicated.
- Some items are indicated as being specific to certain types of research, e.g. animal studies, studies on cell lines. Please indicate 'N/A' (not applicable) for items that do not apply to your study.

References

1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) The ARRIVE guidelines checklist. Animal Research: Reporting In Vivo Experiments. Available from www.nc3rs.org.uk/arrive-guidelines, accessed 29 September 2016
2. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 8: e1000412.
3. National Institutes of Health (NIH) (2014) Principles and guidelines for reporting preclinical research. Available from www.nih.gov/research-training/rigor-reproducibility/principles-guidelines-reporting-preclinical-research, accessed 29 February 2016
4. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.