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**High intensity exercise as a dishabituating stimulus restores counterregulatory responses in recurrently hypoglycemic rodents.**

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## Abstract

Hypoglycemia is a major adverse effect of insulin therapy for people with type 1 diabetes (T1D). Profound defects in the normal counterregulatory response to hypoglycemia explain the frequency of hypoglycemia occurrence in T1D. Defective counterregulation results to a large extent from prior exposure to hypoglycemia *per se*, leading to a condition called impaired awareness of hypoglycemia (IAH); the cause of which is unknown. In the present study, we investigate the hypothesis that IAH develops through a special type of adaptive memory referred to as habituation. To test this hypothesis, we used a novel intense stimulus (high intensity exercise) to demonstrate two classical features of a habituated response, namely dishabituation and response recovery. We demonstrate that following recurrent hypoglycemia, introduction of a novel dishabitatory stimulus (a single burst of high intensity exercise) in male Sprague-Dawley rats restores the defective hypoglycemia counterregulatory response. In addition, the rats showed an enhanced response to the novel stimulus (response recovery). We make the further observation using proteomic analysis of hypothalamic extracts that high intensity exercise in recurrently hypoglycemic rats increases levels of a number of proteins linked with BDNF signaling. These findings may lead to novel therapeutic approaches for individuals with T1D and IAH.

Impaired awareness of hypoglycemia (IAH), defined as 'a diminished ability to perceive the onset of acute hypoglycemia', affects 20-25% of all people with type 1 diabetes (T1D) and increases risk of severe hypoglycemia 6-fold (1). IAH develops primarily in response to repeated episodes of hypoglycemia (2). Individuals with or without T1D, who experience one or more episodes of hypoglycemia have impaired counterregulatory (CRR) hormonal and symptomatic responses during a further episode of hypoglycemia, with the extent of suppression dependent on the depth, duration, and frequency of antecedent hypoglycemia (2). Conversely, CRR can be restored if hypoglycemia is avoided (3). The mechanisms underpinning the development of IAH remain unknown, but are likely a result of changes in specialized glucose-sensing regions of the body such as those found in distinct brain regions like the ventromedial hypothalamus (VMH) (4).

Habituation is type of adaptive memory that occurs in many organisms in response to a repeated, often stressful stimulus (5). Habituation is defined as a 'reduction of the psychological, behavioral or physiological response to a stimulus as a result of repeated or prolonged exposure' (5; 6). Aspects of the physiological responses to repeated hypoglycemia, such as the progressive diminishment of CRR to hypoglycemia following repeated exposure, are consistent with the principal features of a habituated response (5-7) suggesting this may provide an explanation for IAH. In this study we directly address this hypothesis in a rodent model of recurrent hypoglycemia by demonstrating one of the defining features of a habituated process, namely that it is possible to rapidly restore the habituated response by *dishabituation*, the introduction of a novel strong stimulus (5; 6).

## RESEARCH DESIGN AND METHODS

### Animals

Male Sprague Dawley rats (250-300g, Harlan UK) maintained on a 12/12-h day/night cycle and provided with food and drinking water *ad libitum*.

Experimental procedures were approved by the University of Dundee Ethical Review Process and performed in accordance with UK Home Office regulations.

### **Experiment 1: Dishabituation with high-intensity exercise following recurrent hypoglycemia**

To test our hypothesis in rodents, high-intensity exercise (HI) was used as a dishabitulatory stimulus with recurrent hypoglycemia the habituated response. Following 2-weeks of daily handling, rats underwent recurrent insulin-induced (Novorapid®, 0.75-1U/kg IP, NovoNordisk Ltd) hypoglycemia (RH: N=24) or IP volume-matched saline injections (Control: N=16) x3-weekly for 4 weeks (Fig 1A and B). Animals were familiarized (5m/min for 15 mins daily) with the treadmill during the last 2-weeks, with exercise training in the morning and IP injection in late afternoon. Subsequently, vascular catheters were inserted under general anesthesia as previously described (8) and animals recovered over 5 days. On day 5 post-surgery, a further insulin or control injection was administered and then, after feeding freely overnight, on day 6, the animals were allocated to one of the following groups; (i) No exercise, (ii) Low Intensity (LI) exercise (15 mins “walking” pace of 5m/min with 10% incline), or (iii) High Intensity (HI) exercise (5 mins at 5m/min followed by an incremental increase in speed at 2 m/min intervals to 5 mins “running” at 15m/min). Where required, exercise was encouraged using a bottlebrush to the tail. Animals were then returned to their home cages and fed *ad libitum*. The following day all animals underwent a hyperinsulinemic-hypoglycemic (2.8 mmol/l) clamp (8), with sampling for CRR hormones under euglycemic and hypoglycemic conditions (Fig 1C). Animals were euthanized on completion of the study and brains removed for later analysis.

### **Experiment 2: Response recovery to high intensity exercise following recurrent hypoglycemia.**

To examine for response recovery, an enhanced response to the novel stimulus, and gain insight into potential mechanism, a further 2 groups of male Sprague Dawley (SD) rats (n=12-18 per group) were studied and the impact of each exercise modality on counterregulatory hormones, cytokines, BDNF and lactate

measured. Induction of hypoglycemia, familiarization to the treadmill, surgical procedures and exercise were identical to the protocol described for Experiment 1. However, immediately following each of the three exercise protocols blood samples were taken from the carotid artery (N=6-8 per group). The animals were then euthanized.

### **Hormone, cytokine and metabolite analysis**

Assessed as follows: Glucose (Analox GM9 glucose analyzer; Analox Instruments Ltd.), insulin and glucagon (multi-plex ELISA; Bio-plex<sup>®</sup>, Biorad), epinephrine and corticosterone (ELISA; Alpco Immunoassays and De meditic diagnostics respectively), BDNF (DuoSet ELISA; R&D Systems), lactate levels (Sigma Aldrich, UK), cytokines (V-PLEX Pro-inflammatory Panel 2 (rat) ELISA; MSD).

### **Proteomic Analysis**

Proteins extracted from each VMH sample were reduced, alkylated and subjected to trypsin digestion. The tryptic peptides from each animal were labeled with iTRAQ labeling according to manufacturer's manual (AB Sciex). All labeled samples were then pooled together. One half of pooled sample was subjected to high-pH off-line C18 based fractionation (8 fractions), and the other half to strong cation exchanger fractionation (5 fractions). LC-MS/MS was carried out as previously described (9). iTRAQ quantification was carried out using Peaks software (Bioinformatics Solutions Inc.) with IPI-rat database (2012-09-27). All samples were randomized and the analyst was blinded to sample grouping during the processing.

### **Statistical analysis**

All results are expressed as mean  $\pm$  SEM. Statistical analyses were performed using SPSS (Version 21; SPSS). Data were analyzed by one-way analysis of variance (ANOVA) or repeated measures ANOVA followed by post-hoc testing (Bonferonni) to localize significant effects. Statistical significance was set at  $p < 0.05$ .

## **RESULTS**

### **Dishabituation with high-intensity exercise following recurrent hypoglycemia**

Counterregulatory responses to hypoglycemia following LI exercise or No exercise in both control and RH animals did not differ significantly (supplementary material) so were combined into the LI grouping. Plasma glucose profiles were matched during hypoglycemia in all groups (Fig 2A:  $F=0.4$   $df(2,39)$ ,  $p=ns$ ). In contrast, mean glucose infusion rate (GIR) ( $F=9.2$ ,  $df(2,39)$ ,  $p<.05$ ), epinephrine ( $F=34.5$ ,  $df(2,39)$ ,  $p<.05$ ) and glucagon ( $F=10.4$ ,  $df(2,39)$ ,  $p<.05$ ) differed significantly between groups. Post-hoc analysis revealed CRR responses were significantly suppressed in RH+LI rats compared to Control +LI rats during hypoglycemia (Fig 2B-E). In contrast, GIR [2.9(0.6) vs. 4.4 (1.0)  $mg\ kg^{-1}\ min^{-1}$ , Control +LI vs. RH+HI,  $p=ns$ ], epinephrine [7.9(0.4) vs. 6.8(0.6)  $ng\ ml^{-1}$ , respectively,  $p=ns$ ] and glucagon [245(18) vs. 219(31)  $ng\ l^{-1}$ , respectively,  $p=ns$ ] CRRs were not different between Control+LI and RH+HI groups (Fig 2B-E). Corticosterone responses to the hypoglycemic challenge were not affected by any intervention. These findings indicate that a single episode of HI exercise can restore CRR in rats exposed to RH over 4 weeks.

### **Response recovery to high intensity exercise following recurrent hypoglycemia.**

HI exercise resulted in significantly higher levels of epinephrine, glucagon and lactate in both Control+HI and RH+HI groups versus their respective LI studies (Figs 3 A-C). Moreover, consistent with response recovery following habituation, mean (SEM) epinephrine [2.2 (0.3) vs. 1.4 (0.1)  $ng\ ml^{-1}$ ; RH+HI vs. Control + HI,  $p<.05$ ], glucagon [46 (2) vs. 38 (2)  $pg\ ml^{-1}$ ;  $p<.05$ ], and lactate [88 (3) vs. 77 (2)  $ng\ \mu l^{-1}$ ;  $p<.05$ ], responses to HI were all significantly greater following RH (Figs 3 A-C). Interestingly, the profile of cytokine release differed with the expected increase in cytokine release with HI exercise in Control groups (Figures 3E-I), but a more varied response following RH where release of broadly pro-inflammatory cytokines (IL-6, IL-1 $\beta$ , and TNF $\alpha$ ) after HI was significantly suppressed in comparison with Control+HI, whereas the release of anti-



inflammatory cytokines (IL-4, IL-13) was generally increased; the latter increase also evident following low stress LI exercise (Figs 3H and I).

### **Hypothalamic mechanisms of Dishabituation**

Proteomic analysis on VMH samples from RH+LI vs. RH+HI (Fig 4A and 4B), revealed that DNAJB1 (or Heat shock protein 40; Hsp40) and Bassoon (Bsn) were most significantly affected by HI exercise (Fig 3B); confirmed by western blot analysis (Fig 4C). Bsn is involved in glutamatergic signaling as is brain-derived neurotrophic factor (BDNF) (10), and BDNF levels in humans are significantly elevated in response to exercise (11). To determine if BDNF may be involved in dishabituation, plasma BDNF was measured and found to be augmented by HI exercise in RH animals (Fig 3D). In addition, VMH expression of BDNF, TrkB, and pCREB were all significantly increased following RH+HI (supplementary material).

### **DISCUSSION**

This study demonstrates, in an animal model of IAH, that diminished CRR can be rapidly restored by a single bout of HI exercise. This finding supports the hypothesis that diminished CRR following RH results from habituation. Habituation is a form of adaptive learning where there is a decrease or cessation of responses to a stimulus after repeated presentations. Habituation is usually considered in the context of innate behaviors and a reduced response to repeated external or internal stimuli (5; 6). A classical model of habituation is the gill-withdrawal reflex (GWR) in *Aplysia* (12). When the GWR is repeatedly evoked by a tactile stimulus to the siphon, the amplitude of the response shows a marked decrement (habituation). The habituated response can then be restored by presenting a strong tactile stimulus to another part of the animal (dishabituation) (12). Habituation also occurs following repeated physiological stress such as endotoxin exposure or in models of shock (13). In the context of hypoglycemia, low glucose can be considered the internal stimulus, which leads to a reflex counterregulatory response. Recurrent hypoglycemia then leads via habituation to a progressive diminution of this response. This concept of hypoglycemia as an internal sensory cue stimulating a counterregulatory reflex

(motor) response is consistent with our current understanding of the neural circuitry of brain glucose sensing (14).

In the present study, HI exercise was chosen as the dishabituation stimulus, but other novel stressors may prove equally effective. For instance, counterregulatory responses to hypoglycemia and cold-exposure have been shown to influence each other (15). The present findings differ from previous studies where antecedent exercise produced further suppression of CRR to hypoglycemia (e.g. (16)). However, these used continuous aerobic exercise providing a moderate stimulus. Stimulus generalization (or cross-tolerance) between different moderate stressors is a feature of habituation (5; 6); distinct from dishabituation, which requires a novel strong stimulus. Interestingly, prior RH did not suppress the CRR response to exercise, as reported by others (17), but did lead to a change in the inflammatory response to both exercise modalities. The differing intensity of exercises used in the present study likely explains the contrasting findings. This also suggests that the impact of exercise on CRR to hypoglycemia may vary dependent on the type and duration of exercise performed.

To gain some understanding of the mechanisms by which HI exercise acutely restored CRR to hypoglycemia, an exploratory proteomic analysis of VMH tissue was performed. A number of candidate proteins emerged such as Glycogenin 1 (Gyg1) and Adenylate cyclase 10 (Adcy10) that play roles in glycogen turnover which may be relevant to glucose sensing (18), while dipeptidyl peptidase 10 (Dpp10) affects Kv4 channel gating (19). DNAJB1 encodes for heat shock protein 40 that helps protect proteins during cellular stress (20). Bassoon, which was most affected by HI exercise, is a presynaptic cytomatrix protein that contributes to synaptic plasticity and learning (10). Bassoon co-localizes with BDNF in glutamatergic pre-synapses and increased BDNF release helps maintain levels of neural excitation (10). Loss of VMH glutamatergic Steroidogenic Factor 1 (SF1) activity suppresses CRR to hypoglycemia (21), while specific deletion of BDNF in VMH SF1 neurons results in a reduced CRR response to hypoglycemia (22). VMH SF1 neurons also appear to contribute to the metabolic benefits of endurance

exercise (23). Taken together with the findings in the present study, it is possible to speculate that dishabituation may act via BDNF-mediated restoration of glutamatergic activity (Figure 3D). These findings are however observational and need to be directly tested in further studies. In addition, we cannot exclude the possibility that dishabituation occurs in other brain regions such as the thalamus.

Overall, the present study supports the hypothesis that suppressed CRR following RH in T1D is a result of habituation. Consistent with this hypothesis, we have demonstrated that that introduction of a dishabitulatory stimulus, HI exercise, acutely restores the counterregulatory response to subsequent hypoglycemia. If confirmed in humans, these findings may lead to an improved understanding of IAH in T1D and the development of novel treatment strategies designed to restore hypoglycemia awareness.

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**Author Contributions.** AM, JG and JH performed the experiments. RJM designed the studies. AM, JG, JH, MLJA, RJM wrote the manuscript. R.J.M. 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 the accuracy of the data analysis.

**Prior Presentation.** Data from this study was presented as a poster at the 75th Scientific Sessions of the American Diabetes Association, Boston, MA, 5-9 June 2015.

## Figure Legends

**Figure 1. Study design. (A)** Study design for Experiments 1 and 2. Experiment 2 followed exactly the same protocol as Experiment 1, with the exception that animals underwent blood sampling on Day 6 after HI or LI exercise and did not proceed to the hyperinsulinemic hypoglycemic clamp study on Day 7 post-surgery. **(B)** Mean weekly (SEM) glucose at baseline and following insulin injection during recurrent hypoglycemia protocol. After 3-weeks the dose of insulin was reduced from 1U/kg to 0.75U/kg to prevent the development of severe hypoglycemia where animals might require rescue. At the end of each hypoglycemia period, the rats were given free access to food. Sham-injected control rats' glucose levels did not differ significantly from Basal levels in RH group (data not shown). **(C)** Hyperinsulinemic clamp protocol. Fasted rats were stabilized for 90 minutes after catheter opening before insulin ( $20\text{mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) infused. Once animals reached their target glucose a variable 10% dextrose infusion was started and adjusted based on 5-50 minute bench side glucose readings. Glucagon (G), epinephrine (E) and corticosterone (C) were sampled at baseline and during the hypoglycemia clamp.

**Figure 2. Acute exposure to High intensity (Hi) exercise restores defective counterregulatory response to recurrent hypoglycemia (A-E).** **(A)** Plasma glucose during hyperinsulinemic hypoglycemic clamp study. **(B)** Glucose infusion rates (GIR) and **(C)** mean GIR during stable hypoglycemia (90-150mins). Plasma **(D)** epinephrine, **(E)** glucagon, and **(F)** corticosterone responses during hypoglycemia. Control+LI animals shown with white circles/bars, RH+LI exercise black squares/bars and RH+HI exercise grey diamonds/bars. Values shown as Mean  $\pm$  SEM. \*\* $p < .05$ .

**Figure 3. Hormone, metabolic and cytokine responses evoked by low or high intensity exercise in recurrently hypoglycemia or control rats.** Mean  $\pm$  SEM **(A)** Epinephrine, **(B)** Glucagon, **(C)** Lactate, **(D)** BDNF **(E)** IL-6, **(F)** IL-1 $\beta$ , **(G)** TNF- $\alpha$  **(H)** IL-4, **(I)** IL-13. Control+ Li studies represented by white bars, control + HI exercise by light grey bars, RH + LI studies with black bars and

RH+HI studies with dark grey bars.  $\delta = p < 0.05$  Li vs. HI exercise,  $\epsilon = P < 0.05$ , control +Hi vs. RH + Hi.  $\beta = p < 0.05$ , control +Li vs. RH + LI exercise, ND = not detectable. Values Mean  $\pm$ SEM.

**Figure 4. Potential mechanisms underlying dishabituation of VMH glucose sensing.** **(A)** Investigation of the effects of high intensity exercise to VMH proteome using an iTRAQ based proteomic workflow. Proteins extracted from each VMH sample were reduced, alkylated and subjected to trypsin digestion. The tryptic peptides from each animal were labeled with iTRAQ labeling reagents, where report ions 113, 115 117 and 119 were used to label peptide sample from the RH+LI groups, and 114, 116, 118 and 121 those from the RH+HI group. The samples were combined and analyzed using nano-LC-MS/MS. **(B)**. Volcano plot showing the differences of the VMH proteome between the Li and Hi exercise groups. Quantification data was available on 1866 proteins where unique peptides/quantifiable reporter ions are detected. Top differentially expressed proteins with a fold change  $> 30\%$  and a p value  $< 0.05$  (Bonferroni-Hochberg method) are labeled in the chart. **(C)** Western blotting of BSN and Dnajb1 in VMH lysates from RH animals following LI or HI exercises. **(D)** A potential model explaining dishabituation effect. Acute hypoglycemia results in increased glutamatergic synaptic activity, which is suppressed following recurrent hypoglycemia. The dishabituating stimulus then acts to restore glutamatergic synaptic activity, perhaps via BDNF/ Bassoon, leading to restoration of the counterregulatory response.

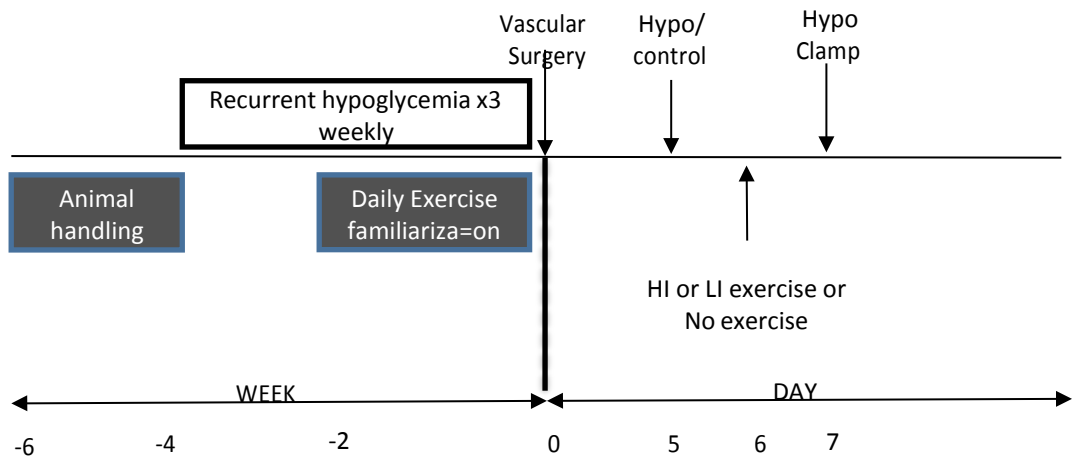
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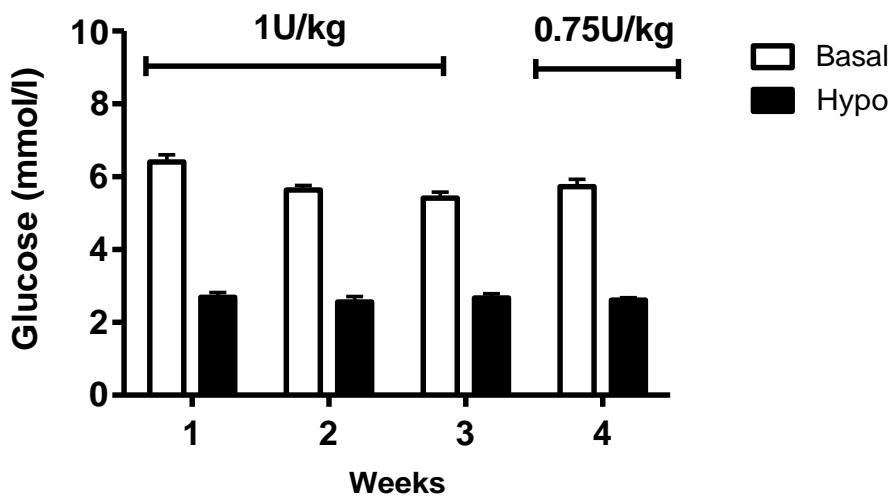
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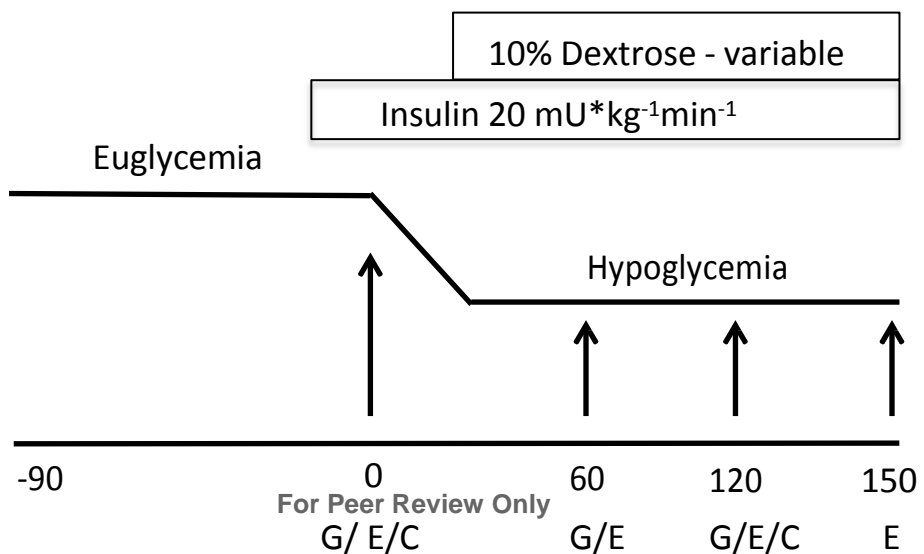
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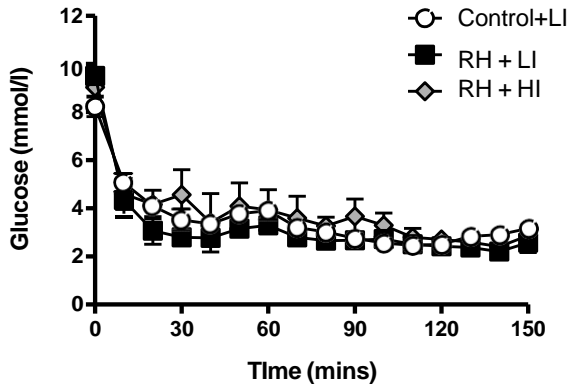


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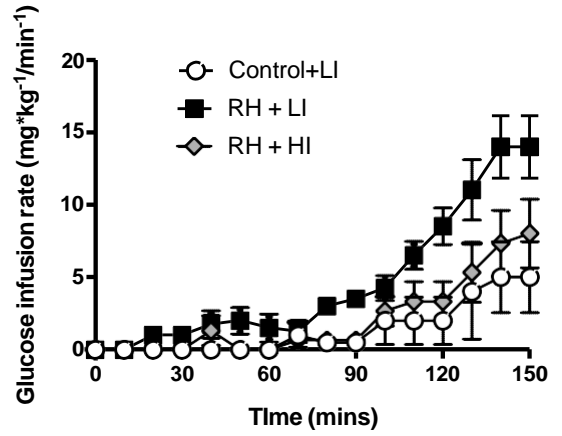




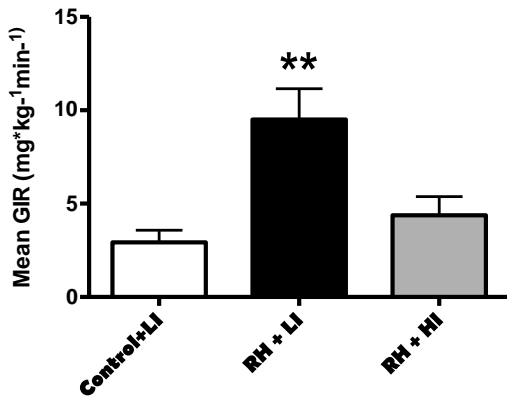
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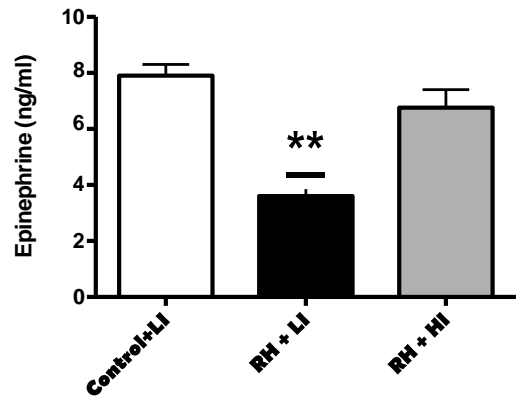
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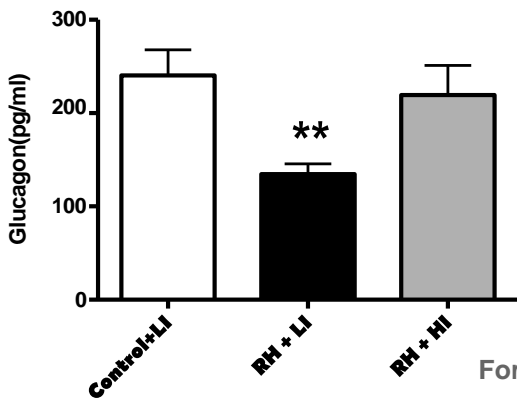
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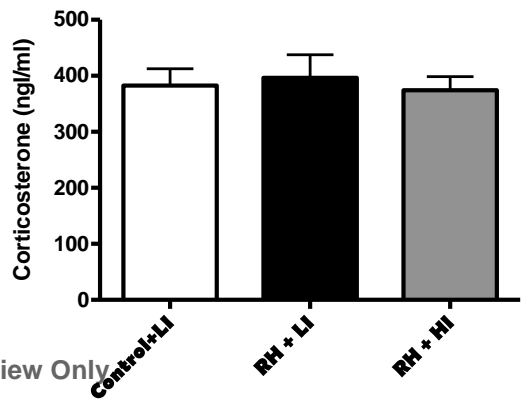
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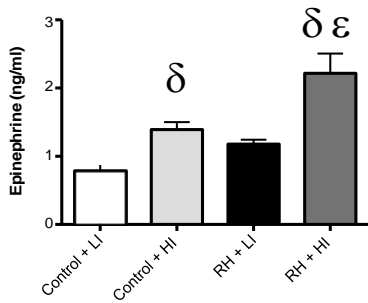
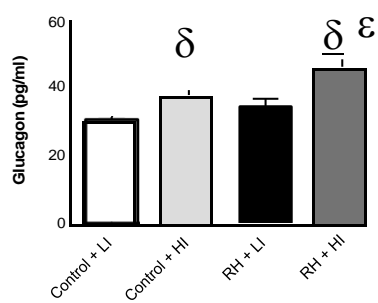
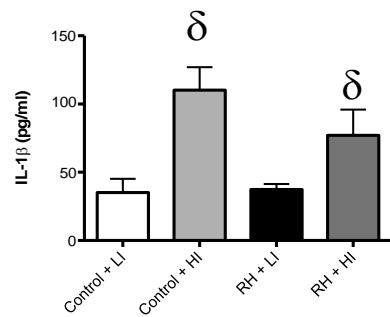
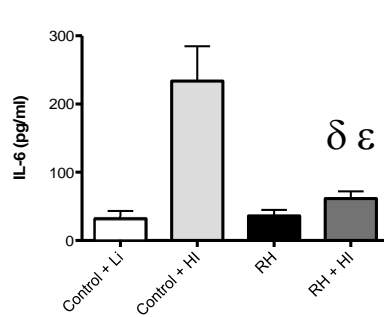
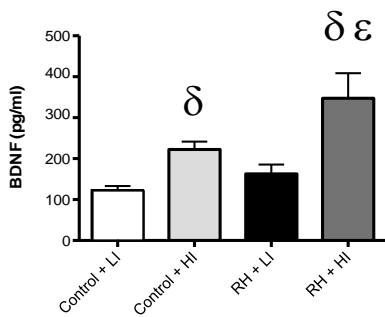
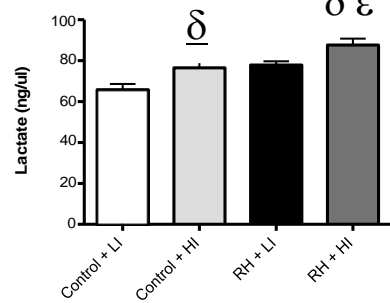
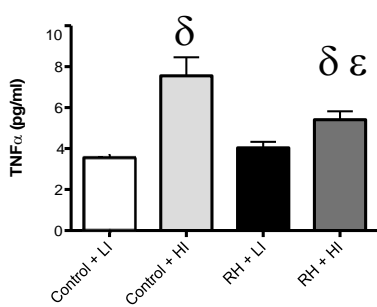
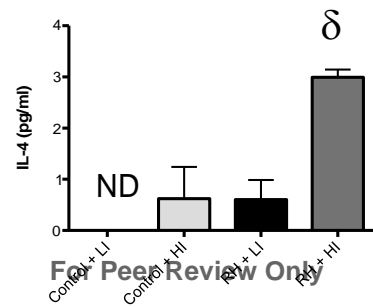
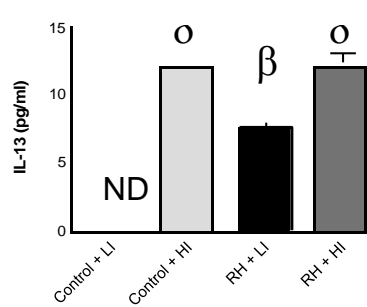


E.



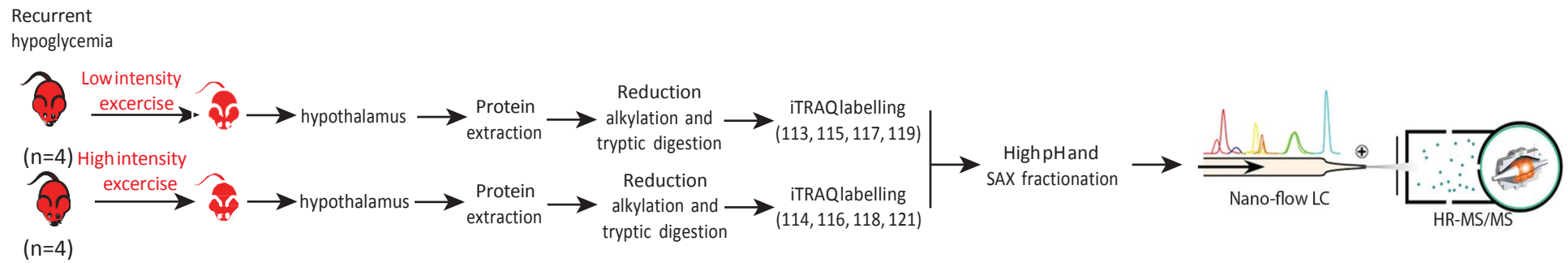
F.



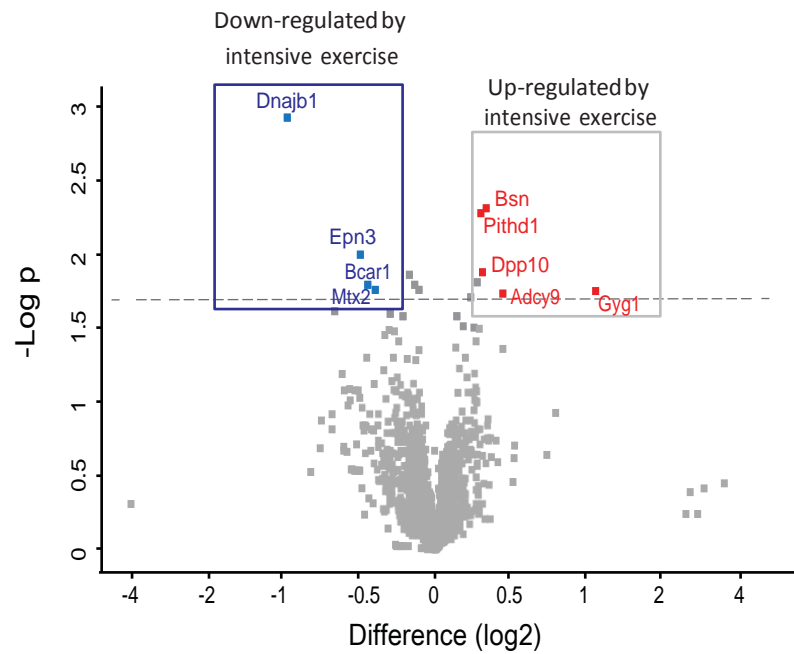
**A.****B. Diabetes****C.****G.****H.****I.**

# Diabetes

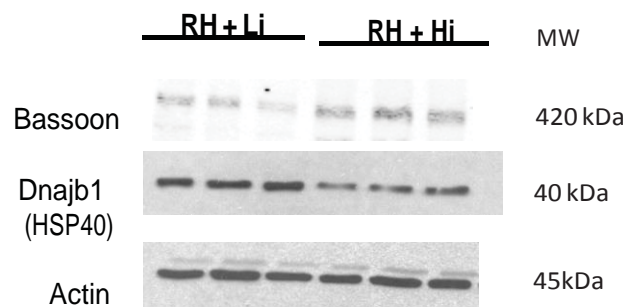
A



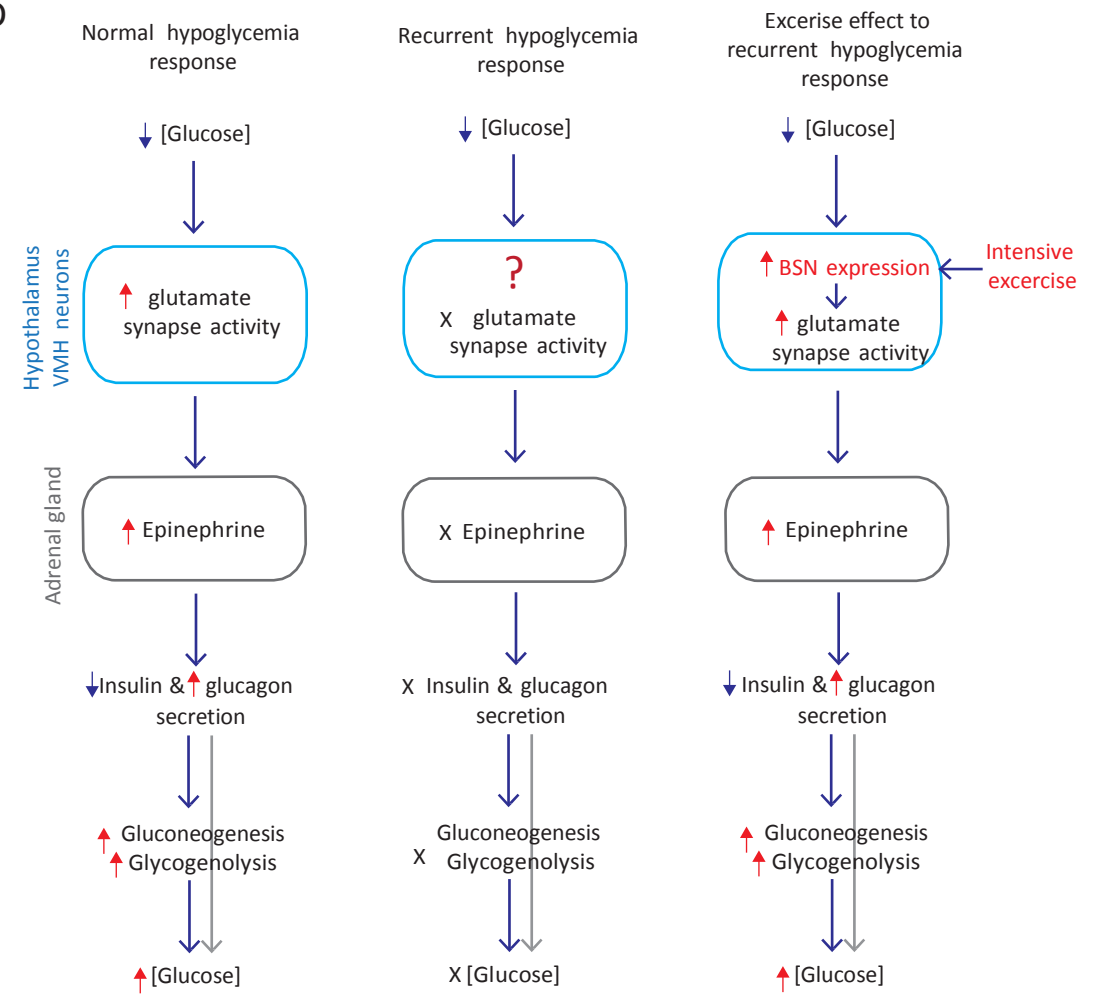
B



C



D

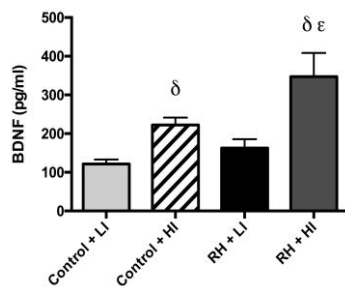


**Supplementary table 1. Comparison of counterregulatory responses to hypoglycemia in rats who had performed either low intensity exercise (LI) or No exercise 24-hours prior to the hyperinsulinemic hypoglycemia clamp study.** Data analyzed using one-way ANOVA with pot-hoc Bonferroni Multiple Comparison Test. In each analysis, ANOVA revealed significant overall effects (all  $p < .05$ ). Post-hoc testing showed significant effects of RH vs. Control in each model, but no significant effect of Li vs. No exercise in either Control or RH rats.

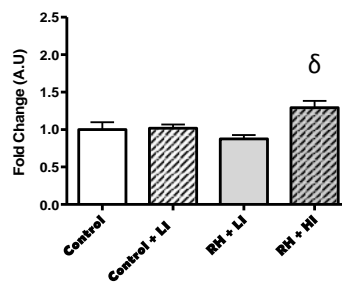
	Control + No exercise (N=8)	Control + Li (N=8)	RH + No exercise (N=8)	RH + Li (N=8)
Mean Glucose Infusion Rate (mg/kg/min)	2.9 (0.6)	2.6 (0.7)	10.2 (1.6)	9.5 (1.6)
Glucagon (ng/L)	241 (27)	250 (25)	132 (24)	135 (11)
Epinephrine (pg/ml)	8.4 (0.5)	7.4 (0.6)	4.2 (0.4)	3.2 (0.3)

### Supplementary data.

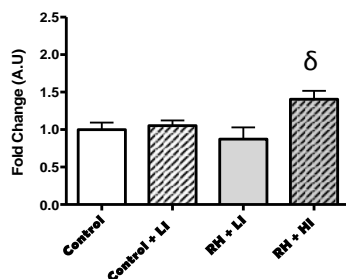
A. BDNF



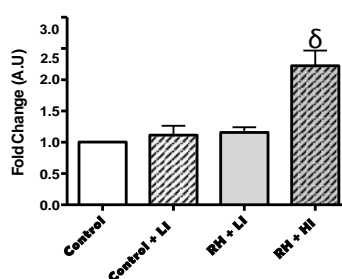
B. BDNF mRNA



C Trk B receptor



D. pCREB



**A.** Changes in plasma BDNF in response to Li and HI exercise in control and RH rats. **B-D.** hypothalamic (VMH) changes in expression of BDNF, TrkB and pCREB under each condition