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Serotonergic neurons are involved in the counter-regulatory response to hypoglycemia

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Running title:

The 5-HT system modulates the CRR to hypoglycemia

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Abstract

Objectives. Intensive insulin therapy provides optimal glycemic control in patients with diabetes. However, intensive insulin therapy causes so-called iatrogenic hypoglycemia as a major adverse effect. The ventromedial hypothalamus (VMH) has been described as the primary brain area initiating the counter-regulatory response (CRR). Nevertheless, the VMH receives projections from other brain areas which could participate in the regulation of the CRR. In particular, studies suggest a potential role of the serotonin (5-HT) network. Thus, the objective of this work is to determine the contribution of 5-HT neurons in CRR control.

Methods. Complementary approaches have been used to test this hypothesis in quantifying the level of 5-HT in several brain areas by HPLC in response to insulin-induced hypoglycemia, measuring the electrical activity of dorsal Raphe (DR) 5-HT neurons in response to insulin or decreased glucose level by patch-clamp electrophysiology; and measuring the CRR hormone glucagon as an index of the ~~counterregulatory response~~CRR to the modulation of the activity of 5-HT neurons using pharmacological or pharmacogenetic approaches.

Results. HPLC measurements show that the 5HIAA/5HT ratio is increased in several brain regions including the VMH in response to insulin-induced hypoglycemia. Patch-clamp electrophysiological recordings show that insulin, but not decreased glucose level, increases the firing frequency of DR 5-HT neurons in the DR. *In vivo*, both the pharmacological inhibition of 5-HT neurons by intraperitoneal injection of the 5-HT1A receptor agonist 8-OH-DPAT or the chemogenetic inhibition of these neurons reduces glucagon secretion, suggesting an impaired CRR.

Conclusion. Taken together, these data highlight a new neuronal network involved in the regulation of the CRR. In particular, this study shows that DR 5-HT neurons detect iatrogenic hypoglycemia in response to the increased insulin level and may play an important role in the regulation of CRR.

Highlights

- 5-HT turnover is increased in several brain areas in response to insulin-hypoglycemia
- Insulin, but not decreased glucose, increases the electrical activity of DR 5-HT neurons
- Inhibition of DR 5-HT neurons reduces glucagon secretion during hypoglycemia leading to an impaired CRR

Introduction

Intensive insulin therapy significantly reduces the onset of ~~hyperglycemia~~ hyperglycemia-related complications in patients with Type 1 or Type 2 Diabetes Mellitus. However, intensive insulin therapy also causes hypoglycemia which represents the major adverse effect of this ~~therapy~~ treatment [1]. Neuroendocrine and autonomic mechanisms known as the counter-regulatory response (CRR) to insulin-induced hypoglycemia protect the brain from hypoglycemia. The CRR involves the release of hormones (e.g. glucagon, epinephrine) which restore normal blood glucose levels by stimulating hepatic glucose production and inhibiting peripheral glucose uptake [1]. Although the physiology of the CRR is well understood, the underlying cellular mechanisms by which the brain modulates the CRR are not fully understood.

Within the brain, it is well established that the hypothalamus, and particularly the ventromedial hypothalamus (VMH), plays a key role in the control of the CRR [2,3]. The involvement of VMH in the regulation of the CRR is based on the presence of specialized neurons called "glucose-sensing neurons" (GSNs) whose electrical activity is modulated in response to changes in extracellular glucose concentration reflecting hypoglycemia [2,4–6]. GSNs use glucose, not only as fuel, but also as a signaling molecule that modulates their electrical activity. There are 2 main populations of GSNs: Glucose-inhibited (GI) neurons increase while glucose-excited (GE) neurons decrease their electrical activity in response to decreased extracellular glucose levels below 2.5 mM, reflecting physiological changes in brain glucose level during insulin-induced hypoglycemia [2,7]. These GSNs are key components in the ~~brain~~ detection of hypoglycemia and the initiation of the CRR by the brain [2,3]. It is important to note that the CRR is initiated in response to insulin-induced hypoglycemia. Most studies ~~of~~ on the

CRR focus solely on the response to hypoglycemia; however, it is important to note that insulin itself also regulates the CRR [8]. VMH neurons also present the ability to detect insulin [8–10]. The effect of insulin in the regulation of the CRR also seems to be a key in view of studies showing that insulin regulates VMH GSNs [11] and that NIRKO mice lacking the insulin receptor in brain neurons present impaired CRR to insulin-hypoglycemia [12].

While the VMH plays ~~a key~~an essential role in the control of the CRR in response to insulin-induced hypoglycemia, it receives projections from other brain areas which could modulate the activity of GSNs and consequently the CRR. In particular, studies have suggested a potential role for serotonin (5-HT) in the regulation of iatrogenic hypoglycemia. The hypothalamus receives serotoninergic projections from the dorsal Raphe (DR) nucleus, the cerebral area containing the cell bodies of 5-HT neurons [13]. Serotonergic tone is increased in certain brain areas during hypoglycemia [14] whereas the electrical stimulation of the DR increases the activity of the sympathetic nervous system and increases blood sugar [15]. Thus, DR 5-HT neurons may detect changes in glucose or insulin levels since they express the glucokinase, a key enzyme involved in the glucose-sensing machinery [16]; or the insulin receptor [17]. These data suggest that the serotonergic system may detect insulin-hypoglycemia and be involved in the regulation of CRR. Thus, we hypothesized that DR 5-HT neurons are activated in response to insulin and/or decreased glucose level to modulate the CRR to insulin-induced hypoglycemia.

To address this hypothesis, we investigated the effect of peripheral injection of insulin-induced hypoglycemia on tissue levels of 5-HT and its metabolites in distinct brain regions including subregions of the hypothalamus, and the *in vitro* application of insulin or the decrease in glucose level on the electrophysiological response of DR 5-HT neurons using patch-

clamp. Finally, we causally addressed the influence of 5-HT neurons in the CRR in inhibiting 5-HT neurons using pharmacological or chemogenetic approaches.

Materials and Methods

Animals. Adult male mice (8-12 weeks) from different strains were ~~group~~ group-housed in conventional housing cages at NutriNeuro's or BFA's animal facilities. Animals were maintained on a 12 hours light-dark cycle with *ad libitum* access to standard chow food and water. All experimental procedures were conducted in accordance with the European directive 2010/63/UE and approved by the French Ministry of Research and local ethics committees (APAFIS#: 8928 and #20317). C57BL/6J were purchased from Janvier laboratory (Le Genest-Saint-Isle, France). Pet1-cre-mCherry mice were obtained by crossing Pet1-cre mice with B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J mice (Jackson Laboratory, Bar Harbor, Maine, USA) as previously described [17].

Intracerebral virus injection in dorsal Raphe nucleus for chemogenetic DREADD (Designer Receptor Exclusively Activated by Designer Drugs) approach. Pet1-cre/mCherry mice (cre/- or -/- WT littermate mice; 8-12-week-old) were anaesthetised with isoflurane (induction 4-5 %, maintenance 1.5%) 30 minutes post-buprenorphine administration (0.05 mg/kg, SC) and placed on a stereotaxic apparatus (Kopf instrument). Adeno-associated virus AAV-2/9-DIO-hM4Di-expressing (6×10^{11} genomes/ml) were injected into the DR (stereotaxic coordinates relative to bregma: -4.5 mm antero-posterior, 0 mm lateral and -3.5 mm dorso-ventral from the dura) at a rate of 50 nl/min for 5 min. Animals were allowed 3-4 weeks to recover from surgery and for optimal virus expression.

Counter-regulation monitoring. Insulin (1.5 U/kg; human recombinant, Actrapid® Roche) was injected intraperitoneally (IP) after 4 hours of fasting. Blood glucose was monitored from 0 to

120 minutes post-insulin infusion *via* tail prick. For measurement of plasma glucagon level, 250 µl of blood was collected in chilled tubes containing EGTA (1.6 mg/ml, Sigma-Aldrich) and aprotinin (250 KIU/ml, Sigma-Aldrich). Plasma glucagon concentrations were determined using commercially available Mouse Glucagon ELISA Kit (Crystal Chem #81518). ~~60~~ Sixty minutes prior insulin injection IP, naïve C57Bl/6J mice received IP injection of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT; 0.1 mg/kg; Tocris) [18] whereas Pet1-cre/mCherry mice injected with the AAV-2/9-DIO-hM4Di in the DR received an IP injection of clozapine-N-oxide (CNO, 1 mg/kg; Sigma-Aldrich) in order to inhibit DR 5-HT neurons and study the impact of the regulation of the CRR.

Tissue sampling and high-pressure liquid chromatography coupled to electrochemical detection (HPLC-ECD). Brain tissue sampling through microdissection and conditioning for HPLC-ECD ~~have been~~were performed as previously described [19]. ~~60~~ Sixty minutes after IP injection of insulin (1.5 U/kg), ~~to quantify the level~~measurement of monoamines levels (5-HT, 5-hydroxyindol-acetic acid (5HIAA), dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC)) and turnover (5HIAA/5HT and DOPAC/DA ratio) were taken.

Brain slice patch-clamp recordings of DR 5-HT neurons. *Ex vivo* patch-clamp recordings were performed on brain slices containing the DR from Pet1-cre-mCherry mice, as previously described [17]. Briefly, mice were intracardially perfused during euthanasia (sodium pentobarbital/lidocaine: 300/30 mg/kg, IP) with ice-cold NMDG solution containing the following (in mM): 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 20 HEPES, 0.5 $CaCl_2$, 28 $NaHCO_3$, 8 D-glucose, 5 L(+)-ascorbate, 3 Na-pyruvate, 2 thiourea, 93 N-methyl-D-glucamine, and 93 HCl 37%; pH: 7.3–7.4; osmolarity: 305–310 mOsm. Brains were quickly removed and 250 µm slices

containing the DR were cut with a vibroslice (Leica VT1000S, Wetzlar, Germany) and transferred at room temperature into artificial cerebrospinal fluid (aCSF) solution (containing the following in mM: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2.5 $CaCl_2$, 2.5 D-glucose, and 25 $NaHCO_3$; pH: 7.3–7.4; osmolarity: 305–310 mOsM) for at least 1 hour. Borosilicate pipettes (4-6 MΩ; 1.5 mm OD, Sutter Instrument) were filled with filtered aCSF. ~~for c~~Cell-attached recordings ~~. Recordings~~ were made using a Multiclamp 700B amplifier, digitized using the Digidata 1440A interface and acquired at 2 kHz using pClamp 10.5 software (Axon Instruments, Molecular Devices, San José, CA, USA). Pipette and cell capacitances were fully compensated and junction potential was corrected off-line. Firing frequency of DR 5-HT neurons was recorded in cell-attached mode. Because DR 5-HT neurons are silent *ex vivo*, phenylephrine (5 µM; Tocris) was perfused to ~~stimulate basal~~induce action potential activity for 5 minutes before application of insulin (300 nM) or change in glucose level (2.5 to 0.1 mM) and recorded for 10 additional minutes. Change in firing rate frequency was analyzed during the last minute of insulin or low glucose application.

Bioinformatics analysis of genes involved in glucose-sensing and insulin signaling in DR 5-HT neurons. Using a recent review of brain insulin signaling and glucose sensing we designed a list of key genes of interest involved in either of these functions (Supp. Figure 1) [3,8,20–24]. Previously-published RNA sequencing data was retrieved from Okaty *et al.* [25] with R software version 4.0.3. The dataset was directly extracted from the available Supplementary materials from Okaty *et al* [25], which initially included 18 pooled fractions and 56 single neuron fractions. We only extracted data from single neuron fractions from the DR, expressed as Counts per million read (CPM). Thus, our final sample consisted of 8 DR 5-HT single neuron fractions, which were the focus of our analyses. In such a sample, the *subset* function was used to

retrieve genes of interest, labelled as gene symbols (nomenclature). For each gene of interest, CPM counts were converted to log_2(CPM+1). For box and whiskers plots, boxes represent the Q1-Q3 interval, while whiskers represent Q1 to minimum and Q3 to maximum values (lower and upper whiskers, respectively), drawn with the *ggplot2* package (version 3.3.6). No statistical analysis was performed on this data, as only one group is represented (8 single neuron fractions from the DR).

~~. Counts per million read (CPM) of 5-HT neurons from the DR were extracted from the database (containing a total of 56 neuronal fractions). A final sample of 8 DR 5-HT neuronal fractions were analyzed. In this sample, the *subset* function was used to retrieve genes of interest with RStudio Team 2020. CPM counts were converted to Log_2(CPM+1). Finally, using a threshold at CPM+1 > 0, we then calculated a mean average expression for each neuronal fraction of individual genes belonging to either glucose sensing or insulin signaling.~~

Statistical analysis. Sample size has been estimated based on previous work from our group or others. No randomization was used. The investigator was not blinded to the group allocation for most experiments. Statistical analysis was performed using ~~*Prism* GraphPad~~ *Prism 9* (San Diego, CA, USA). Data are expressed as mean ± SEM and individual values are plotted on graph when possible, except on Figure 2B-D where data are presented using median in whisker boxes. Outliers were identified using GraphPad Prism 9. After normal Gaussian distribution was ~~assessed~~ verified using the Kolmogorov-Smirnov ~~Shapiro-Wilk~~ test~~,~~ ~~. appropriate~~ Appropriate parametric ~~(~~paired or unpaired Student's t-test ~~as appropriate) tests~~ were chosen to compare two population samples. Two-way ANOVA with repeated measures was used when analysis accounted for two distinct variables.

Results

Insulin increases the activity of the 5-HT system. We first aimed at confirming data from the literature suggesting that insulin-induced hypoglycemia affects the 5-HT system in measuring the 5HIAA/5HT ratio as an estimator of serotonergic activity. Our data show that 60 minutes post-insulin injection, when blood glucose falls at 42.5 ± 3.7 mg/dl (n=7), the 5HIAA/5HT ratio increases in some brain areas including the VMH, lateral hypothalamus or the dorsal hippocampus, cingular cortex or thalamus (Figure 1). Changes in the 5HIAA/5HT ratio is not ~~accompagnied~~associated ~~to~~ with changes in either 5-HT or 5HIAA levels (Supp. Figure 2). Interestingly, we also observed an increase in dopaminergic activity through the measure of the DOPAC/DA (3,4-dihydroxyphenylacetic acid/dopamine) ratio in the VMH (Supp. Figure 3).

Using data mining analyses, we interrogated RNA sequencing data bases from DR 5-HT neurons [25] to determine whether DR 5-HT neurons express the machinery necessary for detecting insulin and/or decreased glucose levels (Figure 2A-D). Analyses have been performed using known genes identified in the literature as playing a key role in either glucose sensing or insulin sensitivity (Supp. Figure 1). First, we confirmed that these neurons express genes ~~characterizing~~ characteristic of the serotonergic phenotype (Figure 2B). Our subsequent analyses revealed that DR 5-HT neurons express a majority of genes known to be involved in glucose-sensing (Figure 2C) or insulin sensitivity (Figure 2D). It should be noted however that some key genes involved in glucose sensing in hypothalamic GSNs are either not expressed or expressed in such low levels as to question physiological relevance (Figure 2C; genes of interest: Glut2, glucokinase, Nos1, Cftr, potassium channels).

The functional effect of insulin or decreased glucose level onto DR 5-HT neurons was assessed using cell-attached patch-clamp recording on Pet1-cre-mCherry mice. As previously shown [17],

insulin (300 nM) increases action potential frequency by 37.5 ± 2.3 % in 80 % of DR 5-HT neurons (Figure 2F; n=10/12 neurons tested). At high level, insulin can activate insulin growth factor-1 (IGF1) receptor. We show that the IGF1 receptor antagonist picropodophyllin does not block the insulin-induced electrical activity of 5-HT neurons, suggesting that insulin mainly acts through the insulin receptor (Supp. Figure 4). On the other hand, decreased glucose level from 2.5 to 0.1 mM failed to alter the firing rate of DR 5-HT neurons (Figure 2E; n=11). Despite being theoretically equipped to sense both insulin and decreased glucose level, these data show that DR 5-HT neurons are only sensitive to insulin and that changes in brain serotonergic activity during insulin-induced hypoglycemia ~~is~~ are due to the effect of insulin ~~onto~~ on 5-HT neurons.

DR 5-HT neurons play a key role in the regulation of the CRR. To test the hypothesis that DR 5-HT neurons play a role in the regulation of the CRR, we inhibited their activity using pharmacological or chemogenetic approaches (Figure 3A). We and others have previously shown that 8-OH-DPAT, a 5-HT1A receptor agonist, completely inhibits the electrical activity of DR 5-HT neurons *in vivo* [18]. As shown in Figure 3B, mice injected IP with 8-OH-DPAT showed significantly lower glycemia post-insulin injection in comparison to controls (Figure 3B). Change in blood glucose in response to 8-OH-DPAT injection is not accompanied ~~with~~ by any change in plasma glucagon production (Figure 3C). To further confirm the role of DR 5-HT neurons in the regulation of the CRR we specifically inhibited these neurons, using chemogenetics by injecting a DREADD inhibitor AAV-DIO-hM4Di in the DR of Pet1-cre/mCherry mice [26]. Figure 3D shows that injection of CNO 30 minutes before the initiation of insulin-hypoglycemia decreases blood glucose level of Pet1-cre/mCherry mice as compared

to wildtype littermates (Figure 3D). Change in blood glucose is associated with a decrease in plasma glucagon concentration in Pet1-cre/mCherry mice treated with CNO (Figure 3E). Together, these data show that the inhibition of DR 5-HT neurons impairs the CRR to insulin-induced hypoglycemia.

Discussion

Our study demonstrates that insulin-induced hypoglycemia activates the 5-HT system to control the CRR. Our data reveal that such an effect is mediated by the action of insulin on DR 5-HT neurons rather than an effect of decreased glucose *per se*. This study brings out a novel neuronal network involved in the regulation of the CRR and new hopes for improving the CRR in patients with Diabetes treated with insulin therapy.

Studies have suggested that the 5-HT system might be involved in the regulation of hypoglycemia in view of data showing that the level of 5-HT is changed in several brain areas in response to insulin-induced hypoglycemia [14,27]. These data ~~are in agreement~~confirm with our findings showing that the ratio 5-HIAA/5-HT is increased in several areas. Our data showing that inhibiting DR 5-HT neurons impairs the CRR highlight an important role of the serotonergic network in the control of the CRR. We found that the inhibition of DR 5-HT neurons using the 5HT1A agonist 8-OH-DPAT [18] or using a chemogenetic approach [26] induces a further decrease ~~of~~ in blood glucose level in response to insulin. In the chemogenetic experiments, this decrease in blood glucose level was associated with an impairment in plasma glucagon release. Surprisingly, there was no significant decrease in plasma glucagon level in 8-OH-DPAT treated animals. This might be due to the fact that ~~acting peripherally~~, 8-OH-DPAT acting peripherally may impact 5-HT1A receptor expressed in the pancreas and may modulate~~s~~ glucagon production directly or through a local modulation of insulin secretion [28,29]. One could also hypothesize that 8-OH-DPAT impacts glucagon signaling, which could explain why despite any significant change in glucagon level, blood glucose level decreases further. In addition, we cannot rule out that the inhibition of the 5-HT system impacts other CRR

hormones including catecholamines or cortisol. Moreover, the further decrease in blood glucose level could also be the consequence of alteration in peripheral glucose uptake or production. Modulation of brain 5-HT activity has been shown to alter peripheral glucose homeostasis [30]. Nevertheless, taken together, these data show that activation of the serotonergic system during insulin-induced hypoglycemia is key for a full CRR. Interestingly, the CRR is impaired type 1 (T1D) or type 2 (T2D) diabetes [31]. We have previously shown that the response to insulin of DR 5-HT neurons is impaired in a model of diet-induced T2D [17]. Thus, the new role of 5-HT neurons in the control of the CRR allow us to hypothesize that the impairment of the response to insulin of these neurons during T2D takes part in the impairment of the CRR.

Despite the fact that the level of 5-HT changes in several brain areas in response to insulin-induced hypoglycemia, no study has shown that 5-HT neurons are directly modulated in response to insulin-induced hypoglycemia. To tackle this question, we 1/ interrogated RNA sequencing databases obtained in DR 5-HT neurons to determine whether these neurons express ~~the~~ genes of the machinery or either low-glucose-sensing or insulin signaling; and 2/ performed patch-clamp electrophysiology to study the response of these neurons to either decreased glucose level or insulin. As we have previously shown, we confirm these findings in the present study in showing that DR 5-HT neurons are activated in response to insulin [17]. This is consistent with the RNAseq analyses showing that the majority of genes involved in the insulin signaling machinery are expressed by DR 5-HT neurons, including the insulin receptor, Irs1, and different kinases of insulin signaling. However, ~~O~~one question remains ~~however~~ on the nature of the ionic channels involved in the response of DR 5-HT neurons to insulin. In the

hypothalamus, diverse studies ~~showed~~ have shown that either ATP-dependent potassium (K_{ATP}) or transient receptor potential canonical (TRPC) channels are involved in the insulin response [9,10,32–35]. TRPC4 and C5, the TRPC isoforms presumably involved in the response to insulin in some VMH neurons, are not expressed in DR 5-HT neurons. Other TRPC channels including TRPC1, C3 and C7 are expressed in DR 5-HT neurons (Log_2 CPM+1: TRPC1: 4.16 ± 0.87; TRPC3: 3.62 ± 1.22; TRPC7: 5.7 ± 1.02) but their sensitivity to insulin is yet to be determined. Regarding K_{ATP} channels, Kir6.2 or Sur1 subunits are expressed but at ~~a~~ relatively low levels. Their involvement needs to be confirmed using pharmacological approached combined to patch-clamp recordings. More profound electrophysiological work needs to be ~~performed~~ carried out to characterize ~~precisely~~ the molecular mechanisms involved in the response to insulin of DR 5-HT neurons. Regarding the possible sensitivity of DR 5-HT neurons to decreased glucose level, the RNAseq analyses show~~s~~ that some genes involved in glucose-sensing are expressed but, if so, at a relatively low level. Most of the key genes necessary for detection of changes in glucose level in VMH GSNs are not expressed or poorly expressed like Glut2 (Slc2a2, glucokinase (Gck), the neuronal NOS (Nos1), K_{ATP} channels subunits, Cftr or other K_{2P}-channels (potassium two pore domain channel). The lack of significant expression of these key players of the glucose-sensing machinery is consistent with our patch-clamp data showing that DR 5-HT neurons are not glucose-sensing. In support of this, we have previously shown that glucose injection during insulin-induced hypoglycemia does not prevent or amplify changes in the electrical activity of DR 5-HT neurons using *in vivo* electrophysiology [17]. Overall, our data show that in response to insulin-induced hypoglycemia, the 5-HT network is activated through ~~to~~ the action of insulin on~~to~~ DR 5-HT neurons.

In agreement with previous studies from the literature [36,37], measurement of monoamines in the brain by HPLC revealed that the ratio DOPAC/DA is also increased in response to insulin-induced hypoglycemia is discrete brain areas, including the VMH. Although microdialysis of glucose into the substantia nigra causes dopamine release [38], there is no clear evidence that VTA dopamine neurons are sensitive to decreased glucose levels. However, ~~like~~ similar to 5-HT neurons, VTA DA neurons express the insulin receptor [39] and are activated by insulin [40,41]. The dopaminergic system may play a role in the regulation of the CRR but this hypothesis is yet to be tested. Changes in dopaminergic turnover may also come from the modulation of dopaminergic neurons outside the VTA. The idea that several brain networks play a role in the regulation of the CRR is supported by many studies showing that GSNs sensing decreased glucose level mimicking hypoglycemia can be found in many ~~places of the~~ brain regions including extra-hypothalamic areas (for review, see [42,43]). The physiological roles of these neurons are not known. They may take part in the regulation of the CRR. Another possibility would be a role played in hypoglycemia awareness, a state of conscientiousness which includes a variety of symptoms (e.g. palpitations, anxiety, confusion) alerting for hypoglycemia and initiating behavioral responses to find energy. This is the case for lateral hypothalamus GI orexin neurons as it has recently been shown [44]. Other brain functions, including memory, motivation could be controlled, in addition to the CRR, by GSNs of these different neuronal networks. Significant work is still needed to fully understand the functions controlled by these specific neurons.

Considering the neuronal networks controlling the CRR, VMH GSNs seem to be a mandatory piece of the puzzle. The CRR is blunted in conditions when VMH GSNs are inhibited, including

T2D [2,3]. Thus, the fact that 5-HT turnover increases in the VMH and that such an increase is ~~key in~~crucial for the full initiation of the CRR would suggest that GSNs are modulated by 5-HT. This hypothesis is consistent with data showing that 5-HT modulates the electrical activity of VMH neurons [45,46] and DR 5-HT neurons project~~s~~ to the VMH [47]. Even though this hypothesis is yet to be tested, we can hypothesize that insulin increases the activity of DR 5-HT neurons and the release of 5-HT in the VMH which, in return, will potentiate the response of VMH GSNs to decreased glucose level, ensuring the full initiation of the CRR (Figure 4). In conclusion, our study highlights a new neuronal network taking part in the regulation of hypoglycemia in showing that the 5-HT system is an important player to control the CRR. Since ~~T~~this system ~~being~~ is the target of numerous drugs already used in human (i.e. antidepressants), our study offers new pharmacological possibilities to enhance the CRR in patients with Diabetes treated with insulin therapy.

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Authors contribution

HM, AC, JL, JK, IR, JB, CM, CGG performed experiments. MDM performed the RNA sequencing database analyses. CGG, MDM, VHR, CM, PDD, BG, JPG, SL edited the manuscript. XF designed the project and wrote the manuscript.

Conflict of interest

The authors declare no competing interests

Supplementary information is available at the journal's website

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Data Availability Statement.

The data that support the findings of this study are available from the corresponding author upon reasonable request. RNA sequencing bioinformatics data taken from Okaty and colleagues (ref 25) is available on the publisher's website at https://doi.org/10.1016/j.neuron.2015.10.007

Figures legends

Figure 1: Effect of insulin-induced hypoglycemia on tissular monoamine level in different brain areas. Ratio 5HIAA/5HT tissue content measured in different brain areas in response to IP injection of insulin (1.5 U/kg, n = 6-7) or saline (n = 6-7). Abbreviations: AMG: amygdala; Cx: cortex; DR: dorsal raphe; HippoC: hippocampus; HT: hypothalamus; STR: striatum; VTA: ventral tegmental area). Data are represented as mean ± SEM. Statistics: Student's unpaired t-test: * p<0.05.

Figure 2: Effect of insulin and decreased glucose level on DR 5-HT neurons activity. A. Workflow of the present RNA expression analyses. Eight 5-HT neuronal fractions were extracted from a previously-published database [25], from which log_2(CPM+1) values were extracted for genes of interest (**A**). Serotonin identity (**B**) genes, low-glucose sensing genes (**C**) and insulin signaling genes (**D**) are plotted, with "nil" indicating no expression in the 8 fractions. Box and whiskers are drawn as Q1-Q3 intervals (boxes) and minimum to maximum values (lower and upper whiskers, respectively). Bars within the Q1-Q3 boxes represent median values. **E, F.** Representative traces of cell-attached mode recording and quantification of firing rate before and after insulin perfusion (n = 8, 300 nM, **E**) or 2.5-0.1 mM glucose decrease (n = 11, **F**) of DR 5-HT neurons from Pet1-cre/mCherry mice. Data are represented as mean ± SEM. Statistics: Student's paired t-test: **p<0.01.

Figure 3: Inhibition of DR 5-HT neurons blunts the CRR to insulin-induced hypoglycemia. A, Timeline for the monitoring of the CRR. **B, C.** Blood glucose level (**B**) and delta plasma glucagon level (difference t0-t60 min post-insulin injection; **C**) of C57BL/6J mice injected IP with 8-OH-DPAT (n = 8, 0.1 mg/kg, IP) or saline 60 minutes before insulin (t0, n = 6). **D,E.** Blood glucose level (**D**) and delta plasma glucagon level (difference t0-t60 min post-insulin injection; **E**) of Pet1-cre/mCherry mice (n = 7) or wildtype littermates (n = 7) injected IP with CNO (1 mg/kg) 60 minutes before insulin (t0). Data are represented as mean ± SEM. Statistics: Two-way ANOVA with repeated measures (B, D): * p<0.05 (B: time: $F_{7,63}$=164.6, p<0.0001, treatment: $F_{1,9}$=11.81, p=0.0074, time*treatment: $F_{7,63}$=2.922, p=0.0103; D: time: $F_{7,70}$=120.8, p<0.0001,

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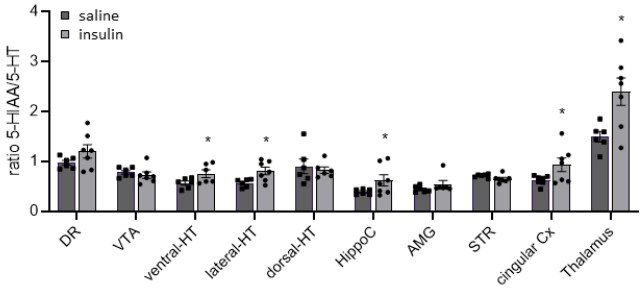
Figure 4: Schematic representation of the hypothesis showing the role of the 5-HT system in the control of the CRR.



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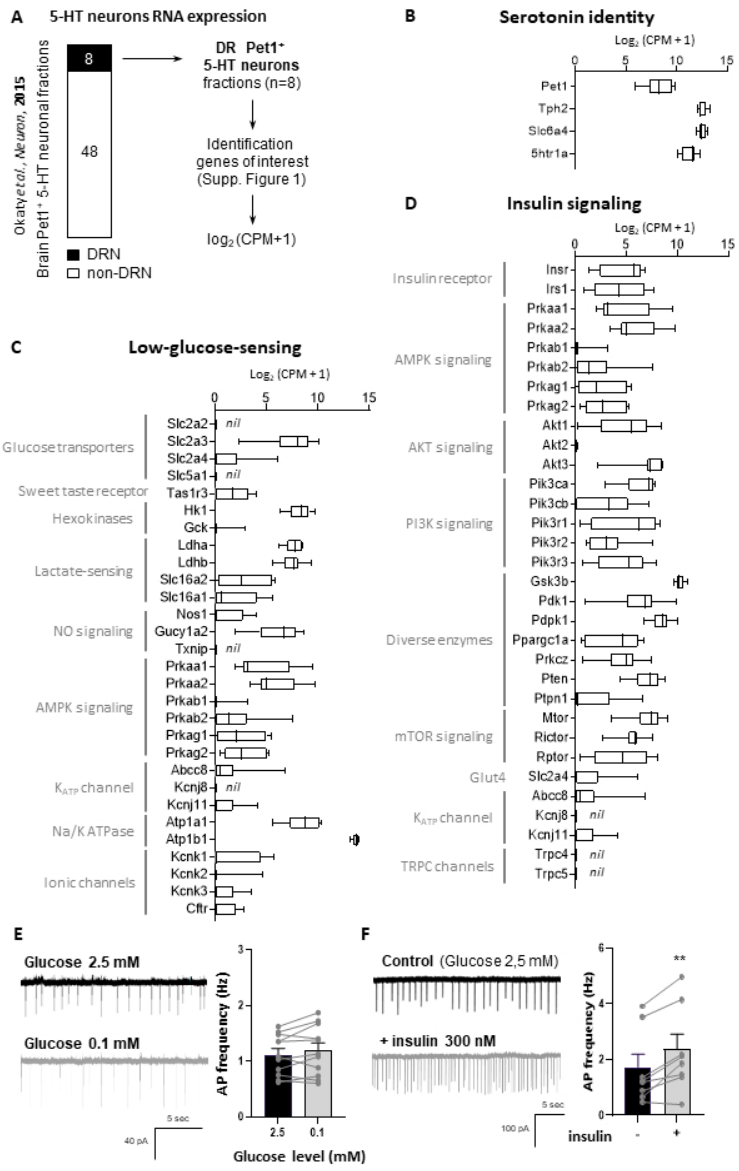

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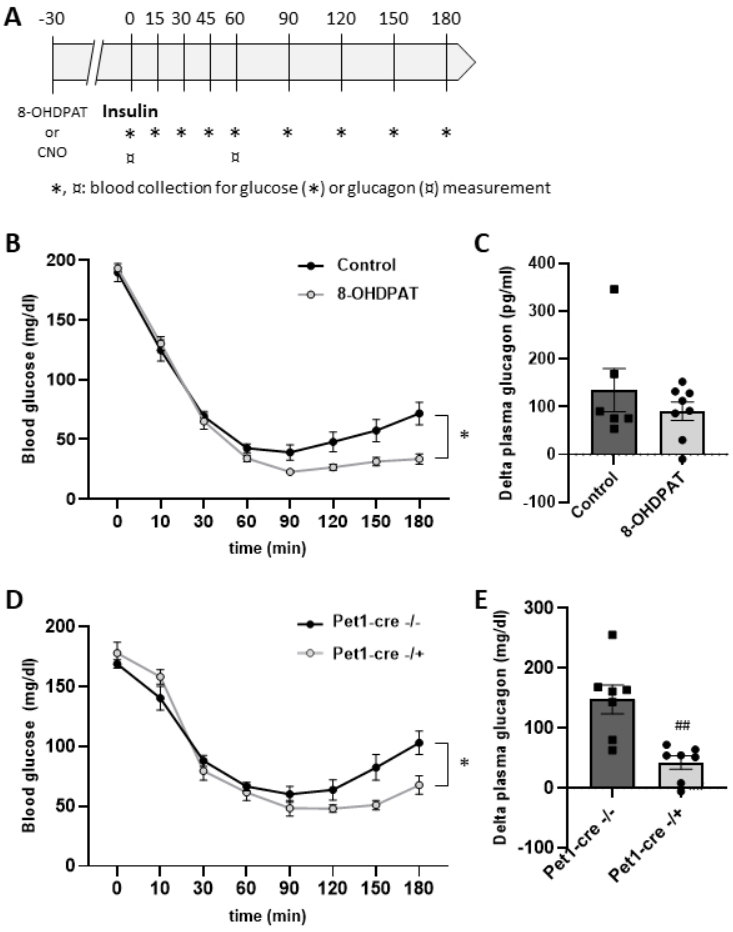

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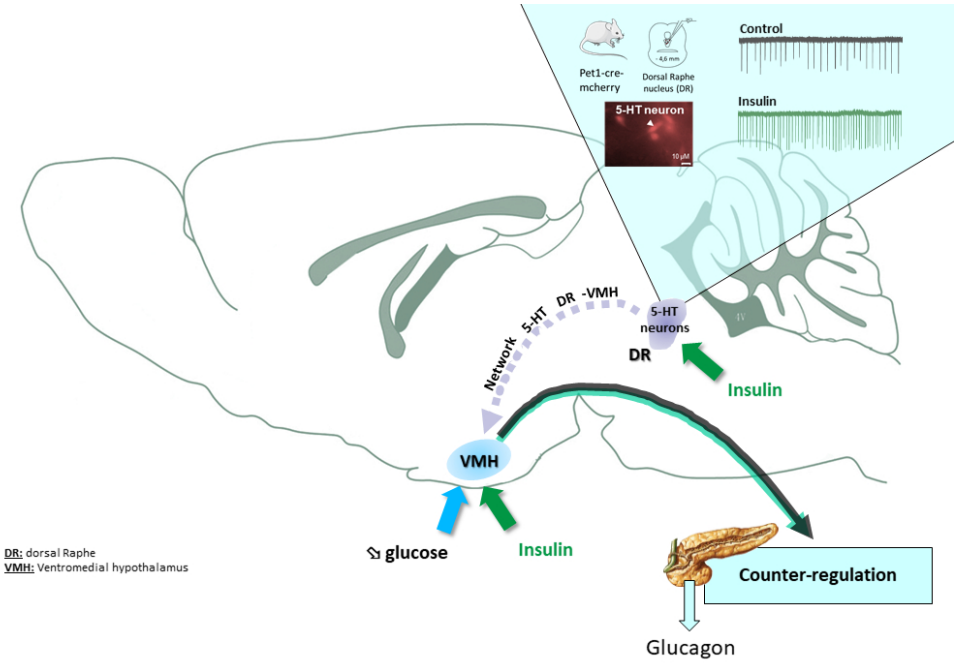

Figure 4: Schematic representation of the hypothesis showing the role of the 5-HT system in the control of the CRR.

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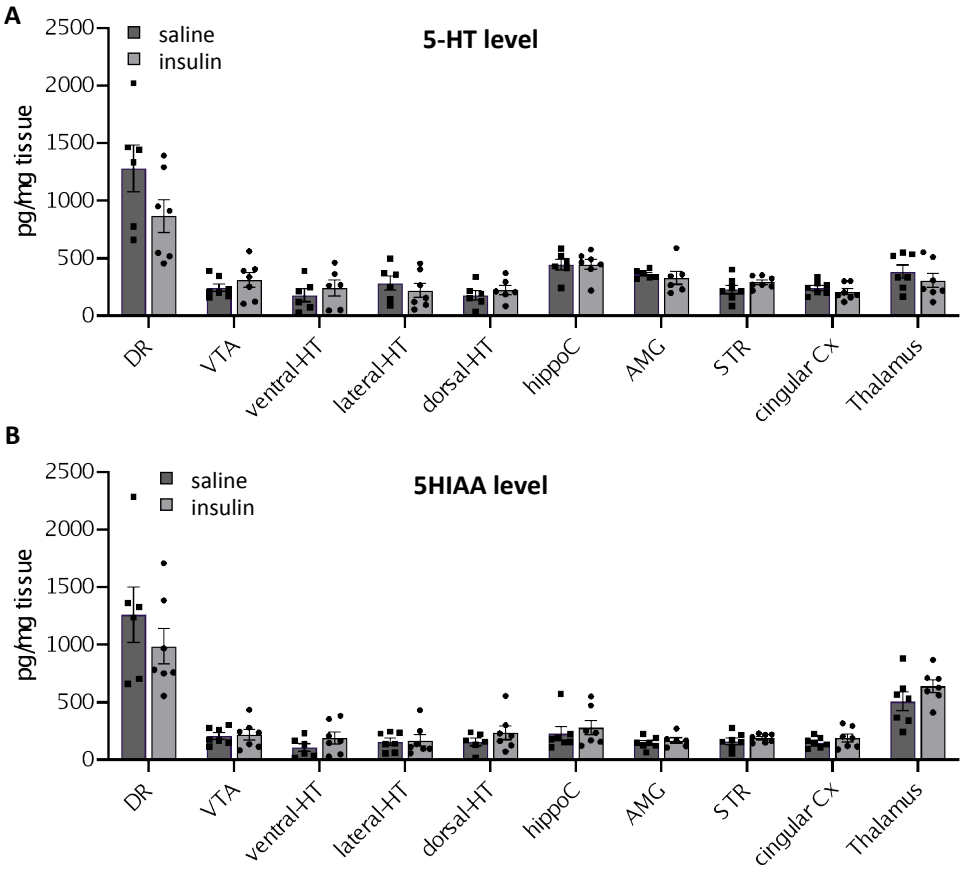
Serotonin identity		
Gene family	**Gene #**	**Gene name**
5HT1A receptor	5htr1a	5HT1A receptor
SERT	Slc6a4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
Tph2	Tph2	tryptophan hydroxylase 2
Pet-1	Pet1	FEV transcription factor, ETS family member, Pet1

Low-glucose signalling		
Gene family	**Gene #**	**Gene name**
Glucose transporters	Slc2a2	solute carrier family 2 (facilitated glucose transporter), member 2 (Glut2)
	Slc2a3	solute carrier family 2 (facilitated glucose transporter), member 3 (Glut3)
	Slc2a4	solute carrier family 2 (facilitated glucose transporter), member 4 (Glut4)
	Slc5a1	solute carrier family 5 (sodium/glucose cotransporter), member 1 (SGLT1)
Sweet taste receptor	Tas1r3	taste receptor, type 1, member 3
Hexokinases	Hk1	hexokinase 1
	Gck	glucokinase
Lactate-sensing	Ldha	lactate dehydrogenase A
	Ldhb	lactate dehydrogenase B
	Slc16a2	solute carrier family 16 (monocarboxylic acid transporters), member 2 (MCT2)
	Slc16a1	solute carrier family 16 (monocarboxylic acid transporters), member 1 (MCT1)
NO signalling	Nos1	nitric oxide synthase 1, neuronal
	Gucy1a1	guanylate cyclase 1, soluble, alpha 1
	Txnip	thioredoxin interacting protein
AMPK signalling	Prkaa1	protein kinase, AMP-activated, alpha 1 catalytic subunit
	Prkaa2	protein kinase, AMP-activated, alpha 2 catalytic subunit
	Prkab1	protein kinase, AMP-activated, beta 1 non-catalytic subunit
	Prkab2	protein kinase, AMP-activated, beta 2 non-catalytic subunit
	Prkag1	protein kinase, AMP-activated, gamma 1 non-catalytic subunit
	Prkag2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit
KATP channel	Abcc8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (SUR1)
	Kcin8	potassium inwardly rectifying channel, subfamily J, member 8 (Kir6.1)
	Kcnj11	potassium inwardly rectifying channel, subfamily J, member 11 (Kir6.2)
Na/K ATPase	Atp1a1	ATPase, Na+/K+ transporting, alpha 1 polypeptide
	Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide
Ionic channels	Kcnk1	potassium two pore domain channel subfamily K member 1
	Kcnk2	potassium two pore domain channel subfamily K member 2
	Kcnk3	potassium two pore domain channel subfamily K member 3
	Cftr	cystic fibrosis transmembrane conductance regulator

Insulin signalling		
Gene family	**Gene #**	**Gene name**
Insulin receptor	Insr	insulin receptor
	Irs1	insulin receptor substrate 1
AMPK signalling	Prkaa1	protein kinase, AMP-activated, alpha 1 catalytic subunit
	Prkaa2	protein kinase, AMP-activated, alpha 2 catalytic subunit
	Prkab1	protein kinase, AMP-activated, beta 1 non-catalytic subunit
	Prkab2	protein kinase, AMP-activated, beta 2 non-catalytic subunit
	Prkag1	protein kinase, AMP-activated, gamma 1 non-catalytic subunit
	Prkag2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit
AKT signalling	Akt1	thymoma viral proto-oncogene 1
	Akt2	thymoma viral proto-oncogene 2
	Akt3	thymoma viral proto-oncogene 3
PI3K signalling	Pik3ca	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
	Pik3cb	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta
	Pik3r1	phosphoinositide-3-kinase regulatory subunit 1
	Pik3r2	phosphoinositide-3-kinase regulatory subunit 2
	Pik3r3	phosphoinositide-3-kinase regulatory subunit 3
Diverse kinases/phosphatases	Gsk3b	glycogen synthase kinase 3 beta
	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1
	Pdpk1	3-phosphoinositide dependent protein kinase 1
	Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
	Prkcz	protein kinase C, zeta
	Pten	phosphatase and tensin homolog
	Ptpn1	protein tyrosine phosphatase, non-receptor type 1
mTOR signalling	Mtor	mamalian target of rapamycin kinase
	Rictor	RPTOR independent companion of MTOR, complex
	Rptor	regulatory associated protein of MTOR, complex 1
Glut4	Slc2a4	solute carrier family 2 (facilitated glucose transporter), member 4 (Glut4)
KATP channel	Abcc8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (SUR1)
	Kcin8	potassium inwardly rectifying channel, subfamily J, member 8 (Kir6.1)
	Kcnj11	potassium inwardly rectifying channel, subfamily J, member 11 (Kir6.2)
TRPC channels	Trpc4	transient receptor potential cation channel, subfamily C, member 4
	Trpc5	transient receptor potential cation channel, subfamily C, member 5

Supp. Figure 1: List of genes involved in serotonin identity, low-glucose sensing and insulin signaling .



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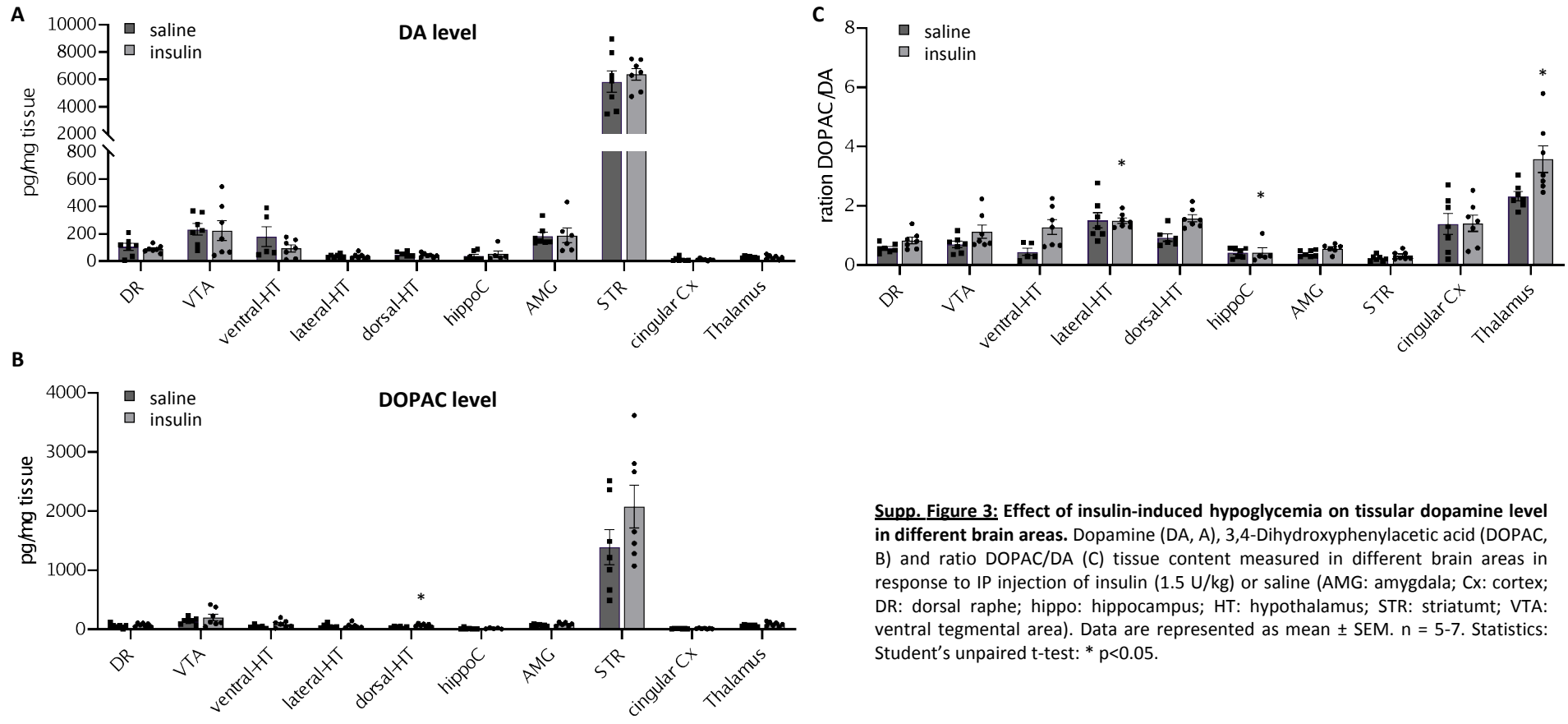
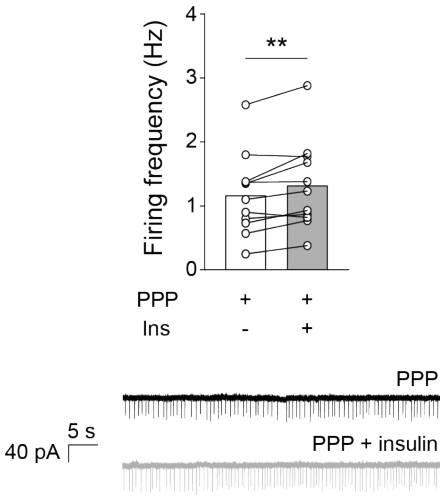


Supp. Figure 3: Effect of insulin-induced hypoglycemia on tissular dopamine level in different brain areas. Dopamine (DA, A), 3,4-Dihydroxyphenylacetic acid (DOPAC, B) and ratio DOPAC/DA (C) tissue content measured in different brain areas in response to IP injection of insulin (1.5 U/kg) or saline (AMG: amygdala; Cx: cortex; DR: dorsal raphe; hippo: hippocampus; HT: hypothalamus; STR: striatumt; VTA: ventral tegmental area). Data are represented as mean ± SEM. n = 5-7. Statistics: Student's unpaired t-test: * $p<0.05$.



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