Perinatal High Fat Diet Exposure Alters Oxytocin and Corticotropin Releasing Factor Inputs onto Vagal Neurocircuits Controlling Gastric Motility

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Abstract

Perinatal high fat diet (pHFD) exposure is known to affect the development of vagal neurocircuits that control gastrointestinal (GI) motility and reduce stress resiliency in offspring. Descending oxytocin (OXT; prototypical, anti-stress peptide) and corticotropin releasing factor (CRF; prototypical, stress peptide) inputs from the paraventricular nucleus (PVN) of the hypothalamus to the dorsal motor nucleus of the vagus (DMV) modulate the GI stress response. However, how these descending inputs, and their associated changes to GI motility and stress responses, are altered following pHFD exposure are unknown. The present study used *in vivo* recordings of gastric tone and motility, *in vivo* assays of gastric emptying rates, *in vitro* electrophysiological recordings from brainstem slice preparations, and retrograde neuronal tracing experiments to investigate the hypothesis that pHFD alters descending PVN-DMV inputs, dysregulating DMV neuronal responses and subsequent gastric motility. Basal gastric emptying rates were found to be significantly delayed in pHFD rats, which also demonstrating an inability to mount an appropriate response to acute stress with a further delay in gastric emptying. Neuronal tracing experiments suggested that pHFD reduced PVN^{OXT} neurons that project to the DMV but increased PVN^{CRF} projections. Whole cell patch clamp recordings of DMV neurons demonstrated that, following pHFD, PVN^{CRF} projections are tonically active, altering GABAergic inputs to DMV neurons. Lastly, blocking DMV CRF receptors in pHFD rats restored the appropriate gastric response to brainstem oxytocin application. Taken together, these results indicate that pHFD exposure leads to an upregulating of CRF inputs to the DMV, resulting in tonic CRF activation on the system and altering gastric motility. These results suggest that pHFD exposure leads gastric dysmotility, leading to a maladaptive gastric stress response and reduced stress resiliency.

Materials and Methods

Ethical Approval

All experiments were performed with the approval of the Penn State University College of Medicine Institution Animal Care and Use Committee, and in accordance with the National Institute of Health regulations. Reporting of animal experiments conforms to the principles and regulations for animal experiment reporting and ethics and conforms with the ARRIVE guidelines.

Animals

To generate the pHFD model, pregnant Sprague Dawley rats (N=8/diet) were placed on either a control (fat, protein, and carbohydrate content 14:27:59% kcal; Purina Mills, Gray Summit, MO) or high fat (60:20:20% kcal; Envigo TD.06414) diet on embryonic day 13 (E13). This date was chosen to start the high fat diet exposure *in utero* when vagal neurocircuits begin to develop[1, 2] as well as to avoid induction of obesity in the dams. Dams remained on their respective diets through gestation and lactation. Litters were restricted to 12 pups after birth, and rat pups were weaned on postnatal day 21 (P21) onto their corresponding diets. Male rats were left to age until at least P28 before starting *in vitro* electrophysiology experiments, and until P42 for *in vivo* gastric motility recordings, gastric emptying assays, and neuronal tracing experiments. Rats were given access to food and water *ad libitum*.

In addition to the two diet groups, subsets of rats were also exposed to an acute stressor prior to gastric motility, gastric emptying, and electrophysiology experiments. These rats underwent a restraint stress which consisted of placing the rat in a cylinder (Plas-Labs; 544-RR) for 2hr ensuring their movement was restricted but caused no other physical duress. Fecal pellet output was then counted and rats were used immediately in subsequent experiments.

Neuronal Tracing experiments

At P46, 8 male rats (n=4/diet from at least 2 different litters) were used for neuronal tracing experiments as described previously[3]. Briefly, rats received an injection of dexamethasone (Sigma D-6645; 1 mg/kg s.c.) to reduce cerebellar swelling. Then, rats were anesthetized using

a rodent cocktail (ketamine, xylazine, and acepromazine (80 mg/kg, 1.6 mg/kg, and 5 mg/kg, respectively, i.p.) and allowed to reach a deep plane of anesthesia, indicated by loss of the foot-pinch reflex. Rats were placed on a homeothermic heating blanket to maintain body temperature of 37C, then positioned on a stereotaxic frame (Kopf Instruments, Tujunga CA) and the dorsal brainstem was exposed via blunt dissection. Following removal of the meningeal membrane, the vagal trigone was exposed. Then, Cholera Toxin B (CTB; List Labs, Campbell, CA, 0.5% in distilled water) was injected into the left and right DMV at the coordinates (in mm) +0.2 to 0.3 rostro-caudal from calamus scriptorus, +/- 0.2 to 0.3 medio-lateral from the midline, and -0.6 to 0.65 dorsal-ventral from the surface of the brainstem. The CTB was microinjected in 60nl volumes using a Picospritzer (Toohey Co., Fairfield NJ). Two injections (medial and lateral) were made in the left and right DVC at each rostro-caudal level, and the pipette left in place for 5min between injections. The overlying musculature and skin were sutured (5/0 Vicryl suture) and rats returned to their home cages. Rats were given carprofen (Zoetis; 1 mg/kg, s.c.) for pain management peri-operatively, and for 3-5 days after surgery. After 12 days, the rats were anesthetized (Inactin, Sigma T133, 150mg/kg i.p.) and, once a deep plane of anesthesia was reached, perfused transcardially using 0.9% saline solution followed by 4% paraformaldehyde in PBS. Fixed brains were dissected, then postfixed and cryoprotected for 3 days in a 4% paraformaldehyde + 20% sucrose solution. 50 micron-thick sections of hypothalamic and brainstem tissue were collected using a freezing microtome, capturing the PVN and DVC, respectively. Tissue sections were kept at -20 C in a long-term storage buffer (50% 0.1M phosphate buffer:25% sucrose:25%ethylene glycol) until used for fluorescent immunohistochemistry.

Brainstem slices were used to visualize oxytocinergic fibers in the DVC as described previously[3]. Briefly, free floating slices were washed 3 times in PBS (0.1M), followed by incubation in immunobuffer (3 ml Triton in 1L TRIS-PBS) for 1 hour. Then, slices were incubated in donkey serum (1% in immunobuffer; Abcam ab7475) for 1 hour to block nonselective binding. Slices were then incubated in goat anti-choline acetyl transferase (Millipore ab144p (ChAT); 1:1000) and rabbit anti-oxytocin (Sigma-Millipore ab911 (OXT); 1:500) primary antibodies for 3 days at room temperature. Following repeated washing in PBS (0.1M) slices were incubated in donkey anti-goat IgG H&L (Alex Fluor 488; 1:500) and donkey anti-rabbit IgG H&L (Alexa Fluor 568; 1:500) secondary antibodies overnight at room temperature. Hypothalamic slides were visualized using the same protocol using goat anti-cholera toxin B (List Labs 703 (CTB): 1:1000) and either rabbit anti-oxytocin (1:500) or guinea pig anti-corticotropin releasing factor (Abcam

ab272391 (CRF); 1:250) primary antibodies. After washing in PBS, brain slices were mounted on subbed slides (Superfrost Microscope Slides; Fisher Scientific) and cover-slipped using Fluoromount-G (Invitrogen).

Immunohistochemical measurements

Immunohistochemical tissues were examined using a Zeiss LSM 900 compact microscope. Confocal images throughout the depth of the 50 micron-thick slices were taken at 20X magnification and compiled to a z-stack. An image was taken of each side (left and right) of the DVC and the PVN.

Sequential scanning and adjustment of the appropriate High-Low settings allowed for optimal intensities. All images were taken at the same settings to allow for direct comparison between samples. The intensity of OXT and CRF fibers within the DMV or NTS was analyzed using ImageJ software (https://imagej.nih.gov/ij/). To determine the intensity of fibers, the DMV or NTS were outlined and background staining was normalized, then mean pixel intensity was calculated. Mean pixel density was calculated in both the left and right side. For each rat, an image was at the intermediate (Bregma -13.9mm) level of the brainstem. The number of CRF+, OXT+, and CTB+ neurons was counted using ImageJ software. Cell counts were made in both the left and right side PVN and throughout the length of the PVN, noted as poster, intermediate, or anterior based on anatomical landmarks. Co-localization of signal was identified by cells with white fluorescence and counted as such.

Protein measurement

Male rats from both diet groups and stress conditions (n=10/group) were selected randomly for serum extraction. Rats were anesthetized with Isoflurane (5% in air) and, once a deep plane of anesthesia was induced, a cardiac puncture was performed to obtain truncal blood samples. The blood was left to clot for approximately 60min at room temperature before serum was extracted after centrifugation (Eppendorf Centrifuge 5417R; 1,500 rpm for 4 minutes). Samples were stored at -20°C until use. Serum samples were run in duplicate using the Enzo Corticosterone ELISA kit (Farmingdale, NY, USA) at a 1:40 dilution (26.99 pg ml^{-1} sensitivity). The optical density of the samples was measured using a SpectraMax 340PC 384 plate reader

interfaced with SoftMaxPRO software at 405nm. The calibrator concentration curve was calculated using an online freely-available immunoassay software package utilizing a 4-parameter logistic curve fitting program (https://www.myassays.com), and unknown concentrations were determined via interpolation.

Adult male rats from both diet groups were randomly selected for cerebrospinal fluid (CSF) extraction. Rats were anesthetized with Inactin (Sigma T133; 150mg/kg i.p.). Once anesthetized deeply (loss of foot-pinch withdrawal reflex), rats were placed on a stereotaxic frame and the dorsal brainstem was exposed via blunt dissection. At this time, 100-200 µL of CSF were extracted using a pipette over a period of approximately 5 minutes. Directly after, the sample was spun down using a centrifuge (Eppendorf Centrifuge 5417R; 1,500 rpm for 4 minutes) and the freshly separated CSF was stored at -80°C until use. CSF samples were run using the Enzo Oxytocin ELISA kit (Farmingdale, NY, USA) and according to manufacture instructions were run neat (15.0 pg ml^{-1} sensitivity). The optical density of the samples was measured using a SpectraMax 340PC 384 plate reader interfaced with SoftMaxPRO software at 405nm. The calibrator concentration curve was calculated using an online freely-available immunoassay software package utilizing a 4-parameter logistic curve fitting program (https://www.myassays.com), and unknown concentrations were determined via interpolation.

Electrophysiology

Whole cell patch clamp electrophysiological recordings were made from rats that were at least 28 days old. Rats in the stressed groups received the 2 hours RS immediately prior to euthanasia. Rats were anesthetized with isoflurane (5% in air) before euthanized via administration of a bilateral pneumothorax. The brainstem was removed and sliced as previously described[4]. Briefly, the brainstems were excised quickly and mounted on a vibratome and submerged rapidly in cold (4°C) oxygenated Krebs' solution. The brainstems were sliced into 300µm sections and placed in warm (30°C) oxygenated Krebs solution for 90min before recording. For each recording, brainstem slice was placed in the perfusion chamber on the stage of a Nikon E600FN microscope. Slices were perfused with warmed Krebs solution (in mM: 126 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, and 10 D-glucose kept at pH 7.4 by bubbling with 95% O2-5% CO2) maintained at 32°C at a rate of 2.0 –2.5 ml/min. DMV neurons were identified by their size and relative location to the dorsal NTS and ventral hypoglossal nucleus. Electrophysiological recordings of DMV neurons were made under bright-

field illumination using 2- to 4-MΩ patch pipettes filled with a potassium chloride intracellular solution (in mM: 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 2 NaATP, and 0.25 NaGTP adjusted to pH 7.4) and a single electrode voltage-clamp amplifier (Axopatch 200B; Molecular Devices). Data were filtered at 2 kHz, digitized via a Digidata 1440 Interface, and stored an analyzed on a PC with pClamp 10 software (Molecular Devices). Recordings with a series resistance of 20MΩ were eliminated from the study.

Miniature inhibitory postsynaptic currents (mIPSCs) were measured in the voltage-clamp configuration, holding the cell at -50 mV. mIPSC's were isolated by perfusing the cell with tetrodotoxin (TTX; 1µM) and kynurenic acid (KynA; 1 mM), a glutamatergic ionotropic receptor antagonist to isolate inhibitory action potential-independent neurotransmitter release. All drugs were made up in fresh Krebs solution on the day of the experiment. Baseline recordings were taken after at least 5min of TTX + KynA application, followed by application of either OXT(Bachem 4016373; 100 nM) or the CRF antagaonist, astressin (Bachem 4026664; 1 µM). Miniature current characteristics, including changes in amplitude and frequency, were assessed using Mini Analysis software.

Gastric motility recordings

A subset of 18 rats (n=9/diet from at least 2 litters) were randomly chosen to undergo gastric motility recordings as done previously[4]. Briefly, rats were fasted for 18 hours before experimentation with water available ad libitum. Rats were anesthetized with Inactin (Sigma T133; 150mg/kg intraperitoneal) and a tracheotomy was performed. An incision was then made along the midline of the thoracic cavity and the ventral stomach was isolated. A miniaturized strain gauge (MSR Neurobiology, Roaring spring, PA) was fixed to the corpus of the stomach, running parallel to the border between corpus and fundus. Rats were placed on a stereotaxic frame (Kopf Instruments, Tujunga, CA) and the dorsal brainstem was exposed via blunt dissection. The meningeal membrane was then removed and the vagal trigone exposed. Rats were then left to rest for at least 1 hour for surgical recovery prior to experimentation. Recordings of the corpus contractile activity was filtered, amplified (QuantaMetrics EXP CLSG-2), then visualized using Axoscope software (Molecular Devices). To ensure proper placement of the microinjection pipette, bicuculline (BIC; 25pmols/60nl) was first injected into the left DMV at coordinates (in mm) +0.2 to 0.3 rostro-caudal from calamus scriptorus, - 0.2 to 0.3 medio-lateral from the midline, and -0.6 to 0.65 dorsal-ventral from the surface of the brainstem (60nl

over 1min). Once an increase in gastric tone and motility was observed following BIC injection, these coordinates were used for subsequent injections.

Baseline corpus motility was recorded for at least 5 minutes before oxytocin (OXT; 150pmols/60nl) was microinjected. Changes to gastric tone and motility were recorded until the trace returned to baseline. After at least 1-hour recovery, astressin (10µg/2µl) was microinjected onto the floor of the 4th ventricle, with two doses given spaced 5 minutes apart. Then, the response to repeat injection of OXT was assessed. Changes to corpus tone and motility were measured as described previously[5]. At the completion of the experiment, the rat was perfused with 0.9% saline followed by 4% PFA and the the brainstem was removed for post-hoc verification of the injection site.

Gastric Emptying

Male rats (n=8/diet from at least 2 litters) were randomly selected to undergo the ^{13}C gastric emptying assay to determine the emptying rate of solid food as described previously[4]. Briefly, rats were first acclimated to the testing procedure by being placed in their respective holding chamber (VetEquip) for approximately 3 hrs/d for 3 consecutive days. Whilst in the chamber, they were fed 1g of pancake (De Waffelbakkers Buttermilk Pancakes). After the 3rd acclimation day, rats were fasted overnight, with *ad libitum* access to water. On test day, rats were placed in their holding chamber for 1hr to allow the carbon dioxide isotope analyzer (Los Gatos Research) to equilibrate. Then, each rat was fed 1g of a pancake that contained 4 µl of [^{13}C]-octanoic acid (Cambridge Isotope Laboratories, Inc). After visually confirming the rats had eaten the entire pancake (in less than 5min or they were omitted from the study), the rats were left to remain in their chamber for at least 4hr. During this time, air from each of the testing chambers was automatically sampled, one at a time, at 30s intervals. At the completion of the gastric emptying study, rats were returned to their cages with full access to food and water. Rats were re-acclimated to their testing chambers 3-4d after testing, and gastric emptying was repeated weekly for 3 baseline measurements. The following week, rats underwent a 2hr restraint stress protocol before being placed in the chambers for data collection.

To analyze these data, for each 30s period of data, the first 10s were removed to ensure a complete flush of the previous air sample from the tubing. The remaining 20s of data were averaged for a single data point at a given time. The concentration of $^{13}CO_2$ was then calculated

and expressed as a change over the baseline. The change of concentration of $^{13}CO_2$ vs time (t) was fitted by a non-linear regression curve using SigmaPlot with the following equation ($y = at^b e^{-ct}$) where y is the percentage of the ^{13}C excretion in the breath per hour (t) and a, b, and c are regression constants estimated for each breath vs. time curve. The gastric half emptying time ($T_{1/2}$) was calculated from a numerical integration procedure using an inverse gamma function. The three baseline $T_{1/2}$ values for each animal was averaged.

Statistical analyses

For immunofluorescent results, an unpaired two-tailed t-test was used to investigate the difference between diet groups (control and pHFD) and their mean fluorescence intensity of fibers or total cell counts per section. This was done at each anatomical level (anterior, intermediate, and posterior) for PVN cell counts.

For protein measurement in CSF samples, an unpaired two-tailed t-test was used to compare the calculated protein concentration (pg/mL) per sample in the two diet groups (control and pHFD). For protein measurement in serum samples, a one-way ANOVA with a post-hoc Bonferroni test was used to compare the 4 experimental groups (control, pHFD, control + stress, and pHFD + stress).

Gastric emptying is expressed as time (min) or as a change from baseline (in mins), with baseline defined as the average half-emptying (T1/2) time of 3 baseline measurements. Results were analyzed using an unpaired two-tailed t-test to identify differences between diet groups for basal rates and as change of baseline after the stressor.

For *in vivo* recordings of gastric motility and tone, each rat served as its own control and the gastric responses were assessed before and after drug application using a paired t-test. Inter-group comparisons were made using an unpaired t-test. Bicuculline was used to confirm an accurate microinjection site, and only those rats that demonstrated an increase in gastric tone and motility in response to Bicuculline microinjection were included in the statistical analyses. A physiologically significant response was considered a change in motility of at least >25%.

For electrophysiological results, the response of any neuron was assessed before and after drug application using a paired t-test, such that each neuron served as its own control. The magnitude of response to an individual drug was compared across groups using a one-way

ANOVA with post-hoc Bonferroni test. Neurons were categorized as responsive or non-responsive based on their pharmacological phenotype where a responsive neuron was one in which a drug induced a change in mIPSC property by greater than 25% magnitude. All electrophysiological recordings were replicated in at least 3 rats.

References

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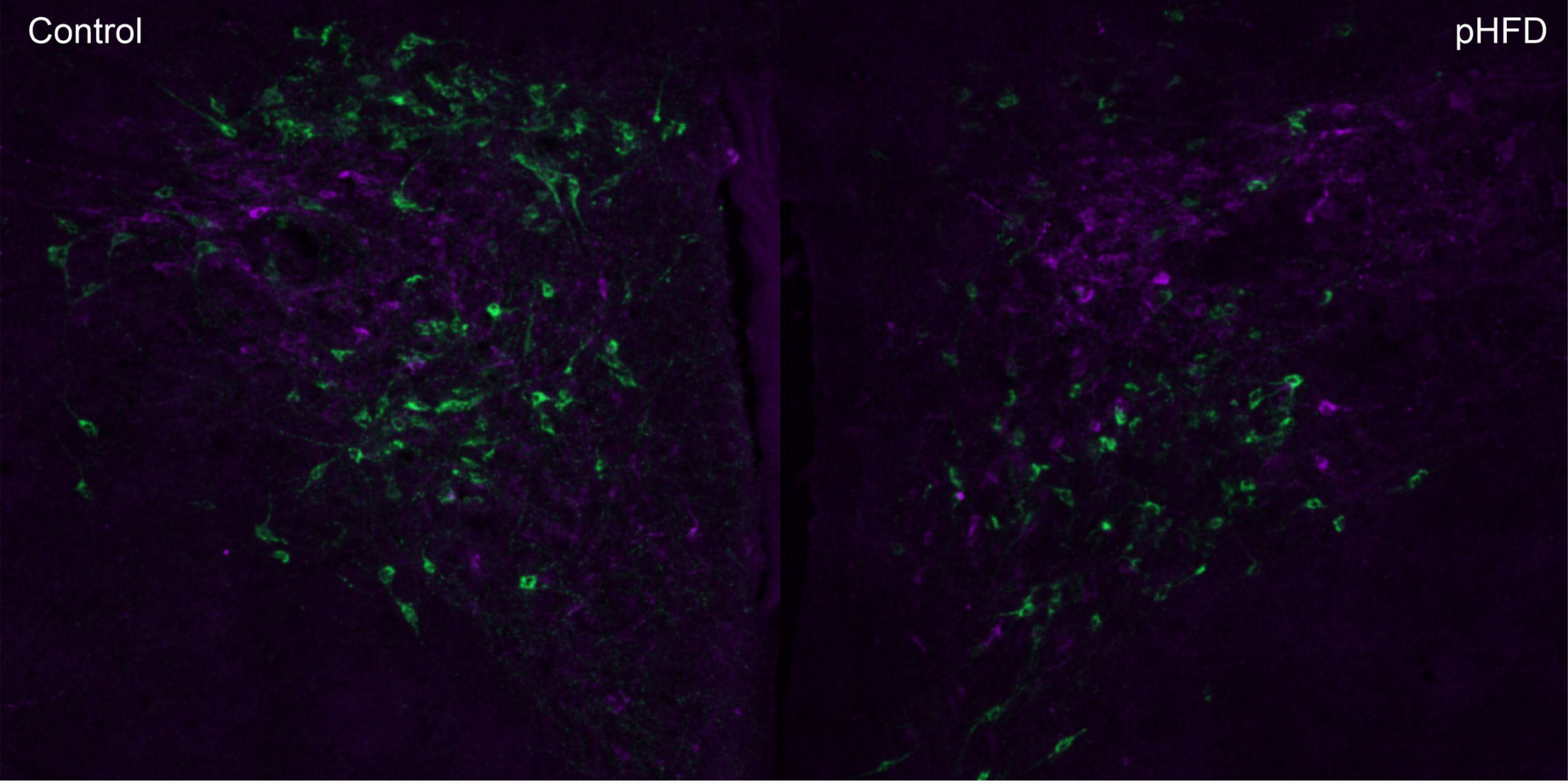
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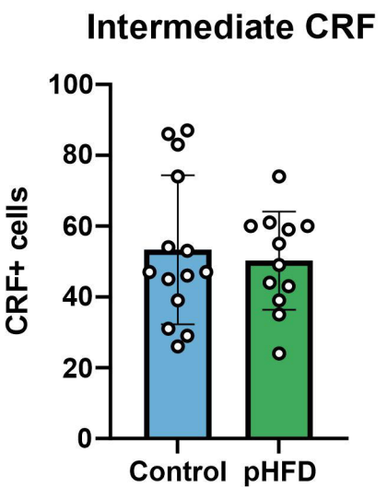
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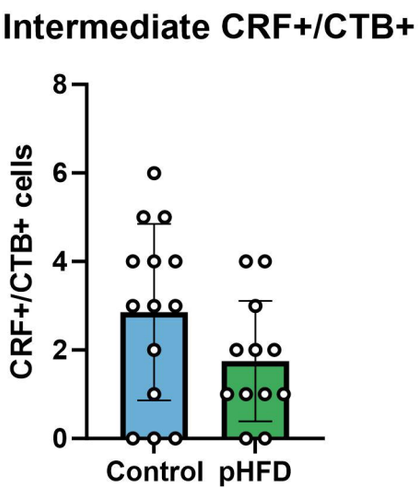
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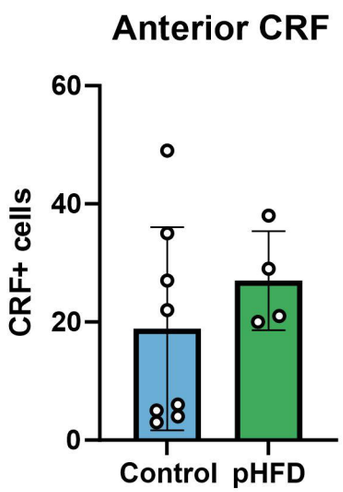
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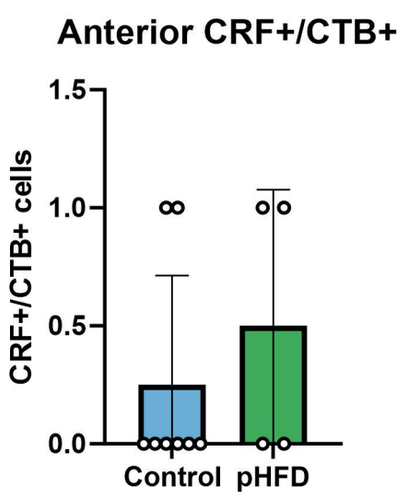
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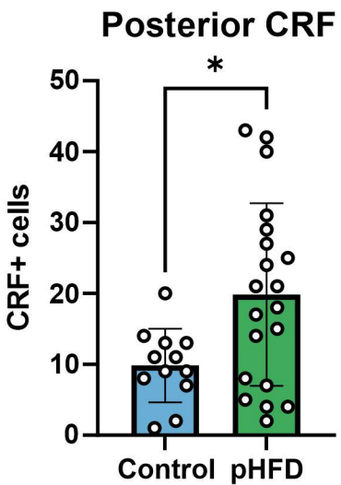
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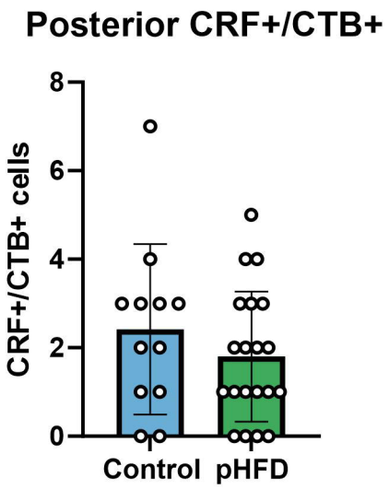
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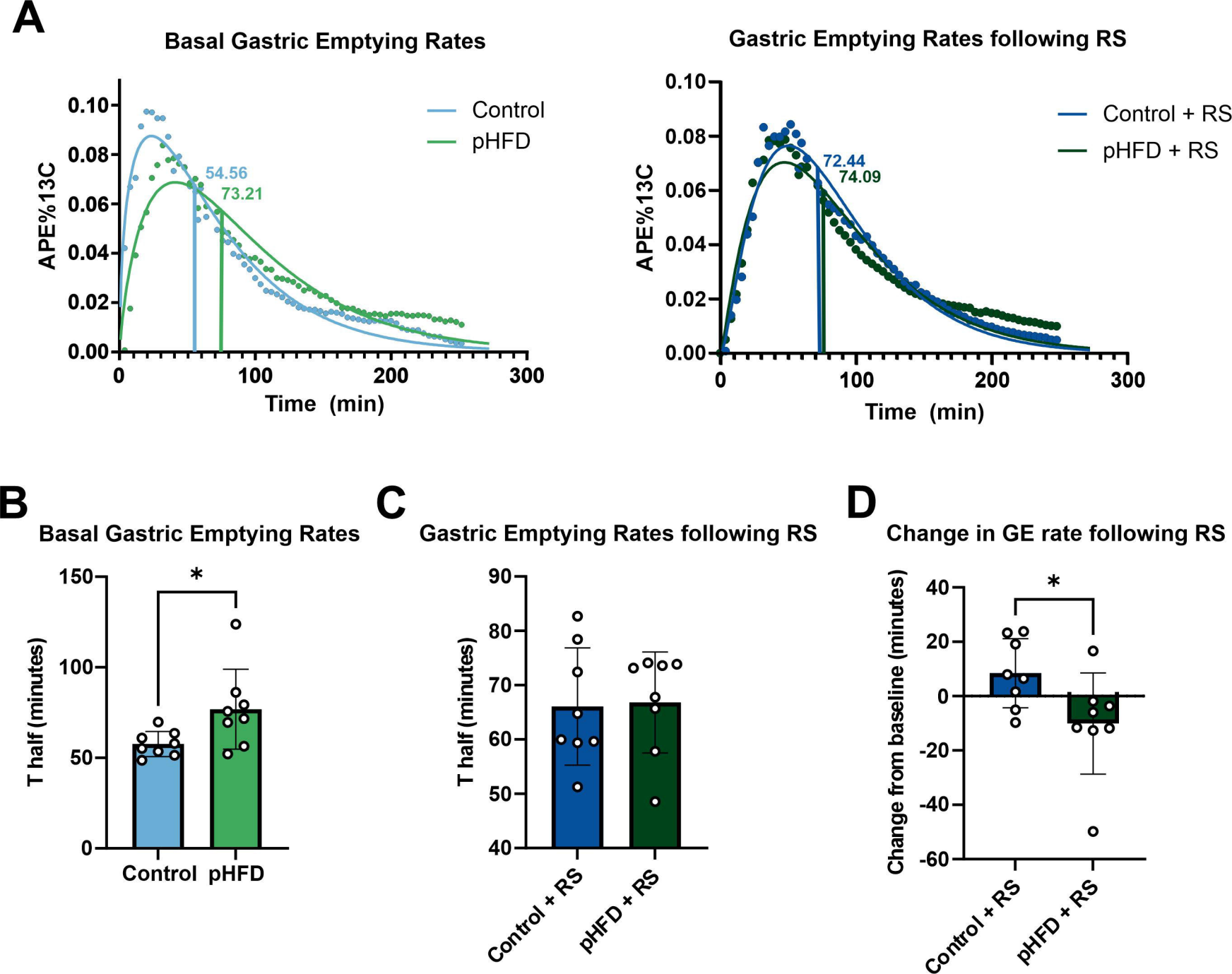
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Time (min)
B
Basal Gastric Emptying Rates
T half (minutes)
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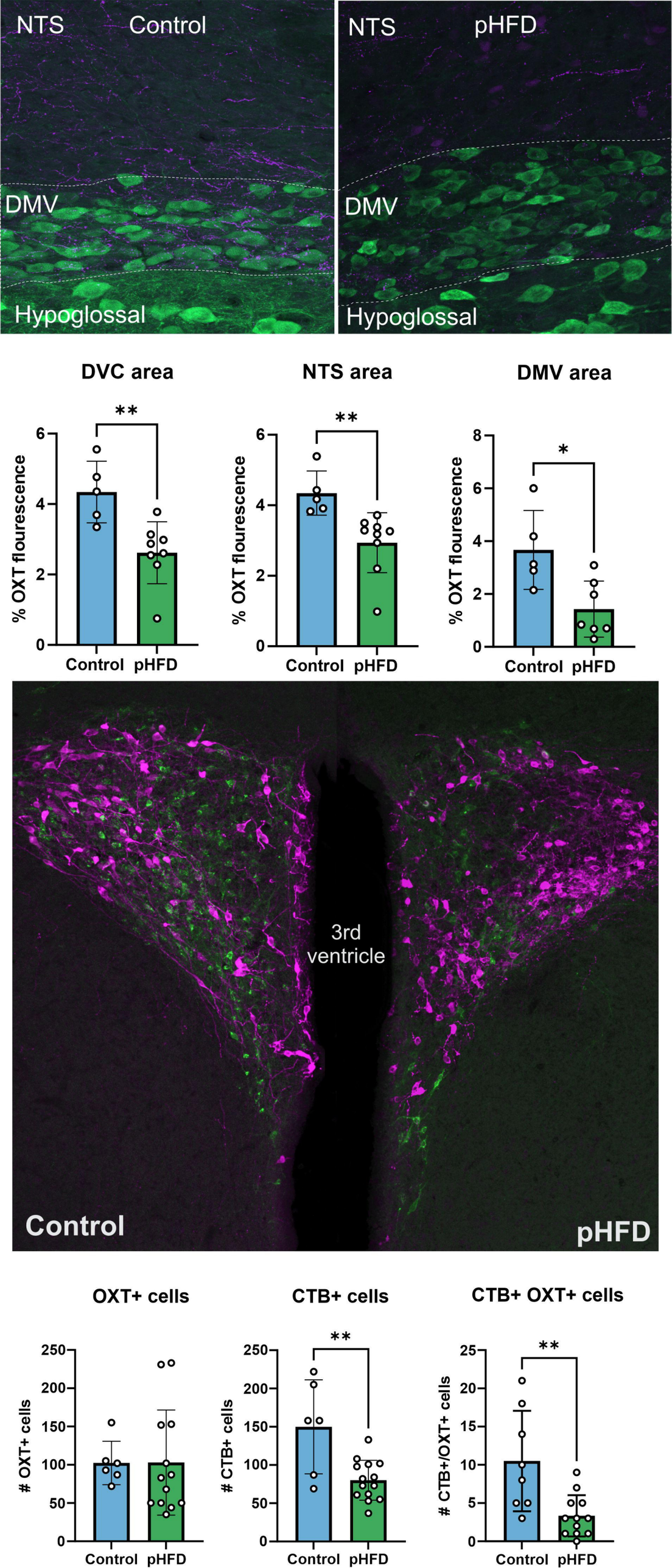
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