The Pennsylvania State University

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EXPOSURE TO A HIGH FAT DIET DURING THE PERINATAL PERIOD ALTERS THE EFFECTS OF CENTRALLY APPLIED $GABA_A$ RECEPTOR ANTAGONISTS ON GASTRIC MOTILITY AND TONE IN RATS

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Abstract:

Parasympathetic motor innervation to the stomach originates from preganglionic motor neurons of the dorsal motor nucleus of the vagus (DMV). Studies have demonstrated that an ongoing GABAergic input to DMV neurons plays a major role in regulating vagal efferent output from the DMV. The aim of this study was to investigate whether a perinatal high fat diet (HFD) affects the GABAergic regulation of vagal efferent output to the stomach. Male Sprague Dawley rats were fed a HFD (60% kcal from fat) from embryonic day 13 until experimentation at 12 weeks of age. Miniaturized strain gauges sutured to the ventral surface of the gastric corpus were used to record gastric motility. The $GABA_A$ receptor antagonists, bicuculline methiodide (BMI; 25, 50, 100pmol) and gabazine (GBZ; 0.78, 3.125, 6.25pmol) were microinjected (60nl) into the dorsal vagal complex (DVC) and the resulting increase in gastric tone was measured at 10 time points after injection (1-10 min) and averaged. A motility index was used to identify changes in motility response and was measured over two five minute periods before and after drug microinjection. Gastric tone responses were represented as a percentage of maximal stomach contraction induced by i.v. injection of the non-selective muscarinic receptor agonist, bethanecol (50µg/kg; 1ml), which itself was unaffected by diet (19.5±3.0g in control vs 25.5±3.6g in HFD; $P>0.05$).

In control rats, BMI induced a dose-dependent increase in gastric tone (2.2±0.34%, 5.4±1.1% and 9.5±3.5% at 25, 50 and 100pmol, n=6, 7, 3, respectively, $P<0.05$ for each) and showed a trend towards a dose-dependent increase following the perinatal HFD (5.3±2.2%, 8±4.1% and 9.7±4.7% at 25, 50 and 100pmol, n= 4,5,7, respectively, $P<0.06$). Similarly, the effect of GBZ to increase gastric tone showed a trend towards dose dependency in control animals (3.65±0.95%, 7.02±2.61% and 14.78±7.8% at 0.78, 3.125 and 6.25pmol, n=5,7,5 respectively). These responses were attenuated in the HFD (4.17±0.8%, 3.3±0.6% and 3.4±0.9% at 0.78, 3.125 and 6.25pmol, n=6,6,4, respectively). Microinjection of BMI increased gastric motility in control and HFD rats with a decrease in magnitude between 25pmol (54.8±9.3%) and 100pmol(26.7±16.5).

In summary, our data suggest that exposure to a HFD during the perinatal period alters GABAergic activity within brainstem and in the neural circuits controlling gastric functions, which may contribute to the dysregulation of homeostatic reflexes, including appetite and metabolic regulation.

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Abbreviations:

5-hydroxytryptamine: 5-HT; Serotonin

Acetylcholine: Ach

Area Postrema: AP

Bicuculline Methiodide: BMI

Cholecystokinin: CCK

Dorsal Motor Nucleus of the Vagus: DMV

Dorsal Vagal Complex: DVC

Gabazine: GBZ

γ-Aminobutyric acid: GABA

γ-Aminobutyric acid type A Receptor: $GABA_A$

Glucagon Like Peptide 1: GLP-1

High Fat Diet: HFD

Inhibitory Post Synaptic Current: IPSC

Gram: g

Milligram: mg

Nitric Oxide: NO

Nucleus Ambiguus: NA

Nucleus of the Solitary Tract: NTS

Pancreatic Polypeptide: PP

Phosphate Buffered saline: PBS

Tractus Solitarius: TS

Vasoactive Intestinal Peptide: VIP

Chapter 1: Background of GABA Circuitry and Obesity

1.1 Vagal Regulation of Gastric Functions

The functions of the gastrointestinal tract are numerous and transient, regulating gastric emptying, motility and secretion. The primary source of innervation to these sub-diaphragmatic organs and viscera is supplied by mixed sensory and motor parasympathetic fibers of the vagus nerve. The motor portion of the vagus arises primarily from neurons within the dorsal motor nucleus of the vagus (DMV) and nucleus ambiguus (NA), which receive various afferent inputs from both thoracic and visceral regions. These afferent fibers originate from bipolar neurons whose cell bodies are located in the jugular and nodose ganglia. Subsequently, the central terminals of these bipolar neurons enter the brainstem via the tractus solitarius (TS) and terminate on neurons of the nucleus of the tractus solitarius (NTS) using glutamate as their primary neurotransmitter (6; 21). Afferent vagal fibers originating in the stomach transduce sensory mechanical and chemical signals via C and Aδ fibers (4). A majority of these fibers are polymodal since they are sensitive to multiple types of stimuli such as chemical neurotransmitters and neuromodulators like 5-hydroxytryptamine (5-HT; serotonin) and cholecystokinin (CCK) as well as mechanical stimuli (27; 39). After passing through the bipolar nodose ganglia, these afferent fibers synapse onto second order neurons at the level of the NTS (6)**.** It is important to note that further afferent signals may be relayed from the GI tract to the lower brainstem via the area postrema (AP) and other regions. For example, pancreatic polypeptide (PP) is released from the pancreas into circulation and may act at the AP while glucagon like peptide (GLP-1), is released from the small intestine and may act on or within the dorsal vagal complex (DVC) to alter visceral functions (25; 28).

The NTS serves as a primary integration center for gastrointestinal, cardiac and respiratory functions. Afferent vagal fibers arising primarily from the stomach project to and synapse at subnuclei medialis, gelatinosus and commisuralis(1; 28; 38). The NTS integrates this sensory information along with inputs it receives from other brainstem and higher CNS centers, and then relays this information to the DMV, among other areas (12). These NTS-DMV projections can be either excitatory or inhibitory principally through glutamatergic or GABAergic connections, respectively (37).

The DMV is mainly comprised of pre-ganglionic parasympathetic motor neurons which project to and innervate the subdiaphragmatic viscera (1; 3; 11). Through these parasympathetic motor neurons, the DMV can induce both excitatory and inhibitory effects within the subdiaphragmatic viscera. While all DMV neurons are cholinergic and excite postganglionic neurons in their target organ of interest via actions on nicotinic acetylcholine (Ach) receptors, two postganglionic pathways exist; an excitatory cholinergic pathway and an inhibitory non-adrenergic, non-cholinergic (NANC) pathway. The excitatory pathway uses Ach to activate muscarinic receptors on the gastric smooth muscle inducing a contraction and an increase in muscle tone and motility. The inhibitory NANC pathway releases nitric oxide (NO) and/or vasoactive intestinal peptide (VIP) to induce gastric relaxation. Inhibition of gastric tone and motility therefore could arise from either activation of the inhibitory NANC pathway or inhibition of the tonically activated excitatory pathway. A further layer of complexity arises since the preganglionic neurons regulating these pathways may be under excitatory (Glutamate,

Glu) or inhibitory (GABA) influence from the NTS.

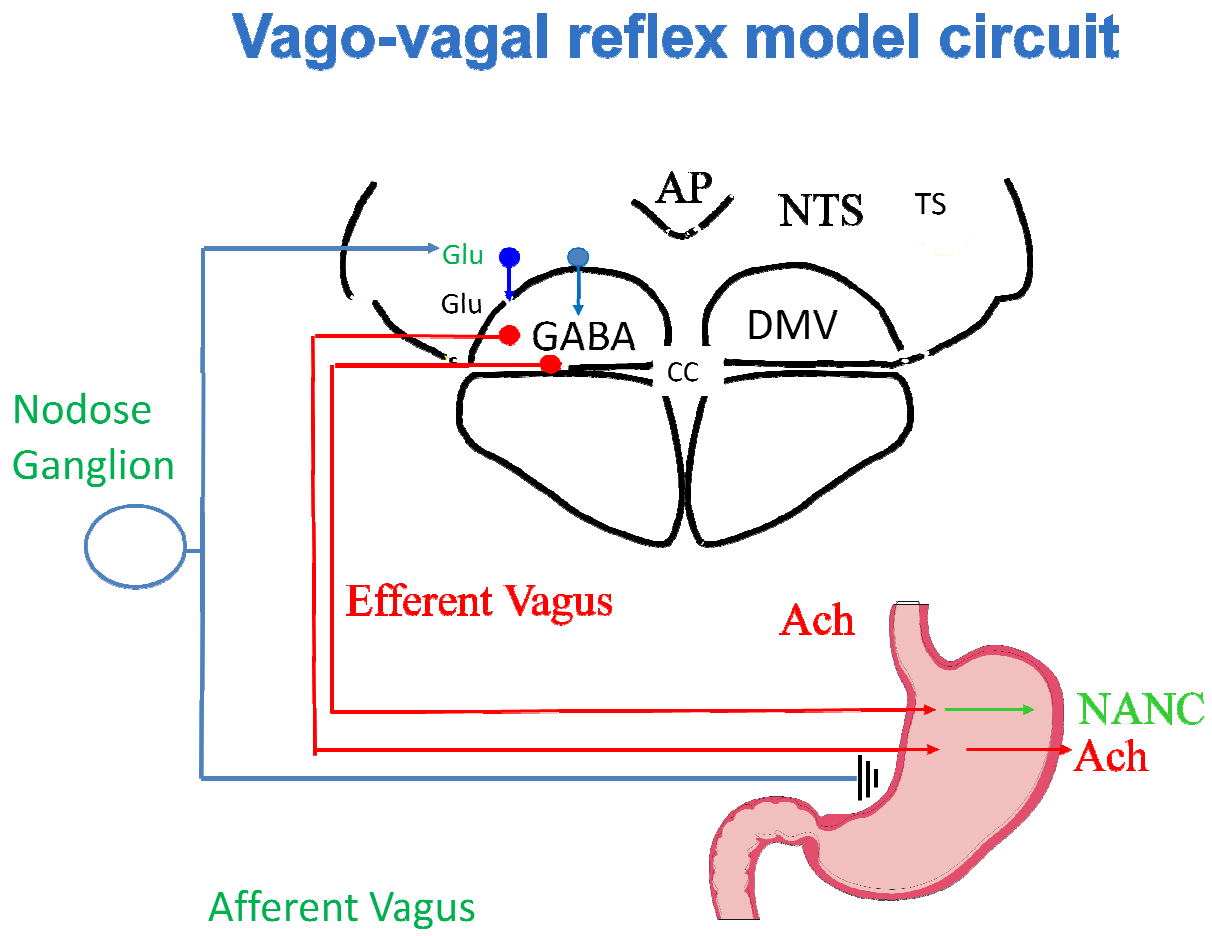

Figure 1.0: Schematic diagram of a gastric vago-vagal reflex circuit. NTS neurons innervate DMV preganglionic neurons using predominately glutamate and GABA as neurotransmitters. All DMV neurons are cholinergic and release acetylcholine to activate nicotinic receptors present on the postganglionic neurons within the visceral target organ, in this instance, the stomach. Postganglionic neurons form one of two pathways, either an excitatory cholinergic pathway, that increase gastric tone and motility via release of acetylcholine to activate muscarininc receptors present on the gastric smooth muscle, or an inhibitory NANC pathway that induces gastric relaxation via release of NO or VIP.

This vago-vagal reflex works to control and regulate gastric motility and tone once activated, the cholinergic pathway increases gastric tone and motility while activation of the NANC pathway decreases tone and motility. DMV neurons regulating gastric functions are under a tonic GABAergic influence from the NTS (35). Inhibition of inhibitory inputs (disinhibition) onto DMV neurons involved in the excitatory cholinergic pathway will increase gastric tone and motility. In this way the excitatory and inhibitory currents acting upon the stomach can finely tune and modulate gastric function.

1.2 GABAergic Transmission

Neurotransmission between the NTS and DMV uses glutamate, GABA and norepinephrine. GABAergic transmission plays a major role in the NTS-DMV synapse (8; 35). γ-Aminobutyric acid (GABA) is one of the principal inhibitory neurotransmitters of the mammalian CNS. GABA effects occur through $GABA_A$ /$GABA_B$ and/or $GABA_C$ receptors. The primary GABA receptor of focus in this study is the ionotropic $GABA_A$ receptor which opens an associated ligand gated chloride ion channel allowing an outward current to hyperpolarize the membrane potential of the target cell.

$GABA_A$ receptors are functionally and pharmacologically diverse and respond to various agonists and antagonists differently due to variations in receptor affinities and efficacy in response to stimuli. They are heterogeneously comprised of a combination of 5 subunits: α, β, and γ. Specific subunits combinations can further confer different functionalities. For example, the $GABA_A$ receptor subunit α1 has been linked to sensitivity to the benzodiazepine, zolpidem. Benzodiazepines are used clinically to treat anxiety, depression, several psychiatric disorders, occasionally they are used as general anesthetics. Zolpidem has been shown to increase the

GABA current decay time (i.e. GABA current gets longer/slower) via interaction with the α1 subunit by increasing the receptors affinity for GABA and increasing the frequency at which the channel opens (13). However, other alpha subunits, specifically α2 & α3, have been linked to increasing receptor site deactivation and are associated with a prolonged GABA current when compared to α1 containing subunit receptors (19). In adult rats, there is a greater expression of α1 subunit containing receptors vs α2/3 subunits. It has been demonstrated that a developmental shift from α 2/3 to α1 subunit containing $GABA_A$ receptor subtypes occurs at approximately postnatal day 21 (17). In rats fed a high fat diet (HFD) during the perinatal period, it is possible that this developmental subunit shift does not occur to the same extent as in normal development**;** α 2/3 subunit shift to predominately α1 subunits will decrease the length of GABAergic currents, hence decreasing the length of GABA mediated inhibition (13; 19). As certain benzodiazepines have subunit specific affinity, it may be possible, in vivo, to establish that rats fed a HFD exhibit an alteration in $GABA_A$ receptor subunit composition. Changes in receptor subunit composition may further play a role in altering tonic and phasic GABA current control of the NTS-DMV synapse, ultimately affecting gastric functions. Unpublished data from Browning, suggest the possibility that an abnormal perinatal environment, i.e. HFD, alters GABAergic output to the DMV neurons innervating the stomach.

Figure 1.1: Traces from gastric-projecting DMV neurons voltage clamped at -50mV. GABAergic Inhibitory Post Synaptic Currents (IPSC) were evoked by electrical stimulation of the adjacent NTS. Note that, relative to IPSC's evoked in control DMV neurons (black), IPSCs in DMV neurons from rats fed a HFD during the perinatal period (red) had a much longer time-course decay. Figure and caption reprinted with permission from Dr. K.N. Browning (unpublished data).

1.3 Perinatal High Fat Diet alters GABA Circuitry

Obesity in adult life can develop as a result of environmental factors in utero (36). Human studies have suggested the possibility that adult onset obesity is correlated to an abnormally high level of nutrient exposure in utero (5). In this project we used a rat model to investigate the underlying developmental mechanisms that may pre-dispose an organism to obesity. Exposure to a HFD during the perinatal period has been shown to increase overall adiposity in male and female rat pups (36). In utero, synaptic differentiation begins at embryonic day 17 while vagal sensorimotor circuits appear at embryonic day 18 (33; 42). Post birth, the vagal neural system remains plastic (until ~P21) and numerous changes in neurotransmitters, neurotransmitter receptors and their subsequent physiological outcomes, may still occur (14). Obesity may result directly from abnormal postnatal development involving various changes in the still plastic neural system of the organism, in fact it has been demonstrated that GABA receptor synaptic densities vacillate transiently during postnatal development (40). For example, GABAergic axon terminals increase in number during postnatal week one (14). Furthermore, GABAergic axon terminal reorganization and placement of GABAergic boutons along with similar developmental changes occurs beginning at postnatal day 10 (31; 34; 41). Therefore, it is plausible that environmental factors during this perinatal period may adversely affect the development of vagal circuitry (unpublished, Browning, Fig 1.1).

Neurotransmitters such as GABA, can act at receptors at a diverse number of locations either at the synapse or extrasynaptically. The resulting responses are termed phasic or tonic responses, respectively. Synaptic/phasic $GABA_A$ receptors are located within the synapse; activation results in generation of an inhibitory post synaptic current. Synaptic/phasic transmissions are likened to "point to point" information communication, and result in the generation of an inhibitory response (24). These synaptic/phasic $GABA_A$ receptors have a different subunit composition in comparison with the extrasynaptic/tonic $GABA_A$ receptors. The synaptic receptors may be composed of α1 and α2 subunits usually in combination with a γ subunit (24).

Extrasynaptically or perisynaptically, GABA may diffuse away from the synaptic cleft activating extrasynaptic/tonic $GABA_A$ receptors (18). These extrasynaptic/tonic $GABA_A$ receptors usually, although not always, contain the δ subunit along with varying combinations of subunits α4 and/or α6 and β2 and/or β3 (2; 20; 23). The δ subunit confers an extreme level of GABA sensitivity to the extrasynaptic/tonic $GABA_A$ receptors not normally associated with synaptic/phasic $GABA_A$ receptors (22; 34). This makes these extrasynaptic/tonic receptors particularly proficient in responding to low concentration of GABA contained within the extrasynaptic space.

Current information suggests that not all extrasynaptic/tonic $GABA_A$ receptors are active at a given time (18). At low doses, the $GABA_A$ receptor antagonist gabazine (GBZ) inhibits preferentially the phasic GABA receptors, leaving the extrasynaptic/tonic receptors relatively unaffected (32). It has however, been demonstrated that at high concentrations GBZ antagonizes extrasynaptic receptors as well (10).

GABA primarily is released in an action potential dependent manner, however, there is long standing evidence indicating that small quantities of GABA are released into the synaptic/extrasynaptic space in an action-potential independent manner (32). It has long been known that BMI antagonizes both synaptic/phasic and extrasynaptic/tonic $GABA_A$ receptors And some studies suggest that BMI also blocks calcium channels (7; 20; 30). In summary, BMI blocks both synaptic/phasic and extrasynaptic/tonic $GABA_A$ receptors while GBZ preferentially blocks the synaptic/phasic $GABA_A$ receptor thus, a comparison of the effects of these two antagonists can be used to determine the proportion of synaptic/phasic vs extrasynaptic/tonic $GABA_A$ receptor activation in a physiologically relevant setting (24).

Chapter 2: Specific Aims and Hypothesis

2.0 Specific Aim #1

Ongoing GABA input from the NTS to the DMV plays a major role in regulating gastric tone and motility. The tonic GABA current activates extrasynaptic $GABA_A$ receptors; phasic GABA currents activate synaptic $GABA_A$ receptors. Tonic and phasic GABA currents can be blocked with the $GABA_A$ receptor antagonist BMI which has similar affinity for both synaptic/phasic and extrasynaptic/tonic receptors. Conversely, GBZ has a much higher affinity for the synaptic/phasic $GABA_A$ receptors, leaving the extrasynaptic/tonic current unaffected. In this series of experiments we aim to elucidate the relative contribution of tonic and/or phasic GABA currents on gastric tone and motility. We predict that both BMI and GBZ microinjected into the dorsal vagal complex (DVC) will increase gastric tone and motility. If the stomach is mainly controlled by the extrasynaptic/tonic GABA currents, then BMI microinjection will increase gastric motility and tone to a greater extent than GBZ. However, if the stomach is predominantly under the control of phasic $GABA_A$ receptor generated current, GBZ will exert a greater or equal effect of BMI to increase gastric tone and motility. If the stomach is under the control of both phasic and tonic currents equally, we hypothesize that the increase in gastric motility and tone in response to BMI microinjection would be greater than that in response to GBZ. These experiments will be conducted in vivo in anesthetized rats fed normal chow diet and through microinjection of BMI or GBZ into the DVC. Gastric motility and tone will be measured through data collected by a miniaturized strain gauge sutured to the gastric corpus to measure tension produced by circular smooth muscle contraction/relaxation on the stomach. DVC Microinjection of either BMI or GBZ, will illustrate the effects ongoing activation of phasic and tonic GABAA receptors exerts upon vagal efferent motoneurons controlling gastric functions.

2.1: Specific Aim #2

It has been shown that pups exposed to a perinatal HFD are more likely to develop obesity later in life (36). A perinatal HFD appears to induce a change in GABAergic currents in the DMV (Browning unpublished), possibly due to alteration in the ratio of tonic vs. phasic $GABA_A$ receptor activation in the DMV. During development the GABA system is highly plastic. It is possible that environmental factors, i.e. exposure to a HFD, will induce reorganization of this plastic system. We will test the hypothesis that in rats fed a HFD in the perinatal period there will be a reorganization of GABAergic currents through changes in synaptic/phasic $GABA_A$ receptors. We predict that these plastic changes will induce two effects; A) in HFD rats, microinjection of GBZ will exert a greater effect on gastric motility and tone in comparison to BMI, due to the preferential action of GBZ at synaptic/phasic $GABA_A$ receptors. This observation may be most visible at the lower doses of GBZ as at higher doses GBZ may begin to act extrasynaptically, B) In comparison to control animals, we expect that in HFD rats, microinjection of BMI or GBZ will induce a proportionally greater response due to an increased activation of the synaptic/phasic receptors regulating gastric motility and tone. These experiments will be conducted in vivo using rats fed a perinatal HFD and through microinjection of BMI and GBZ. Gastric motility and tone will be measured through data collected by a miniaturized strain gauge sutured to the gastric corpus, to measure tension produced by circular smooth muscle contraction/relaxation on the stomach. Microinjection (of either BMI or GBZ) into the DVC will illustrate the effects that alterations in the proportion of phasic vs tonic $GABA_A$ receptor activation has upon vagal efferent motoneurons controlling gastric function.

Chapter 3: Methodology

Methodologies described in this study have been based closely on and/or adopted from previously described methods (26). All experiments were performed using male Sprague-Dawley rats obtained from Charles River (Horsham, PA).

3.1 Rats, Anesthesia & Strain Gauge Placement

Pregnant dams were fed a HFD (60% kcal from fat; Harlan Teklad Diet 06414) starting at embryonic day 15. Control rats were fed standard chow. Pups received the same diet (control or HFD) starting from weaning (post-natal day 21) for 12 weeks prior to experimentation. Procedural methods for control and HFD rats were as follows:

Male Sprague Dawley rats were fasted overnight with ad libitum access to water. On the day of the experiment, rats were anesthetized using thiobutabarbital (Inactin;125 mg/kg control and 150mg/kg HFD); to produce an irreversible plane of anesthesia that does not compromise autonomic GI function (9). For control rats, anesthetic was given via intraperitoneal injection in the lower right quadrant. HFD rats received a slightly different anesthetic protocol involving induction with isoflurane (2.5-3% isoflurane, 600ml 0_2/min) followed by tapered Inactin infusion with simultaneous isoflurane withdrawal (See Section 3.5). To ensure proper depth of anesthesia, the foot pinch withdrawal reflex was tested. If required in control rats, isoflurane (2.5-3%, 600ml/min) was used to induce a suitable plane of anesthesia prior to surgery. Rats were then placed on a homoeothermic blanket control unit (38^0C) in a supine position to maintain body temperature homestasis and a 2-4 cm midline incision was made. A 6 x 8-mm strain gauge **(**RB Products, Madison, WI**)** was placed on the stomach aligned with the circular smooth muscle and

then sutured (4/0 silk suture) to the corpus of the stomach. The strain gauge lead extended out through the inferior portion of the abdominal laparotomy and the signal was amplified via Quanta Metrics EXP CLSG-2 and recorded with Axoscope software (Molecular Devices, Sunnyvale, California). After closing the abdominal laparotomy, a tracheal tube (7-8cm, PE10) was inserted and connected to a rodent ventilator (Harvard Apparatus Model 638, Holliston, MA) at a rate of 98 breaths per minute. The anterior aspect of the neck was shaved, the musculature was blunt dissected and a cannula (PE 50) was inserted through the internal jugular vein. The cannula was used for intravenous infusion of the muscarinic antagonist, bethanecol, used to induce maximal gastric contractility for verification of strain gauge placement. Rats were then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA).

3.2 Exposure of Fourth Ventricle Floor

The floor of the fourth ventricle was exposed through blunt dissection of the musculature and in removal of the overlaying pial and arachnoid membrane. Glass micropipettes with a tip diameter of 50μm (World Precision Instruments, Sarasota, FL) were pulled (Narishige, Japan, Model PE 21). Through the use of a micromanipulator (Kopf Instruments, Tujunga, CA), pipettes were placed into the DVC at 0.1-0.2 mm rostral to calamus scriptorius, 0.1-0.2mm mediolateral and 0.73 mm dorsoventral to the surface of the medulla, according to the atlas of Paxinos and Watson.

3.3 Drug Injections & Measurement of Gastric Tone

BMI or GBZ were microinjected at various doses (100, 50, 25pmol in 60nl PBS for BMI ; 6.125, 3.25, 0.78pmol in 60nl PBS for GBZ) after gastric tone/motility stabilized (~45 min) following surgery. Fluorescent microspheres (Fluoresbrite carboxy NYO; Polysciences, Warrington, PA, USA) were included in the injectate for post hoc verification of injection sites. The micropipette meniscus was identified via a monocular microscope attached to the stereotaxic apparatus. Multiple injections were made on the same rat at slightly different sites within the DVC (0.1-0.2 mm rostral to calamus scriptorius, 0.1-0.2mm mediolateral and 0.73 mm dorsoventral to the surface of the medulla) with an interval period of 60-120 minutes between injections. Baseline gastric tone and motility were determined from the average traces measured in 10min and 5 minutes, respectively, prior to drug injection. To measure gastric tone post injection, measurements were taken every minute for a period of ten minutes and averaged. Motility data were analyzed as a motility index (Sec 3.4). Gastric tone/motility values were compared to tone/motility prior to microinjection using a student's t-test with statistical significance set at $P<0.05$. The dose –dependent effects of BMI vs. GBZ were analyzed using a 2-way ANOVA with significance set at $P<0.05$. At the end of the experiment bethanecol (50μg/kg in 1ml) was given i.v. to determine maximal gastric contraction and strain gauge placement accuracy. Bethanecol is a non-selective muscarinic receptor agonist, which can induce maximal gastric contraction. Vehicle (PBS) was also microinjected within the DVC to determine the value of the pharmacological response. Microinjections of $GABA_A$ receptor antagonists and vehicle were also made in areas outside the DVC and the effects on gastric tone and motility were examined to determine the specificity of drug effects within the DVC.

3.4 In Vivo Motility Data Analysis

Strain gauges were calibrated with a 0.5g weight weekly. The following formula was used to calculate the motility index:

$$\text{Motility Index} = (N_1 x 1) + (N_2 x 2) + (N_3 x 4) + (N_4 x 8)$$

Where N is the number of motility peaks within a given time span. Each peak was weighted differently depending on the size of the response (in mV). Gastric motility responses were grouped as 25-50mg, 51-100mg, 101-200mg and signals above 201mg for N_1 through N_4 respectively. In each animal, a motility index was calculated before and after drug microinjection. These motility indices are referred to as the pre-injection and post injection motility indices, respectively.

3.5 Brainstem Removal, Slicing, Imaging & Statistical Analysis

At the end of the experiment rats were perfused transcardially with saline (5ml) and 4% paraformaldehyde in PBS. The brain stem was removed and post-fixed in a solution of 20% sucrose and 4% paraformaldehyde. After ~ 72 hours, coronal brain stem slices were cut at a 50μm thickness with a cryostat. Slices were then mounted on subbed slides and air dried. Later the slides were rehydrated in PBS, cover slipped and the injection sites were identified using an E400FN fluorescence microscope. Statistical procedures for determining significance of injection responses in the rats were determined using a students t-test and ANOVA.

3.6 Drugs

Unless otherwise stated, all chemical and drugs were obtained from Sigma Chemicals Co. (St. Louis, MO).

Chapter 4: Results

4.0 Observations

While gastric tone is reasonably stable across rat species, it is known that Sprague Dawley rats, in comparison to Wistar and Lewis rats, display a greater variation in gastric motility. However, Sprague Dawley rats have consistent gastric motility when measuring intragastric pressure and generally, obesity studies are more commonly performed in this strain.

Gastric motility and tone data were collected on a total of 56 male Sprague Dawley rats (control n=28; HFD n=28). All rats were weighed prior to experimentation. HFD rat weights (555±6.10g) were significantly greater than age matched controls (241±5.40g; $P<0.05$). Further, HFD rats that received BMI microinjections (533±4.40g) were significantly heavier than control rats receiving BMI (259±5g) and HFD rats that received GBZ (578±7.80g) weighed significantly more control than rats receiving GBZ (227±2.60g)**.** There was no significant difference in weights of HFD rats that received BMI (533±4.4g) or GBZ (578±7.80g) or in control rats that received BMI (259±5.00g) or GBZ (227.4±2.60g) ($P>0.05$, Figure 4.0).

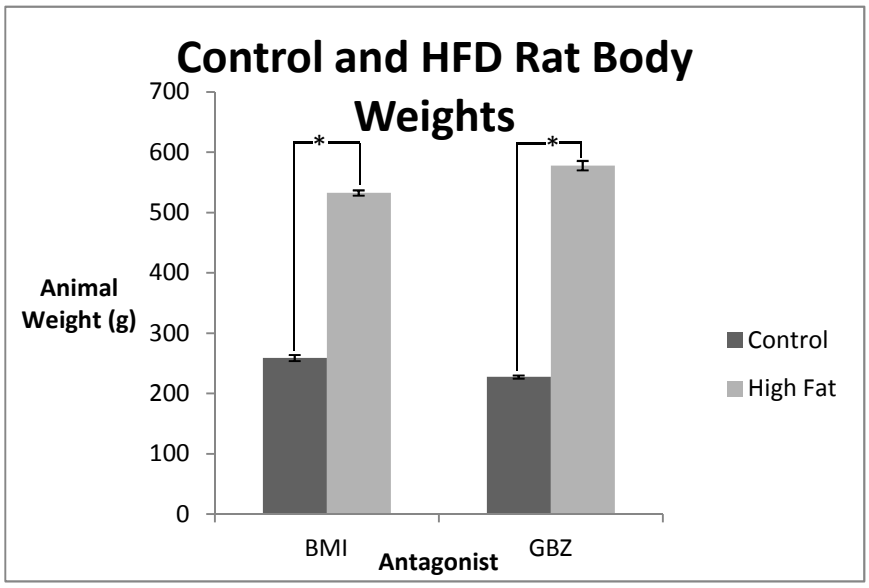

Fig 4.0: Graphical illustration of the body weights of rats that received BMI or GBZ microinjection. HFD rats were significantly heavier than control rats. (*) Indicates significance ($P<0.05$).

The baroreceptor reflex is an integral component of systemic neuronal regulation of blood pressure. Arterial baroreceptors consisting of both myelinated A and non-myelinated C fiber types, comprise this system and are located within the carotid sinus and aortic arch (15). The C fibers specifically are responsible for initiating and regulating this reflex in an abnormal physiological environment (29). In a study examining the effects of HFD (33%kcal fat) induced obesity, it was determined that obese rats illustrated a significant decrease in baroreceptor response to stimulus. In the present study it was pertinent to design a new protocol taking into account these specific changes in the homestatic reflex**.** When anesthetizing rats fed a HFD it was determined that administration of isoflurane (2.5-3%) with simultaneous induction of Inactin (0.15-0.33µl/min) over a period of 180-300 minutes provided an adequate level of anesthesia for the surgical protocol to begin without compromising cardiovascular or respiratory function (M. Andresen, personal communication).

4.1 Preface

In all experiments it was necessary to induce maximal contraction of the gastric corpus as a means of allowing responses to be represented as a percentage of the maximal contractile ability of the stomach. Bethanecol is a muscarinic agonist and, via intravenous infusion (50µg/kg), can induce a maximal contraction of the stomach. In control rats, bethanecol induced an average increase in gastric tone of 0.71 ±0.073g with an average time to return to baseline of 13.3 ± 3.8min. HFD rats exhibited a similar average increase in gastric tone of 0.67 ± 0.057g with a return to baseline time of 10.5 ± 2.70min (Fig 4.1A) ($P>0.05$).

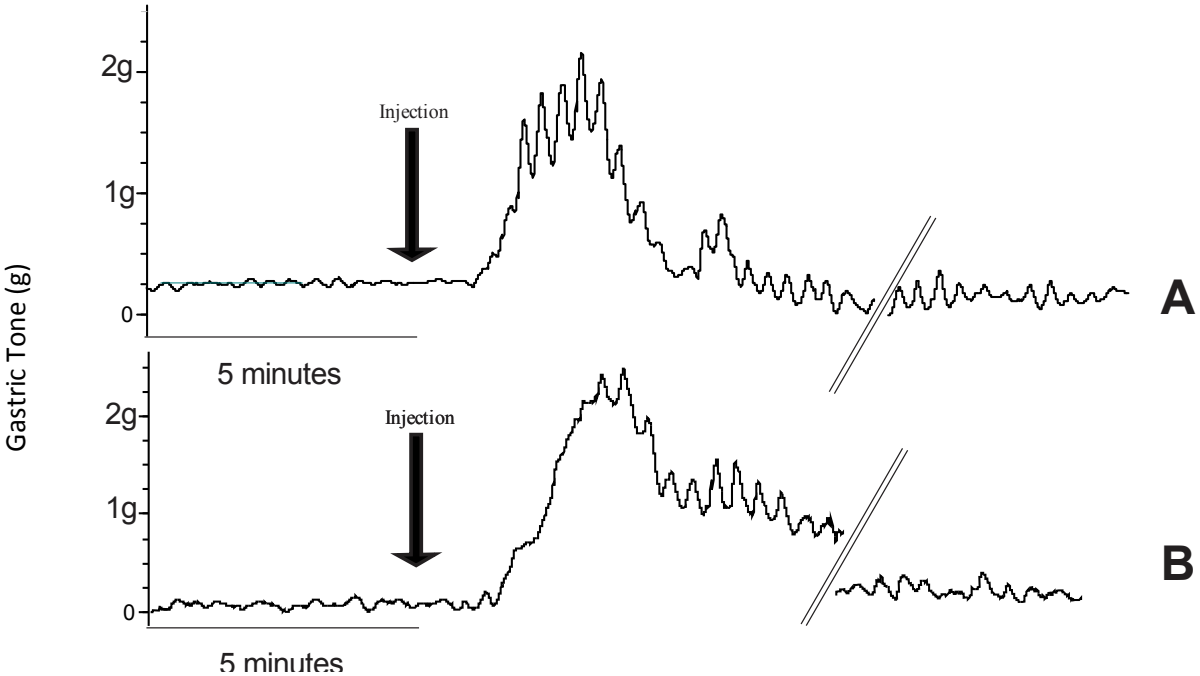

Fig 4.1.A: Intravenous infusion of the muscarininc receptor agonist bethanecol (50µg/kg) induces a maximal increase in gastric tone and motility in both control and HFD rats. Representative tone/motility recording demonstrating a maximal stomach contraction induced through intravenous bethanecol infusion. There was no significant difference in responses between the two groups or between rats that received BMI or GBZ. Bethanecol response in (A) control rat and (B) in a HFD rat. (//) Represents refractory period for the response to return to baseline. These data suggest that HFD does not affect the maximal contractile ability of the stomach.

All drugs were dissolved in vehicle, phosphate buffered saline (PBS; NaCl, KH_2PO_4). To exclude the possibility of vehicle induced effects, PBS (60nl) was microinjected into coordinates within the brainstem corresponding to sites of drug microinjection (N=5, 3 in control and HFD rats, respectively) as well as corresponding sites on the contralateral portion of the brain (N=2, 3 in control and HFD rats, respectively). The average increases in gastric tone in response to PBS microinjection in control (0.21± 0.34%) and HFD (0.15±0.05%) was not significant ($P>0.05$; Fig 4.1B).

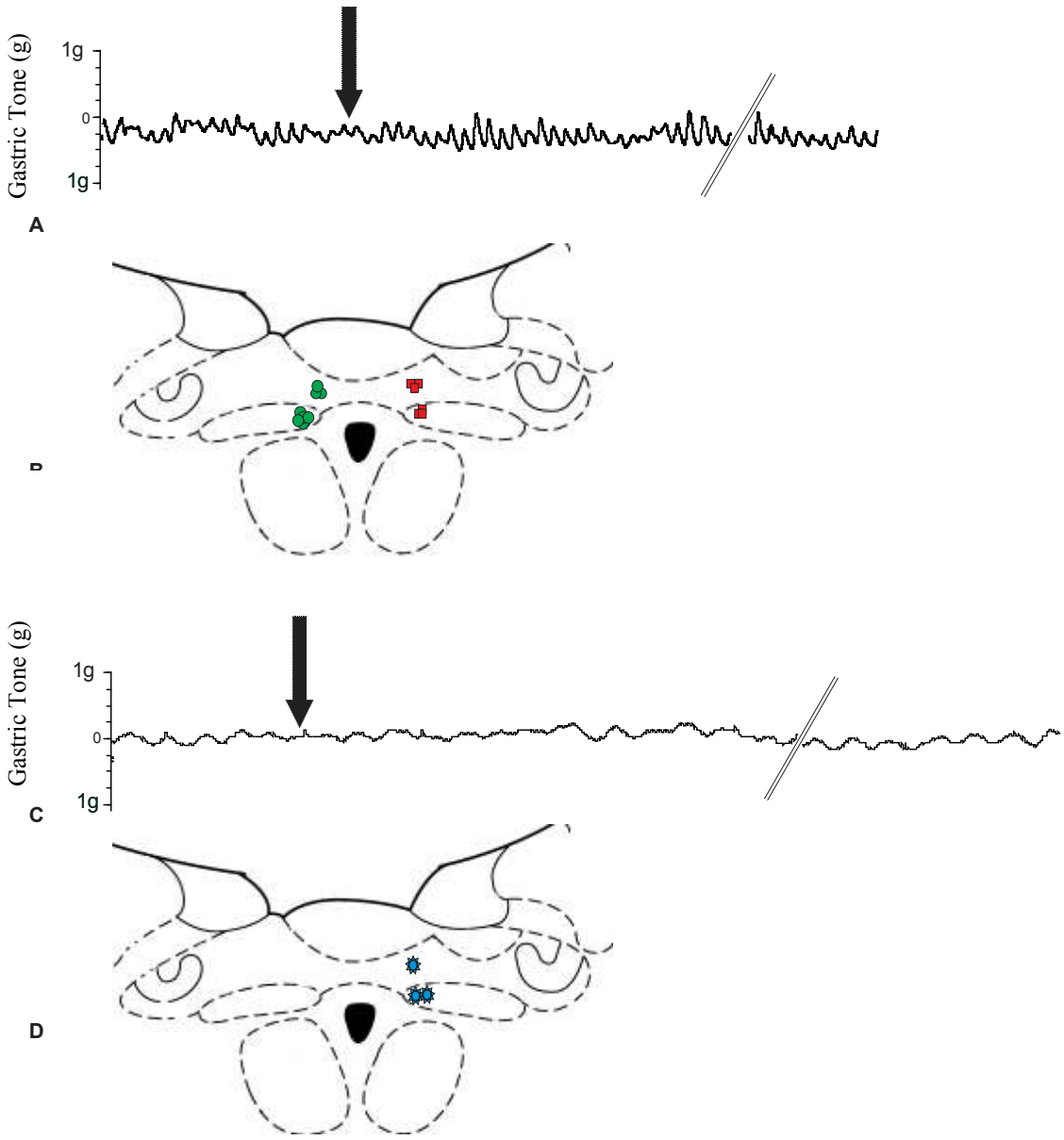

Fig 4.1.B: Representative trace of PBS microinjection and corresponding injection sites. (A) PBS (60nl) was microinjected into the DVC. Microinjection did not induce significant changes in gastric motility or tone in control and HFD rats (N=5, 3 in control and HFD rats, respectively). (B) Map of drug microinjection sites. (C) Contralateral injection of PBS (60nl) has no significant effect on gastric tone or motility. (D) Contralateral injection site map in three discrete locations corresponding to actual

microinjection sites (N=2, 3 in control and HFD rats, respectively). (//) Represents the refractory period for the response to return to baseline.

4.2 Gastric Tone In Response to Bicuculline Microinjection

An objective of this study was to determine whether a HFD affected the GABAergic regulation of gastric motility and tone. Microinjection of BMI into the DVC in control rats induced a dose dependent increase in gastric tone when evaluated as a percentage of maximal stomach contraction induced by bethanecol. The largest response occurred following microinjection of 100pmol BMI (9.51±3.5%; N=3). The middle dose of BMI (50pmol/60nl; N=7) caused an increase in tone of 5.40±1.20% with the lowest dose of 25pmol/60nL (N=6) increasing gastric tone to 2.24±0.34% of maximal possible contraction (Fig 4.2). In control rats, the low dose induced a gastric contraction that was significantly lower than the middle (50pmol) and high (100pmol) dose. Likewise, the high dose exerted a significantly greater effect on gastric tone than did the middle dose ($P<0.05$).

In the HFD, microinjection of BMI induced an increase in gastric tone when evaluated as a percentage of maximal stomach contraction induced by bethanecol. The 100pmol/60nl dose elicited an average increase in gastric tone of 9.78±4.70% (N=7). The 25pmol/60nl (N=4), increase tone an average of 5.40±2.30%. The middle dose 50pmol/60nL (N=5) caused an increase in tone of 8.00±4.10%. There is a trend ($P=0.06$) towards a difference between the effects of BMI on gastric tone when comparing the effects in rats fed a control vs. a HFD following 25pmol BMI microinjection (Fig 4.2).

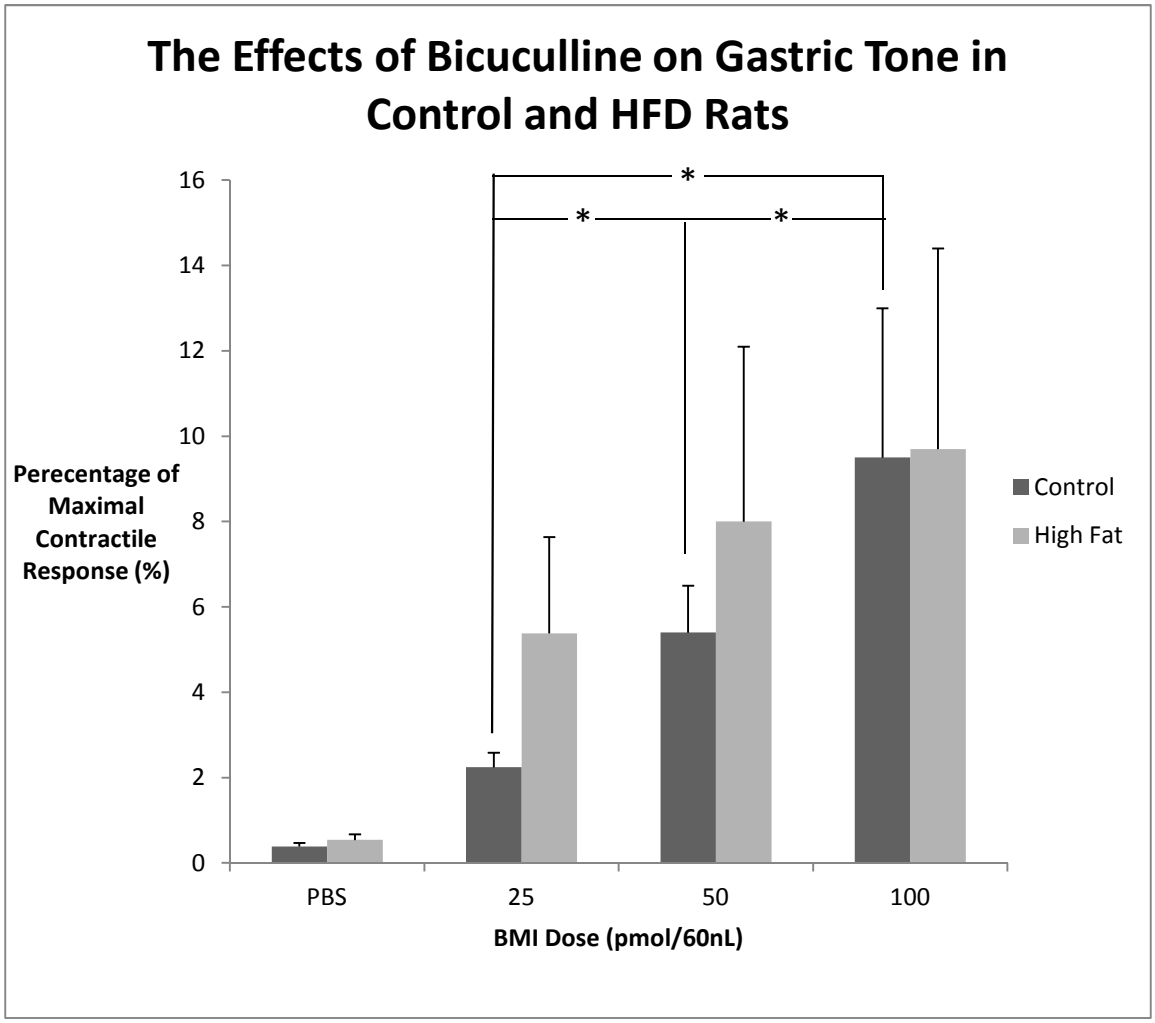

Fig 4.2: Microinjection of the $GABA_A$ antagonist BMI increases gastric tone in control and HFD rats. This is a graphical representation of changes in gastric tone expressed as a percent of the maximal contractile response induced by peripheral application of the muscarinic agonist bethanecol. Microinjection of BMI induced a dose-dependent increase in gastric tone in control rats. In comparing control vs. HFD rats, there is a trend towards significance ($P<0.06$) These results confirm that A) $GABA_A$ receptor activation in the DVC exerts significant effects upon gastric tone and B) there is a trend towards BMI having a greater effect on gastric tone in HFD rats ($P<0.06$ in 25pmol control vs. HFD). (*) $P<0.05$. 25pmol (N=6,4); 50pmol (N=7,5); 100pmol (N=3,7) in control and HFD rats, respectively.

4.3 Gastric Motility in response to Bicuculline Microinjection

Gastric motility alterations in response to BMI microinjection in the DVC were also recorded in control and HFD rats. The method for determining the motility index for a given animal is described above (see Section 3.5). In control rats, pre-injection motility indices ranged 44.6±7 to 88.8±24.4. There were no significant differences in the increase in gastric motility in response to microinjection of 25, 50 and 100pmol BMI. However, motility indices were altered from 55.5±21 to 88.35±35.5 in the 25pmol (N=6) dose; 88.8±24.4 to 116±39.8 in the 50pmol (N=7) dose; from 44.6±7.6 to 59±8 after injection of the 100pmol (N=3), dose. These responses were calculated as a percentage increase in comparison to the pre motility index. Percent increases of 54.8%±9. 3, 34.%7± and 26.7%±16.5 occurred for 25, 50 and 100pmol BMI injection doses, respectively. The changes in motility indices post injection were not significantly greater in comparison to the pre motility indices.

In the HFD pre injection indices ranged, from 40.9±5 to 57.4±19.1. Following BMI microinjection, motility indices increased from 56.8±24 to 65.2±24.6, from 57.4±19.1 to 64±21.6 and from 40.9±5 to 47.4±4.6 for microinjection doses of 25 (N=4), 50 (N=5) and 100pmol (N=7), respectively. The responses were calculated as a percent increase from the pre-motility index. For the 25, 50 and 100pmol injection doses, the percent increases were 21.7±14.6%, 18±7.8% and 22.2±12.8%, respectively. The changes in motility indices post injection were not significantly greater in comparison to the pre motility indices. The lowest dose of BMI induced a greater change in motility index in comparing control and HFD rats ($P<0.05$).

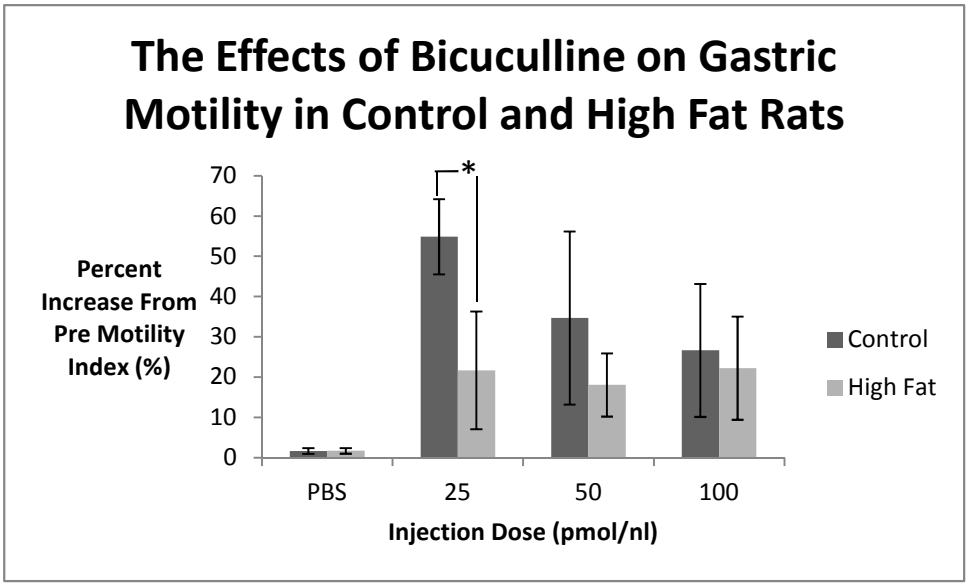

Fig 4.3: Microinjection of the $GABA_A$ antagonist BMI altered gastric motility in control and HFD rats. This is a graphical representation of changes in gastric motility when expressed as a percentage increase between the pre and post motility indices. These data suggest that A) although the effects of BMI are not dose-dependent, $GABA_A$ receptor activation in the DVC plays a role in regulating gastric motility. B) The effects of BMI are reduced following a HFD possibly suggesting that in the HFD, GABA release in the DVC is increased rendering BMI less effective. 25pmol (N=6, 4) 50pmol= (7, 5) 100pmol (N=3, 7) in control and HFD rats, respectively. (*) $P<0.05$.

4.4 Gastric Tone in Response to Gabazine Microinjection

An additional aim of this study was to determine whether a HFD affected the GABAergic regulation of gastric motility and tone in response to exogenous application of the $GABA_A$ receptor antagonist, GBZ. Microinjection of GBZ induced no significant difference in injection dose between the control or HFD groups. Microinjection of GBZ into the DVC consistently induced an increase in gastric tone in both control and HFD when evaluated as a percentage of maximal stomach contraction induced by bethanecol. In control rats, the highest dose of GBZ (6.25pmol; N=5; 14.8±7.80%) trended ($P<0.09$) towards inducing the greatest response when compared to the lowest dose(0.78pmol; N=5; 3.70±0.90%). The median dose (3.125pmol; N=7; 7.00±2.60%) exerted a response half that of the high dose.

In the HFD, GBZ similarly induced an increase in gastric tone. The lowest dose of GBZ (0.78pmol; N=6) induced a change in gastric tone of (4.14%±0.8). Rats at the middle (3.125pmol, N=6) and high (6.25pmol, N=4) doses produced a similar effect (3.3%±0.6, 3.4%±0.9), respectively (Fig 4.4).

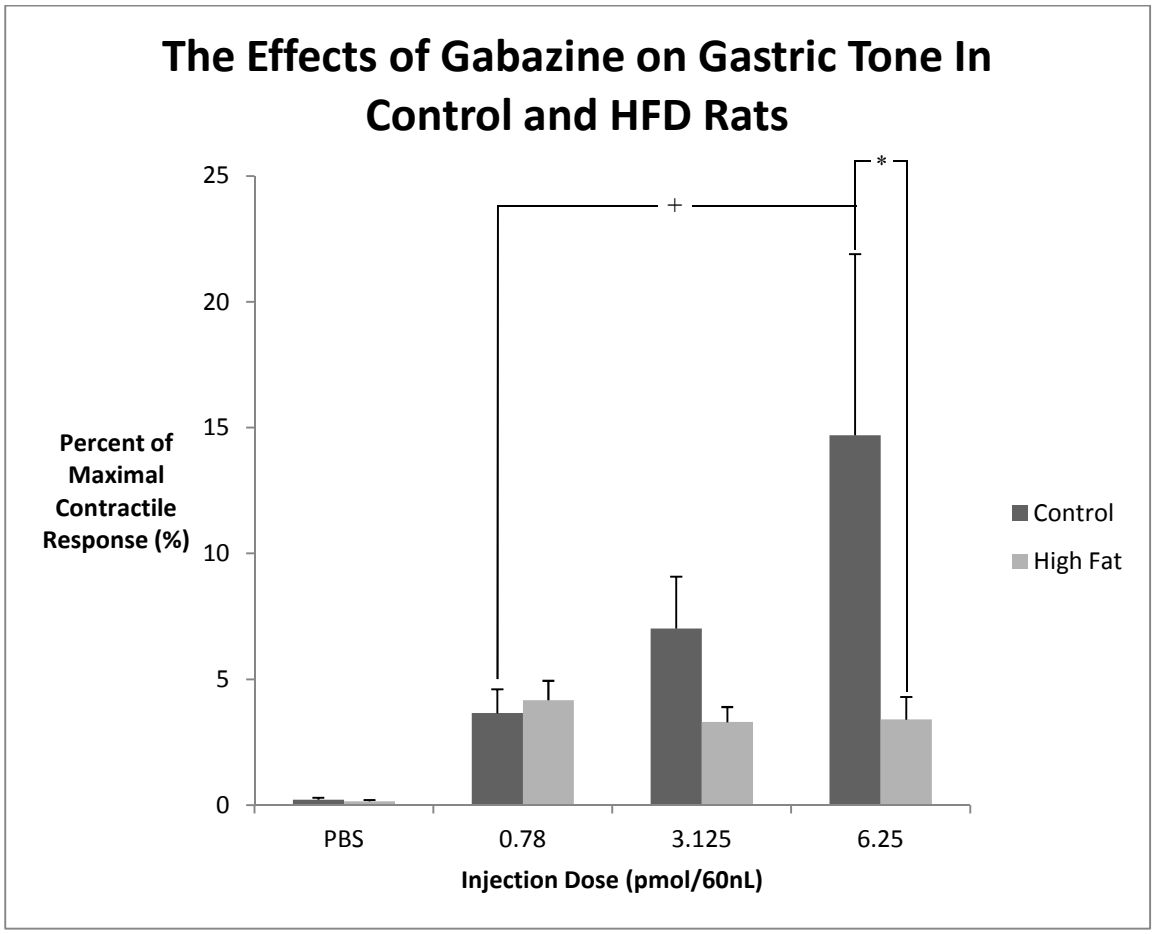

Fig 4.4: Microinjection of the $GABA_A$ antagonist GBZ increases gastric tone in control and HFD rats. This is a graphical representation of changes in gastric tone expressed as a percent of the maximal contractile response induced by peripheral application of the muscarinic agonist bethanecol. These results suggest that; A) activation of phasic/synaptic receptors in the DVC regulate gastric tone in control animals, B) the effects of GBZ are not dose-dependent in HFD rats, although activation of $GABA_A$ receptors still partially regulate gastric tone in these rats. C) Effects of GBZ are decreased following HFD, possibly suggesting that GABA release within the DVC is increased, rendering GBZ less effective. 0.78pmol (N=5, 6), 3.125pmol (N=7, 6) and 6.25pmol (N= 5, 4). (*) $P<0.05$. (+) $P<0.1$

4.5 Gastric Motility in Response to Gabazine Microinjection

Motility indices were calculated for both control and HFD rats in response to GBZ injection. Pre motility indices ranged 33.4±3.20 to 67.4±20.4 in control rats. Specifically, the pre motility indices for control rats were 33.4±3.2, 49±9.2 and 67.4±20.4 with post-injection indices of 39.6±2.6, 59.4±11.6 and 90.2±26.1 for injection doses of 0.78 (N=5), 3.125 (N=7) and 6.25pmol (N=5), respectively. Responses were calculated as a percentage increase in comparison to the pre motility index. For 0.78, 3.125, and 6.25pmol injection doses, the percent increases were 16.8±12.5%, 24.6±13.8% and 38.3±14.5%, respectively.

Motility indices in HFD rats were calculated in a similar manner. Pre injection indices for high fat rats were 50.8±13.9, 50.5±10.8 and 51.8±16.1 while post injection MI were 59±16.2, 65.8±15.9 and 66.3±20.3 for injection doses of 0.78 (N=6), 3.125 (N=6)and 6.25pmol (N=4), respectively. Fig 4.5 illustrates the response to injection calculated as a percentage of the pre motility index. The lowest dose (0.78pmol/60nl)) correlated with a motility change of 17.8±12.5%. The median dose (3.125pmol/60nl) induced a change of 28.7±11.3% while the highest dose (6.25pmol/60nl) induced a change in motility (33.7±13.7%).

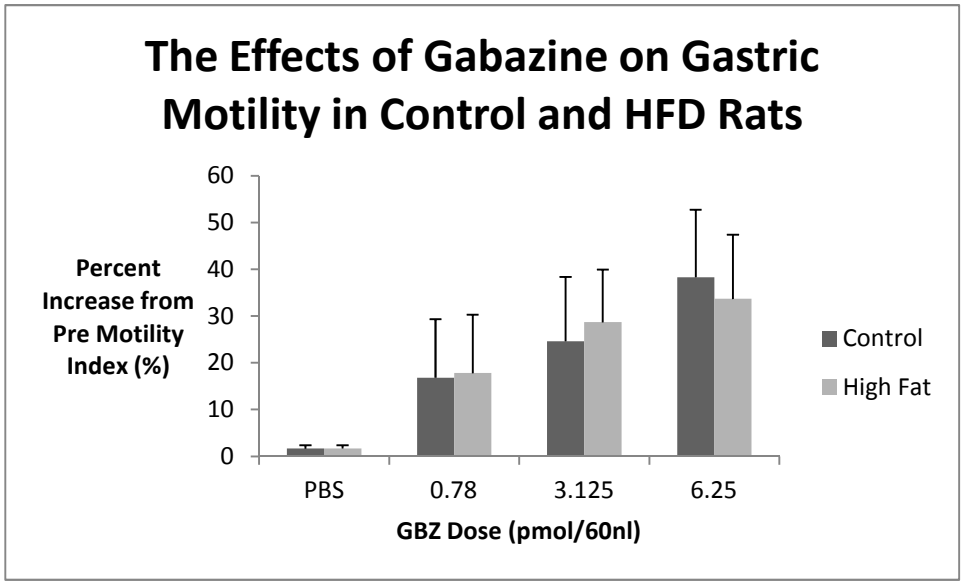

Fig 4.5: Microinjection of the $GABA_A$ antagonist GBZ increases gastric motility in control and HFD rats. This is a graphical representation of changes in gastric motility when expressed as a percentage increase between the pre and post motility indices. These results suggest that; A) antagonism of $GABA_A$ receptors by GBZ within the DVC plays a role in regulation of gastric motility. B) Since the effects of GBZ on gastric motility in control rats trends towards a significantly greater effect to increase gastric motility compared to BMI ($P<0.09$), these results suggest that the effects of GABA to regulate gastric motility are more dependent upon activation of synaptic/phasic receptors in comparison to extrasynaptic/tonic receptors. 0.78pmol (N=5, 6) 3.125pmol (N=7,6), 6.25pmol (N=5,4) in control and HFD, respectively.

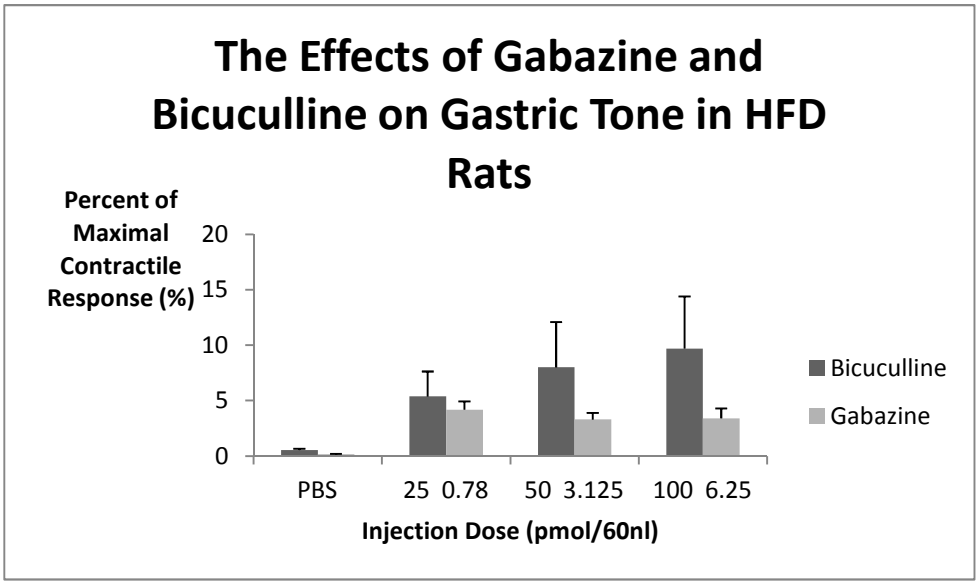

Fig 4.6: Graphical comparison of the effects of BMI and GBZ on gastric tone in HFD rats. In HFD rats, the effect of BMI to increase gastric tone appears greater than that of GBZ. These results suggest that A) both phasic and tonic receptors activated by ongoing GABA release are involved in the regulation of gastric tone and B) that extrasynaptic receptors may play an increased role in regulating gastric tone in the HFD.

Control	Tone Response (g)	Tone Response (%)	Pre Motility Index	Post Motility Index	Percent Increase (%)
25pmol N=6	0.02 ±0.011*	2.24±0.34	55.5±21.2**	88.0±35.8	54.8±9.30
50pmol N=7	0.06 ±0.14	5.43±1.12	88.8±24.4	116±39.8	55.7±37.5
100pmol N=3	0.08±0.02	9.51±3.54	44.6±7.60	59.0±18.0	26.7±16.5

HFD	Tone Response (g)	Tone Response (%)	Pre Motility Index	Post Motility Index	Percent Increase (%)
25pmol N=4	0.05±0.01	5.38±2.25	56.7±24.0	65±24.6	21.7±114.6
50pmol N=5	0.06±0.01	8.00±4.14	57.4±19.1	64±21.6	18.1±7.9
100pmol N=7	0.07±0.01	9.70±4.71	40.9±5.00	47±4.00	22.4±12.8

Table 1: Summarized data of the effects on gastric tone and motility in control and HFD rats elicited by BMI microinjection within the DVC. Each number represents the averaged data for all rats within each group. Differences in tone response (g) reflect differences in strain gauge sensitivities used across experimental groups. (*) Denotes significance comparing the 25pmol dose to both 50 and 100pmol doses in control rats, respectively. (**) Denotes significance when comparing 25pmol control and HFD rats.

Control	Tone Response (g)	Tone Response (%)	Pre Motility Index	Post Motility Index	Percent Increase (%)
0.78pmol N=5	0.05±0.01	3.67±0.95	33.4±3.2	39.6±2.60	16.8±12.5
3.125pmol N=7	0.06±0.01	7.02±2.0	49.0±9.20	59.4±11.6	24.6±13.8
6.25pmol N=5	0.06±0.01	14.7±7.20	67.4±20.4	90.2±26.1	38.3±14.5

HFD	Tone Response (g)	Tone Response (%)	Pre Motility Index	Post Motility Index	Percent Increase (%)
0.78pmol N=6	0.78±0.34	4.17±0.77	50.8±13.9	59.0±16.2	17.8±12.5
3.125pmol N=6	0.35±0.14	2.89±0.39	50.5±10.8	65.8±15.9	28.7±11.2
6.25pmol N=4	0.64±0.15	2.67±0.89	51.7±16.1	66.3±20.3	33.7±13.7

Table 2: Summarized data of the effects of GBZ microinjection within the DVC on gastric tone and motility in control and HFD rats. Each number represents the averaged data for all rats within each group. Differences in tone response (g) reflect differences in strain gauge sensitivities used across experimental groups.

Chapter 5: Discussion

5.0 Study Rationale & Summarized Results

GABA is the predominant inhibitory neurotransmitter of the CNS. Within the DVC, GABA can act upon $GABA_A$ and/or $GABA_B$ receptors to induce cellular hyperpolarization and subsequent neuronal inhibition. Inhibition of DMV neurons by GABA results in gastric inhibition via withdrawal of cholinergic tone (i.e. inhibition of the tonically active cholinergic pathway). The $GABA_A$ receptor plays a pivotal role in control of vago-vagal reflexes within the DVC. It has been shown that the $GABA_A$ receptor antagonists GBZ and BMI can antagonize the phasic or both phasic and tonic GABA currents within the DVC, respectively (7; 18; 24). A study comparing the effects of microinjection of BMI and GBZ within the DVC on gastric tone and motility has yet to be conducted in vivo. The aim of the present study, therefore, was to examine the effects of BMI and GBZ on gastric tone and motility in both control rats and rats fed a HFD.

The effects of microinjection of the $GABA_A$ receptor antagonists, BMI and GBZ, on gastric tone and motility were measured via miniature strain gauges sutured to the gastric corpus (see Fig 4.2; 4.4, respectively). Prior to microinjection, all rats were administered bethanecol (i.v.) to assess the maximal contractile ability of the stomach, there were no significant differences in maximal contractility of the gastric corpus between control and HFD rats in both

drug groups (BMI; GBZ). This indicates that the HFD, or subsequently the obesity induced by the HFD, does not alter the contractile ability of the gastric corpus.

The increase in gastric tone in response to microinjection of 25pmol BMI was significantly less than that induced by 50 or 100pmol BMI ($P<0.05$). Additionally 100pmol BMI induced a significantly greater increase in gastric tone compared to 50pmol. This indicates a dose dependent response to BMI in control rats.

In the HFD rats, responses trended towards being increased relative to control rats at 25 and 50pmol doses. In contrast, 100pmol BMI induced a similar increase in gastric tone in both control (9.50±3.50) and HFD (9.70±4.70) rats.

5.1 Bicuculline Responses Following the HFD

The data regarding changes in motility, however, introduce an alternative explanation. In response to microinjection of 25pmol BMI, control rats demonstrated a 60% greater increase in gastric motility compared to the HFD rats. These data suggest an alternative theory to the one described above namely, GABA release is increased following the HFD. An increase in ambient GABA levels within the DVC would decrease the magnitude of inhibition induced in response to lower doses of antagonist. This would lead to a markedly smaller increase in gastric motility post BMI microinjection in HFD rats. Recently, a study comparing the ambient GABA levels in rats fed a standard chow and a HFD were tested. Between control and HFD rats, there was a significant increase in ambient GABA levels exhibited in the HFD (16). In addition, another study discerned that in rats fed a HFD, there was a significant increase in $GABA_A$ receptor

binding densities in brain regions involved in cognition (42). Data in the present study support these findings since HFD rats had an increased response to antagonist suggesting a greater degree of receptor activation by GABA. Previous studies coupled with data presented in this study (See figure 4.2, 4.3; 4.4, 4.5) point to an increase in ambient GABA levels within the DVC or transient shifts in subunit composition of the $GABA_A$ receptor in HFD rats.

5.2 Gabazine Responses and the HFD

In both control and HFD rats, microinjection of GBZ into the DVC increased gastric tone and motility. While not reaching statistical significance, in control rats there was a trend towards a dose-dependent effect that may become significant with the addition of more experimental numbers. In the HFD rats, however, there was no evidence of any dose-dependency. Indeed, in HFD rats, the effects of GBZ are similar across the dose range administered with similar increases in gastric tone of 4.7, 3.3 and 3.4% in response to 0.78, 3.125 and 6.25pmol, respectively. For example; GBZ technically exerts an equal effect on gastric tone at all doses used within this study. Potentially if lower doses were used, the effects of GBZ may exhibit some degree of dose-dependency.

At low doses, GBZ preferentially acts on synaptic/phasic $GABA_A$ receptors. Blockade of synaptic/phasic receptors leaves only extrasynaptic/tonic receptors available for participation in the regulation of the gastric tone as seen in Fig 4.4. In contrast, BMI will block both the synaptic/phasic and extrasynaptic/tonic $GABA_A$ receptors with equal affinity. In comparing the effects of GBZ and BMI in control rats, there is a trend ($P<0.09$) towards GBZ inducing a greater increase in gastric tone than BMI. This indicates that in control rats, activation of synaptic/phasic receptors predominates in the regulation of gastric tone. However, in HFD rats, BMI induces a

significantly greater increase in gastric tone than GBZ (See figure 4.6). From these data it is possible to conclude that both the phasic and tonic receptors play a role in regulating gastric tone.

Gao makes note of two receptor subtypes associated with GBZ sensitivity that mediate the tonic GABA current within the DVC(18). Type 1 (T1) receptors are GBZ insensitive and saturated at normal physiological concentrations. The Type II (T2) receptors however, are active only in the presence of nipecotic acid (a non-selective GABA transporter inhibitor) (18). GABA transporters are activated upon increased GABA concentrations within the synapse. We originally hypothesized that a HFD may alter ambient GABA levels within the DVC directly. If these levels are increased in a HFD, than at higher doses, extrasynaptic diffusion of GABA may alternatively bind to these activated T2 receptors in addition to the extrasynaptic δ subunit containing receptors. At this time we cannot be sure of the effect of T2 receptor activation on gastric motility and tone leaving two possible scenarios; A) activation of T2 receptors play a prominent role in tonically regulating gastric motility and tone. Scenario: T2 receptors are activated in the presence of increased GABA, or increased ambient physiological levels of GABA in HFD rats. Exogenous administration of GBZ will block this additional population of receptors, removing the ongoing inhibitory effect of GABA thus resulting in increased gastric tone. B) T2 receptors play little to no role in regulating gastric motility and tone. In this scenario if GBZ has a preferential action at these T2 receptors, GBZ will pharmacologically reduce the action of GABA but these actions will have no physiological outcome, at least with respect to gastric tone and motility. As of yet, we have no way of knowing what role these receptors play in motility and tone, as such, these possibilities present an obstacle and remain to be investigated.

5.3 Summary & Conclusions

BMI induced a dose dependent increase in gastric tone in both control and HFD rats. At higher doses, the induced increase in gastric tone was similar between control and HFD rats, however, at the lowest dose used (25pmol), BMI induced a greater increase in gastric tone in HFD rats. The reason for such a difference remains to be elucidated. It is possible that such differences may reflect either changes in the subunit composition of $GABA_A$ receptors (the HFD prevented the normal developmental shift from α2/3 receptors to α1 receptors prolonging the actions of GABA) or the HFD resulted in increased activation of extrasynaptic/tonic $GABA_A$ receptors (which would be blocked by administration of BMI but not GBZ).

In response to GBZ microinjection there was a trend towards a dose-dependent increase in gastric tone in control rats. GBZ microinjection in HFD rats demonstrated no such dependence with the highest doses failing to exert a significant response in comparison to the lowest dose. BMI responses to microinjection in HFD rats showed a trend toward dose dependence. While HFD rats responded to GBZ with a decrease change in tone relative to control rats, in contrast BMI induced an increased effect on gastric tone in the HFD. These data point to, A) an increased role of the tonic/extrasynaptic receptors (as BMI has a greater effect than GBZ) or B) an increased release of GABA in an action potential dependent manner, which would increase the activation of the phasic receptors, causing GBZ to exert less of an effect.

As of now, the motility studies in both GBZ and BMI microinjection remain relatively inconclusive. However, we do present evidence supporting that: A) Both synaptic/phasic and extrasynaptic/tonic $GABA_A$ receptors are involved in the regulation of gastric tone and motility. B) Gastric tone is regulated differently from gastric motility. C) HFD during the perinatal period alters GABAergic regulation of brainstem neurocircuitry controlling gastric tone and motility without affecting the contractility of the stomach itself.

5.4 Future Directions

Further studies will allow the elucidation of the role of brainstem $GABA_A$ receptors in the regulation of gastric functions. In vivo motility recordings are still the primary means by which a physiological response can be measured, however, in conjunction with both electrophysiological and immunohistochemical studies, it may be possible to determine the location and type of receptors (including subunit composition/variations) present within brainstem neurocircuits and their subsequent alterations following perinatal exposure to a HFD.

In addition, it would be integral to determine the developmental timing window at which vagal neurocircuitry is altered in exposure to the HFD, and whether these changes are permanent. Through the use of a combination of these experimental techniques/approaches, it may be feasible to determine the effects of perinatal dietary manipulation on brainstem neural patterning and the subsequent effects this may have on physiological outcomes, such as obesity.

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