The Role of Neuropeptide-Y in Conveying the Degree of Glycemic Challenges in the Rat Paraventricular Nucleus of the Hypothalamus

NEUR 494 Honors Thesis
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Abstract

The paraventricular nucleus of the hypothalamus (PVH) is critical in regulating neuroendocrine activities by modulating the hypothalamic-pituitary-adrenal (HPA) axis through innervations by Corticotrophin-releasing-hormone (CRH) neurons. The neurons in the medial parvicellular dorsal section of the PVH (PVHmpd) are of particular interest due to their unique ability to synthesize a diverse range of neuropeptides that have modulatory outputs on the HPA axis. This study aimed to further understand the role that Neuropeptide Y (NPY), a regulator of CRH neurons, plays in conveying the severity of glucoprivation, or a lack of cellular access to glucose. We administered saline, insulin and 2-deoxy-glucose (2DG) to cause varying deficits in cellular glucose and fluorescently labeled NPY as well as several other peptides and enzymes of interest through an immunocytochemical protocol. We aimed to establish a negative control for the PVHmpd by imaging at the border between the PVHmpd and the PVHdp, PVHpml, and PVHmpv. Our findings reveal that glutamate terminals on this bordering region express Vesicular Glutamate Transporter 2 (VGlUT2), which transports glutamate into releasable vesicles, differently under varying degrees of glycemic stress. In accordance with previously gathered data about expression of peptides in the PVHmpd, we found the bordering regions to differ in their expression of certain neuropeptides and enzymes.
**Introduction**

All stressors, both physiological and psychological, threaten homeostasis, and the hypothalamic-pituitary-adrenal (HPA) axis is fundamental in the subsequent neuroendocrine response. Corticotrophin-releasing-hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVH) modulate the HPA axis, and respond to all stressors by initiating the release of adrenocorticotropic-releasing hormone (ACTH) from corticotropes in the anterior pituitary (Aguilera & Liu, 2012). The majority of the neurons in the medial parvicellular dorsal section of the PVH (PVHmpd) have a distinct ability to synthesize a diverse range of neuropeptides that have modulatory outputs on the HPA axis (Watts, 2005). ACTH travels through the bloodstream and results in the release of glucocorticoids from the adrenal cortex. Glucocorticoids are essential for the body’s normal response to stress and aid in the utilization of energy reserves, as well as many other regulatory functions intended to restore homeostasis.

Glucoprivation, or reduced cellular availability of glucose, can be caused by hypoglycemia (low blood sugar), and as a stressor is of particular interest as the brain needs a continuous supply of glucose to function properly, and because of its applications in understanding how glucoregulatory disorders, including diabetes mellitus, affect brain function (Wittman, 2008). Discovering how specific information about a stressor, such as the degree of the glycemic challenge, is relayed through the brain to provoke a response would greatly aid in understanding how the nervous system interacts with the glycemic challenges.
presented by glucoregulatory disorders. Future treatments for these disorders may rely on an understanding of how stimulus intensity, like the degree of glucoprivation, is communicated.

Glucoprivation and hypoglycemia trigger a series of systemic glucoregulatory responses that attempt to restore brain glucose and protect its reserves. Catecholaminergic (CA) neurons in the hindbrain are crucial for the resulting neuroendocrine response. Dopamine beta-hydroxylase (DBH) is the enzyme that converts dopamine to norepinephrine, while phenylethanolamine N-methyltransferase (PNMT) is the enzyme that converts norepinephrine to epinephrine (Wittman, 2008). Both DBH and PNMT are found only in CA neurons, which innervate the PVHmpd.

A lack of glucose can be induced in vivo through the injection of either insulin to cause hypoglycemia or 2-deoxy-glucose (2DG) to cause cytoglucopenia, a more severe glycemic challenge than hypoglycemia (Wittman, 2008). Both the administration of insulin and 2DG cause an increase in the circulation of corticosterone which inhibits CRH neurons as part of a negative feedback system.

Neuropeptide Y (NPY), which has been shown to be a regulator of CRH neurons, exerts its effect through CA neurons, which innervate the hypothalamus through origins in both the hypothalamus and hindbrain (Evangelia et al., 2005). The majority of NPY is colocalized with CA neurons in the hindbrain (Gujar et al., 2014) and previous research indicates that CA/NPY modulates the activity of CRH neurons in response to glucoprivation (Wittman, 2008). Hypothalamic
innervation by hindbrain NPY neurons contributes to glucoprivic feeding, and it has been shown that these neurons are responsive to glucoprivation (Wittman, 2008).

While it has been established that hindbrain CAs are necessary for the full response to glycemic challenges (Evangelia et al., 2005; Khan et al., 2011), the mechanisms by which the intensity of the stimulus is conveyed are not yet fully understood. It likely involves interactions between CAs and other various signaling mechanisms, including glutamate, GABA, and peptides such as NPY (Wittman, 2008).

NPY may interact with a series of neuropeptides and neurotransmitters to convey stimulus intensity, a number of which we will explore in this study. Vesicular Glutamate Transporter 2 (VGLuT2) is the transporter that moves glutamate into releasable vesicles, and signals the presence of glutamatergic terminals (Wamsteeker & Bains, 2010). Phospho-ERK1/2 (pERK) is a protein kinase and is associated with cellular activation as it responds to upstream growth and differentiation signals, so consequently it can indicate appositions that are communicating with the soma of neurons (Watts & Khan, 2013; Mandal et al., 2014). Phospho-Synapsin I (pSynI) is important for fine-tuning neurotransmitter release by interacting with vesicles to influence plasticity and can indicate terminal activation (Cesca et al., 2010). Its role in fine-tuning neurotransmitters may be important in relation to NPY because it may allow NPY to scale certain patterned stress response signals in the PVHmpd in an intensity-dependent manner based on the degree of glucoprivation. The presence of both
pSynI and VGluT2, which work with vesicles at the terminal, is necessary for indicating an active presynaptic terminal, however, if VGluT2 is found without pSynI, it will only indicate the presence of glutamate vesicles at the terminal. If a region contains neither pSynI nor VGluT2, then we can only consider it to be some type of pre-synaptic structure, but not necessarily a terminal. A combination of VGluT2 and pERK would indicate the presence of an activated glutamatergic terminal.

Understanding the role of NPY in these pathways is highly relevant to research involving dysfunction in the stress response. While its precise role is not fully understood, the NPY that is colocalized with hindbrain CAs may be involved in regulating the intensity of the stress response resultant from varying glycemic challenges. It is of great importance in understanding the regulation of stress response pathways if the action of NPY can be specifically defined. Therefore, I will be investigating the hypothesis that NPY is involved in conveying intensity of the stimulus to the CRH neurons.

We expect to find that in conveying stimulus intensity, NPY will be found to have a higher degree of colocalization with CAs when there is a more severe deprivation of glucose systemically, as caused by 2DG in comparison with insulin. This would mean that in animals treated with 2DG there would be an increase in NPY colocalized with DBH and PNMT, to demonstrate an association with an increase in CA activity, as well as with pERK, VGluT2, and pSynI, to demonstrate an association with increased synaptic activity, compared to animals treated with insulin. I would also expect to see greater terminal activation
as indicated by higher levels of pSynI and VGluT2. The purpose of this study will be to establish a negative control around the border of the PVHmpd by imaging regions directly adjacent to it, including the medial parvicellular ventral section (PVHmpv), posterior magnocellular lateral section (PVHpml) and the dorsal parvicellular section (PVHdp). The PVHmpv relays signals from leptin in the pathway that stimulates adaptive thermogenesis in brown adipose tissue (Xu & Xie, 2016). Lateral to the PVHmpd is the PVHpml, which contains a high concentration of vasopressin-secreting cells (Simmons & Swanson, 2009). Dorsal to the PVHmpd is the PVHdp, which sends its projections to autonomic centers of the brainstem and encompasses CRH neurons from nearby regions in the PVH (Simmons & Swanson, 2009).

We expect that these three regions should have different neuropeptide and CA activity than the PVHmpd. Establishing a negative control for the PVHmpd will allow further research to compare results with this study in order to provide evidence that their measurement profiles for the PVHmpd are distinct from surrounding regions and that they have measured the PVHmpd and not these surrounding regions.

**Methods**

**Animal Preparation**

Six adult male Sprague-Dawley rats (~300g) were fitted with jugular catheters, and divided into three groups receiving injections of either saline, insulin (2U/mg/kg) or 2-deoxy-glucose (2DG, 250mg/kg) directly into the catheter.
Thirty minutes later, anesthesia was administered directly down the catheter of all groups, and rats were perfused immediately. Blood glucose was tested immediately before and thirty minutes after experimental procedures.

**Immunocytochemistry**

Eight sets of 20µm coronal sections were cut through the entire PVH and processed for immunocytochemistry (ICC) using antibodies against NPY, DBH, PNMT, VGluT2, pSynI, and pERK1/2 (Table 1). Adjacent sections were Nissl stained to determine cytoarchitecture, and to confirm PVHmpd location.

Sections were washed in tris-buffered saline (TBS) for 30 minutes at room temperature, then incubated in the primary antibody solution for 72 hours at 4°C. Sections were again washed in TBS, followed by an overnight incubation with a fluorescently-conjugated secondary antibody, or incubation in biotin solution as appropriate. After sections from series 3 were stained for the primary antibody, they were incubated in a solution of donkey anti-sheep biotin for 8 hours at room temperature, so that the biotin would bind to NPY, before overnight incubation in the secondary solution. Alexa-488, the fluorescent molecule, conjugated to streptavidin, a molecule that binds biotin, was used as the fluorescent marker for sheep NPY in this series. After incubation, these molecules formed a complex in which NPY was bound to biotin, which connected to a streptavidin/alexa-488 complex.
**Table 1** Primary and secondary antibodies used for each series

<table>
<thead>
<tr>
<th>Series</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Mouse anti-DBH</td>
<td>Donkey anti-mouse Alexa-488 (Jackson ImmunoResearch, 715-605-150)</td>
</tr>
<tr>
<td></td>
<td>(Millipore, MAB308)</td>
<td></td>
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<tr>
<td></td>
<td>Rabbit anti-PNMT (Dr. M. Bohn, Northwestern University)</td>
<td>Donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152)</td>
</tr>
<tr>
<td></td>
<td>Sheep anti-NPY (Millipore, AB1583)</td>
<td>Donkey anti-sheep Alexa-647 (Jackson ImmunoResearch, 713-605-147)</td>
</tr>
<tr>
<td>3</td>
<td>Sheep anti-NPY (Millipore, AB1583)</td>
<td>Biotin Donkey anti-sheep Alexa-647 (Jackson ImmunoResearch, 713-065-147) + streptavidin 488 (Jackson ImmunoResearch, 713-065-147)</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-PNMT (Dr. M. Bohn, Northwestern University)</td>
<td>Donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152)</td>
</tr>
<tr>
<td></td>
<td>Guinea pig anti-VGluT2 (Chemicon International, AB5907)</td>
<td>Donkey anti-guinea pig Alexa 647 (Jackson ImmunoResearch, 706-605-148)</td>
</tr>
<tr>
<td>4</td>
<td>Mouse anti-phospho-ERK1/2 (Cell Signaling, 9106)</td>
<td>Donkey anti-mouse Alexa-488 (Jackson ImmunoResearch, 715-605-150)</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-PNMT (Dr. M. Bohn, Northwestern University)</td>
<td>Donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152)</td>
</tr>
<tr>
<td></td>
<td>Sheep anti-NPY (Millipore, AB1583)</td>
<td>Donkey anti-sheep Alexa-647 (Jackson ImmunoResearch, 713-605-147)</td>
</tr>
<tr>
<td>5</td>
<td>Mouse anti-DBH (Millipore, MAB308)</td>
<td>Donkey anti-mouse Alexa-488 (Jackson ImmunoResearch, 715-605-150)</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-pSynI (Acris Antibodies, APO2514PU-S)</td>
<td>Donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152)</td>
</tr>
<tr>
<td></td>
<td>Sheep anti-NPY (Millipore, AB1583)</td>
<td>Donkey anti-sheep Alexa-647 (Jackson ImmunoResearch, 713-605-147)</td>
</tr>
</tbody>
</table>

Each series is presented showing the primary and secondary antibody used for labeling. Series 1 was used for Nissl staining to determine cytoarchitecture.
**Imaging**

Images were captured using a Zeiss 700 Laser Scanning Confocal Microscope, equipped with a Zeiss AxioImager Z1 camera (Carl Zeiss MicroImaging, Inc., Thorwood, NY). Images were captured using a 40x oil-corrected objective (numerical aperture 1.3), and through the Zeiss Zen software (Zen Black, version 2011) an optical zoom of 1.6 was employed. For all laser lines, the Airy unit was set to ~1.0. 3D images at the border between the PVHmpd and the PVHdp, PVHpml, and PVHmpv were captured by taking images across the X, Y, and throughout the Z axis (~0.4µm). Images were collected by selecting the section with the clearest and most intact PVH region per animal from atlas level 26 as defined by Swanson (2004) in *Brain Maps: Structure of the Rat Brain*.

**Image Analysis**

Images were analyzed using Volocity software (PerkinElmer, version 6.3, Waltham, MA). Changes in the overall number of objects, as well as the degree of colocalization of NPY with DBH, PNMT, VGluT2, and pSynl were measured. NPY appositions to phospho-ERK1/2—labeled soma were also analyzed. Objects were defined as three dimensional (3D) fluorescently-labeled structures, with a size threshold of greater than 0.5µm and less than 2µm. Appositions to labeled soma were analyzed according to methods published by Bouyer and Simerly (2013). Intensity thresholds were set to exclude light-noise at two standard deviations above mean background brightness. Signals outside these
parameters do not mark anatomical structures of interest, so those values were excluded after filtering for noise.

The number of objects was used to compare changes in structure following varying glycemic challenges. Colocalization was established by quantifying the normalized number of 3D structures that expressed two or more fluorophores.

**Statistical Analysis**

Statistical analysis was done using JMP Pro (version 12.1.0). Results were expressed as the mean ± SEM. Statistical analyses were made using unpaired t-tests for two group comparisons. For comparison between all groups under varying hypoglycemic conditions, a one-way ANOVA was used. A $p$ value of less than 0.05 was considered significant in all tests.
Results

*Nissl staining was used to determine cytoarchitecture*

**Figure 1** Nissl staining of PVH section

Nissl staining of a PVH section (A) as compared with an atlas depiction by Swanson (2004) in *Brain Maps: Structure of the Rat Brain* (B).

*Blood glucose measurements demonstrated that the intended effect of the glycemic challenge was achieved in each treatment*

Blood glucose and circulating corticosterone were tested immediately before and thirty minutes after experimental procedures to ensure the desired effect had been achieved. The saline treatments maintained blood glucose at the same level before and after treatment while the insulin produced a hypoglycemic effect that was significantly different than the 2DG treatment, which produced a much higher blood glucose value than the insulin treatment (Fig. 2).
**Figure 2** Blood glucose and circulating corticosterone before and after treatment

Blood glucose and circulating corticosterone measurements were taken immediately before (T0) and thirty minutes after (T30) administration of treatment.
Number of VGluT2 objects differed significantly between saline and insulin groups

In order to examine how the number of glutamatergic terminals bordering the PVHmpd changes in response to the degree of glucoprivation, the number of VGluT2 objects in each treatment were measured (Wamsteeker & Bains, 2010). After injection of 2DG, insulin, or saline, there was a significantly higher number of VGluT2 objects in the saline condition than in the insulin condition (Fig. 3). This data was collected as the average of two animals per treatment. There were no significant differences in the number of VGluT2-expressing objects between the other groups.

Figure 3 VGluT2 objects in each treatment

Significant difference between the means of VGluT2-expressing objects in the saline and insulin conditions (A). VGluT2-expressing objects displayed in each condition in Volocity imaging software (B-D). Error bars were not displayed due to the availability of two animals for each condition.

Number of colocalized objects of NPY and DBH was trending towards significance between 2DG and insulin groups

In order to examine how CA activity in the regions bordering the PVHmpd change in relation to NPY levels during varying degrees of severity in regards to
the lack of glucose, the number of NPY objects colocalized with DBH was measured (Wittman, 2008). After injection of 2DG, insulin, or saline, the number of colocalized NPY and DBH-expressing objects between the 2DG and insulin treatments was trending towards significance (Fig. 4). This data was collected as the average of two animals per treatment. There were no significant differences between the other groups.

**Figure 4** Colocalized NPY and DBH objects

![Figure 4](image)

Difference between the 2DG and insulin treatments of colocalized NPY and DBH objects was trending towards significance (A). Colocalized objects expressing both NPY and DBH displayed in each condition in Volocity imaging software (B-D). Error bars were not displayed due to the availability of two animals for each condition.

**Number of NPY, DBH, PNMT, pERK, and pSynl objects did not differ significantly between groups**

A difference in NPY-expressing objects between groups may have indicated that the severity of the glycemic challenge is conveyed to the HPA axis through modulatory shifts in NPY levels, so the number of NPY objects between groups was tested (Wittman, 2008). Significant differences between groups in the numbers of DBH and PNMT objects independently would indicate how CA activity changes between varying levels of glycemic stress, so the numbers of
these objects were compared. The protein kinase, pERK, was examined because it functions as a general indication of activation as well as an indicator of appositions stemming from the soma of neurons (Watts & Khan, 2013; Mandal et al., 2014). Neurotransmitter release may be fine-tuned by pSynl, so the number of objects expressing it was examined (Cesca et al., 2010). All of these were measured in the regions bordering the PVHmpd to establish a negative control. After injection of 2DG, insulin, or saline, there were no significant differences in the number of NPY, DBH, PNMT, pERK, or pSynl expressing objects between groups.

*Number of colocalized objects of NPY and PNMT, NPY and VGluT2, NPY and pSynl, and NPY and pERK did not differ significantly between groups*

Understanding the way that NPY, PNMT, VGluT2, and pSynl interact, and under what conditions, is essential in creating a profile of how the PVHmpd is modulated in response to variations in glucoprivation. The number of colocalized objects of these neuropeptides and enzymes was examined in the region bordering the PVHmpd to create a negative control. After injection of 2DG, insulin, or saline, there were no significant differences in the number of colocalized NPY and PNMT, NPY and VGluT2, NPY and pSynl, and NPY and pERK and pSynl expressing objects between groups.
Discussion

The purpose of this study was to build a negative control for the bordering regions of the PVHmpd. Therefore, it was anticipated that the results of these measurements would contradict the expected results of a study performed on the PVHmpd itself.

VGluT2 indicates the presence of an active glutamatergic terminal (Wamsteeker & Bains, 2010). We found that when experiencing hypoglycemia induced by the injection of insulin, animals showed fewer VGluT2 terminals in the region bordering the PVHmpd than in the control condition. VGluT2 does not increase in response to hypoglycemia, but rather it decreases which may indicate a disinhibitory effect. Hypoglycemia might mobilize other peptides which serve to mitigate the level of VGluT2, thus causing its decrease and a downstream circuit to be disinhibited to respond to the glycemic challenge. Perhaps VGluT2 still plays a role as glutamate may activate other regions of the brain that are involved in the stress response, but are not necessarily neuroendocrine. These results support that glutamate is widely used in many different pathways, and that it’s precise function in modulating the stress response has yet to be understood. As a negative control, it would be expected that measurements of VGluT2-expressing objects in the PVHmpd would differ from these results.

Determining how NPY and DBH interact in the stress response will help determine if and how NPY might convey stimulus intensity in regards to glycemic stress (Wittman, 2008). In the periphery of the PVHmpd we have established a negative control to be compared later with data from the PVHmpd itself. Objects
expressing both NPY and DBH were found in greater numbers in the 2DG treatment as compared to insulin. This value was trending towards significance, which may suggest that while NPY and DBH objects do not independently change in number between conditions, their level of association does so that they may work together to convey stimulus intensity. NPY could be increasing the activity of DBH to produce more norepinephrine in these peripheral regions to the PVHmpd. Similar analysis of the PVHmpd may yield differing results.

It has been shown that DBH-containing neurons innervate CRH neurons (Wittman, 2008), and that DBH expression increases when stressed under glucoprivic conditions (Li et al., 2006). PNMT has been shown to decrease in hypothalamic areas after immobilization stress in rats (Saavedra & Torda, 1980). It has been shown that when injected with insulin and 2DG, rats significantly increased PVHmpd pERK expression (Khan et al., 2011). The lack of significant changes in PNMT, DBH, and pERK-expressing objects between groups on the bordering region of the PVHmpd support the hypothesis that the PVHmpd is unique in these responses.

NPY, DBH, PNMT, pERK, and pSynI may all have roles in modulating the stress response (Wittman, 2008; Evangelia et al., 2005; Khan et al., 2011), but neither the number of objects expressing these, nor the number of colocalized objects of NPY and PNMT, NPY and VGluT2, NPY and pSynI, and NPY and pERK changed significantly between groups in the regions bordering the PVHmpd. A change in NPY-expressing objects in particular might have suggested an activity-dependent increase in peptide synthesis. We would also
expect to see an increase in the number of pSynI-labeled terminals in the PVHmpd which would suggest an increase in the number of vesicles available for activity-induced exocytosis. Colocalized objects may indicate co-activation or associated activity in the PVHmpd, but the bordering regions showed no activity that would indicate this. It would be strong evidence that the PVHmpd’s activity is distinct from the surrounding regions should further analysis show significance in measurements of the PVHmpd itself.

The overall goal of this study is to provide more insight regarding the roles that various signaling mechanisms play in regulating an appropriately-graded CRH neuroendocrine response to glycemic stressors, specifically focusing on the role of NPY. By measuring the the PVHmpv, PVHmpl, and PVHdp, which are regions bordering the PVHmpd, we have established a negative control for activity in the PVHmpd. The results of this study will be used in conjunction with further analysis dedicated to measuring the expression of neuropeptidergic and CA activity in the PVHmpd.
References


