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Neuropeptide Y: A potential marker for a life-history transition in the red-sided garter snake (*Thamnophis sirtalis)*

by

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An undergraduate honors thesis submitted in partial fulfillment of the

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and

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Thesis Adviser

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Portland State University

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Introduction

Organisms must know at what point in time to exhibit particular sets of behaviors, such as feeding, courting, or hibernating, to enhance their individual fitness. These periods are referred to as a life history stage and the timing of which is crucial, especially in extreme environments where windows of opportunity are narrower than in more temperate climates (Wingfield et al., 1998). Organisms coordinate external cues from the environment and internal cues to time such events. Along with knowing when to undergo certain life history stages is when to transition from one life history stage to another life history stage, the mechanisms of which are poorly understood. This study explores a possible mechanism influencing the transition from mating behavior to feeding behavior in the well-studied red sided garter snake, *Thamnophis sirtalis parietalis,* a transition that is critical for optimization of individual fitness and reproductive success (Schneider 2004, Schneider at al., 2013).

These red sided garter snakes live in an extreme environment in Manitoba, Canada. They inhabit a den by the thousands for eight months during winter dormancy where they are more or less inactive. This is followed by an intense mating period in the spring after emergence from the den that lasts for approximately one month. The duration of mating is sexually dimorphic for these organisms as females will mate for approximately 24-48 hours before transitioning to foraging behavior, while males will mate for up to two weeks before transitioning to foraging behavior (Shine et al., 2001). After transitioning, foraging and feeding behavior persists for the next three months during summer before returning to the den for

hibernation. The focus of this project is the transition from mating to feeding behavior in the spring, and a possible underlying mechanism driving this transition.

In a previous study performed by this lab, it was shown that corticosterone, a stress hormone, is elevated in snakes exhibiting courting behavior, and lower in feeding snakes. Additionally, as corticosterone levels were experimentally lowered with metyrapone, a glucocorticoid synthesis inhibitor, snakes were shown to be induced into exhibiting feeding behavior (Lutterschmidt and Maine, 2014).

Corticosterone is hormone that has numerous roles, including regulating metabolism, energy mobilization, and reproductive organization, and is also crucial in facilitating life history transitions in many organisms (Landys et al., 2006, Moore and Jessop, 2003, Wada 2008). Although it was shown to influence the transition from mating to feeding in the red-sided garter snake, it is known that there are many chemical messengers effecting this life history transition, including neuropeptide-Y (NPY), a potent neurohormone responsible for appetite and feeding behavior in *T. sirtalis,* as well as all taxa studied to date (Matsuda et al., 2012, Mercer et al., 2011, Morris and Crews, 1998, Schneider et al., 2013). In the metyrapone experiment described above it was shown that as corticosterone levels decrease, there is an increase of NPY cell numbers within the brain. It was hypothesized for this project that NPY would be significantly higher in feeding snakes than mating snakes, and is an additional hormone influencing this behavioral transition in the spring.

Methods

For this study, lab members, including Master's student Ashley Maine and Dr. Lutterschmidt, had to go to Manitoba, Canada and perform field work to obtain specimens. Mating snakes were collected at the den in early spring during their intense mating period. The feeding snakes were collected at a road approximately 1 km away from the den along their migratory path to the summer feeding grounds. The exhibited behavior of each snake was further confirmed with a Y-maze in which the snake has the option of choosing a worm trail or pheromone trail, displaying feeding or mating behavior, respectively (LeMason and Master, 2001). After the behavior confirmed, the snakes were sacrificed and neural tissue extracted and fixed in paraformaldehyde.

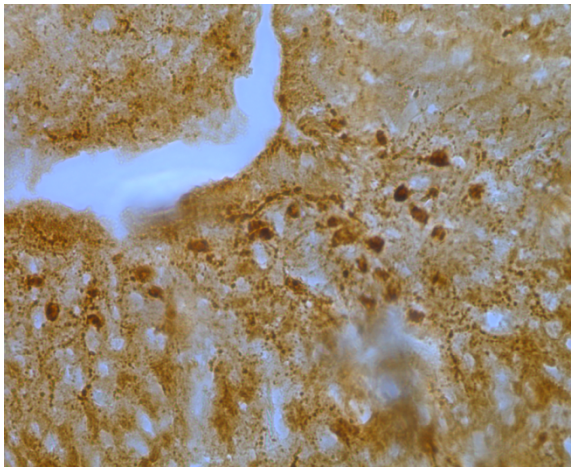
As this study was just one aspect of Ashley Maine's Masters Project, it was of utmost interest to discover what was occurring with the hormones in her study, including NPY, as snakes returned to the den for winter dormancy in the fall. As such snakes were collected in the fall from the road and den as mentioned above. The number of specimens collected in each scenario-- spring mating, spring feeding, fall road, fall den-- were 24 each, resulting in a total number of 96 specimens for this experiment.

It is important to note that this project with NPY was only one of five studies being performed on these 96 organisms. Back in the lab at Portland State University the neural tissue was sliced onto microscope slides in the cryostat for preparation of immunohistochemistry assays. Five sets of slides were made per organism, (each set labeled A, B, C, D, and E), and each set is available for use of separate experiments. This is due to the slices being very thin, 25 nm, and cells within the brain are approximately 125 nm; to avoid double counting of cells and get

the most experimental use out of these organisms five projects are going to be performed, this particular project with NPY being just one of them (using only set C).

The next part of the project required performing a massive immunohistochemistry assay on all of the slides containing neural tissue on one set from the 96 organisms, totaling around 500 slides. This part was quite laborious: it required long hours in the lab, making large quantities or buffers for washes in between applications of solutions to the slides which had to be performed delicately with micropipettes and occasionally cover slipped with Parafilm. An immunohistochemistry assay is a way to visualize cells secreting a particular peptide in a tissue, which after staining will look like solid dark ovals called immunoreactive cells (-ir) (See Figure 1). After staining the cells can be counted to quantify the levels of peptide within the tissue. The assay works by first applying a blocker, usually goat or rabbit plasma, which bind many of the proteins within the tissue that are commonplace to many organisms. This is done to decrease non-specific binding of the primary antibody, which is applied next after washing the tissue. The primary antibody is strongly specific to the protein of interest, NPY in this case. The primary antibody was confirmed to be strongly NPY specific with a preadsorption test which was performed prior to this experiment. In a preadsorption test, decreasing amounts of NPY are added to the primary antibody prior to application to neural tissue. At the highest amount of NPY added to NPY-specific antibody, no NPY-ir cells are visualized due to the added NPY saturating all of the binding sites of the primary antibody. Conversely, as the amount of added NPY is decreased to none, there will be more and more NPY-ir cells visualized on the tissue.

Figure 1: Photomicrograph of NPY-ir cells in the cortex.

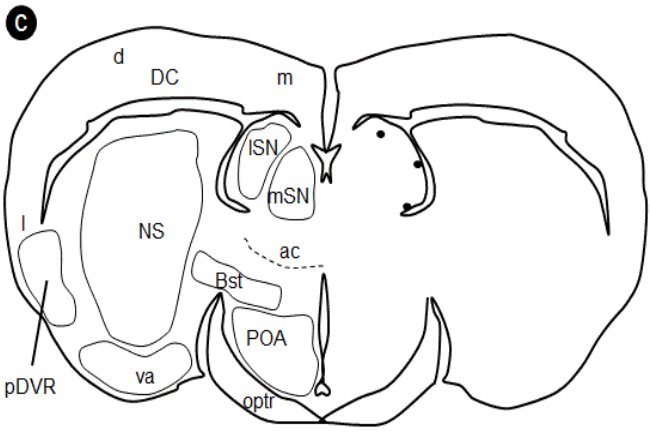


Since it is important for the primary antibody to bind to all of the NPY in the tissue, the slides were cover slipped with Parafilm and incubated for 48 hours with the primary antibody. After incubation and washing the secondary antibody was applied to the tissue. The secondary antibody binds specifically to the primary antibody and contains a chemical component on it, many biotin molecules strongly bound to avidin molecules, each with horseradish peroxidase enzyme attached on the most external aspect of the molecule. This complex is then exposed to 3,3-diaminobenzidine (DAB), which is a chromogen agent that will oxidize in the presence of the peroxidase enzyme and yield a bronze colored precipitate. DAB is highly carcinogenic so proper safety precautions and waste protocols were performed when using this chemical. Since the stain with DAB did not yield a strong enough coloring of NPY-ir cells, an amplification step using biotinylated tyramide was performed. This extra step did create somewhat more background, but clearly distinguishable NPY-ir cells, which was critical for the next stage in this project. The slides were then washed in ethanol and cover slipped using glass covers and para glue.

The next stage in this project was challenging; it required counting and recording all of the NPY-ir cells in three brain regions, the cortex, nucleus sphericus, and pore-optic area

(POA)/hypothalamus, under the microscope after imaging each section. The brain regions were mapped according to anatomical brain sections adapted from (Martinez-Marcos et al., 2001, Martinez-Marcos, 2005, and Khromer et al., 2010) (See Figure 2). Each region had to be counted by a single individual to ensure consistency. The slides were coded to hide sex, season, and location of each organism to keep the counter blind of this information so as not to skew the data. If there were any missing or uncountable sections due to tearing/folding from the assay, the mean using the preceding and proceeding sections was used. If there were more than one consecutive sections missing, either the organism was removed from the study, or an alternative statistical analysis was performed which accounts for this type of situation. I was responsible for counting the cortex, which took me just over six months. The Master's student I worked with, Ashley Maine, counted both the nucleus sphericus and the POA/hypothalamus within 8 months, and that was in addition to all of her duties as a first year Master's student (difficult classes, teaching, other projects in the lab), which was quite impressive.

Figure 2: Three brain regions were quantified shown on the diagram below: the cortex (DC), the nucleus sphericus (NS), and the pre-optic area/hypothalamus (POA).



Results/Conclusion

After all of the data was obtained, statistical analysis of each brain region was performed by Ashley Maine. In the cortex and the POA/hypothalamus a two-way ANOVA followed by a Turkey's multiple comparisons test was performed and in the nucleus sphericus a two-way ANOVA was performed, all with location and season as factors (See Figure 3).

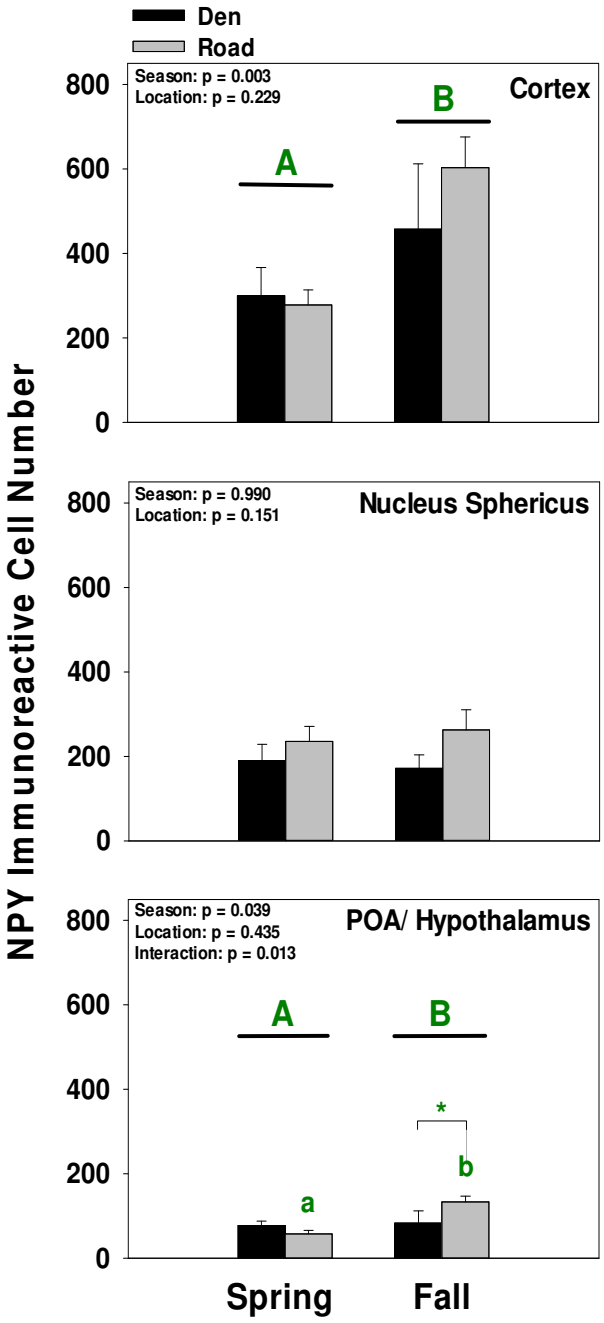
The cortex is the homologue of the mammalian hippocampus, which is involved with spatial navigation. In this study there were significantly more NPY-ir cells in the fall than in the spring. This seasonal difference is likely due to the fact that these organisms have been migrating and moving around the summer feeding grounds for the past three months. They have been using their spatial navigation to and around food, which makes sense as to why NPY cell numbers were higher in the fall compared to spring, in which they had just been hibernating for 8 months.

The nucleus sphericus is the homologue of the mammalian amygdala, which is involved in sexual behavior. There was a trend for snakes collected at the road to have more NPY cell numbers, but this did not reach significance. As this brain region is involved primarily with sexual behavior, this result was not surprising. The reason for visualization of NPY in this region may be due to NPY's neuromodulatory role influencing sexual and social behaviors (Hostetler et al., 2013).

The POA/hypothalamus is responsible for hormone production which governs hunger, sleep, and circadian rhythms, among other processes. In this region there was not only a significant seasonal difference with more NPY-ir cells in the fall, but also within the fall there

were significantly fewer NPY cells in snakes collected at the den versus the road. Since these organisms have been feeding for the past three months it was not surprising that there were more NPY cells in the fall. It was very interesting, however, that there were significantly fewer NPY cells at the den than the road, as this data suggests these organisms are shutting down feeding behavior as they get to the den, which is only 1 km away from the road.

Figure 3: Graphical representation of statistical analysis of NPY-ir cell numbers in the cortex, nucleus sphericus, and POA/hypothalamus at road and den in spring and fall.



Summary/Future Directions

Overall there is a significant seasonal difference in NPY-ir cell numbers. Specifically, snakes have significantly more NPY cells during the fall in the cortex and hypothalamus, regions governing spatial navigation and feeding behavior, respectively. This suggests NPY influences migratory and feeding behavior. Fall snakes have been eating for three months, so it is not surprising that these snakes have more NPY cells.

Within the POA/hypothalamus, snakes on their way *to* the den (collected at road) during the fall have significantly higher NPY cell numbers than those collected *at* the den. These data suggest that snakes have potentially switched off feeding behavior once they reach the den. This switch occurs rapidly as road collected snakes are only 1 km away from the den. This makes sense as these organisms are preparing for an 8 month winter dormancy, but the rate at which it occurs is perplexing. This is especially exciting data as there have not been any other studies looking at how NPY influences preparation for hibernation.

As the focus of this study was the transition from mating to feeding behavior in the spring, it is noted that although there were no significant differences in neural NPY in mating versus feeding snakes in the spring, that NPY is still important in influencing the life history of these organisms, specifically shutting down feeding behavior in preparation for winter dormancy in the fall. Additionally as the collection of feeding snakes in the spring was very close to the den, 1 km, it is thought that perhaps it takes longer to reach significantly higher neural NPY levels. As such the lab team went to the field this spring and collected specimens further away from the den along the migratory path to the summer feeding grounds.

As previously mentioned, there are many chemical messengers affecting this transition from mating to feeding. Another neuropeptide Ashley Maine is looking at is arginine vasotocin (AVT), which is the homologue of mammalian arginine vasopressin. It is mostly known for its role in regulating ion homeostasis, but has also been shown to be responsible for sexual behaviors in many taxa (Godwin 2010, Goodson 2001, Wilczynski et al., 2005). The immunohistochemistry assay for AVT has been performed and manual counting of AVT-ir cells is currently in process. The hypothesis is that there will be more neural AVT in mating snakes than feeding snakes. It will be interesting to see if AVT has more of an impact on this transition than NPY. Regardless, it is known that corticosterone plays a crucial role in the life history transition from mating to feeding behavior in *T. sirtalis.* Additionally, the data from this project shows NPY does influence feeding behavior, and that NPY is critical for switching off foraging and feeding in preparation for winter dormancy. The understanding of such life history transitions at the neuroendocrine level is crucial for developing a model of how hormones influence life history transitions among organisms.

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Animal Care

"Experimental protocols were approved by the Portland State University Animal Care and Use Committee (protocol number psu110824.1) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This research was

performed under the authority of Wildlife Scientific Permit WB12691 issued by the Manitoba Department of Conservation" (Lutterschmidt and Maine 2014).

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