THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF ANIMAL SCIENCE

DETAILED MAPPING OF ADIPONECTIN RECEPTORS IN THE BRAIN OF THE WHITE-THROATED SPARROW (*ZONOTRICHIA ALBICOLLIS*)

KELLY PANDOLPHO
Spring 2014

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Veterinary and Biomedical Sciences
with honors in Animal Sciences

Reviewed and approved* by the following:

Paul A. Bartell
Associate Professor of Avian Biology
Thesis Supervisor and Honors Advisor

Ramesh Ramachandran
Associate Professor of Molecular Endocrinology
Faculty Reader

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

The role of adiponectin in the white throated sparrow and other avian species is poorly understood, despite all of the research regarding adiponectin in both humans and mice. The main objective of this experiment was to determine the locations of adiponectin receptors AdipoR1 and AdipoR2 in the brain of the white-throated sparrow with the intention of providing more information about adiponectin in avian species. I hypothesized that the majority of the receptors would be located in the diencephalon region of the brain, a region most notably involved with hormone secretion and regulation. We found that our results were inconclusive due to limitations with immunohistochemistry, primary antibody choice, and with the narrow-range of available antigen retrieval methods for fresh-frozen tissue. Regardless, this experiment, with proper modifications, will be invaluable in the push to understand how adiponectin regulates biological functions in the avian species.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank Dr. Bartell for allowing me the chance to research in his lab, for his guidance, and for his support. I would also like to thank Dr. Ramachandran for assisting me with my research and reading my paper, as well as the Schreyer Honors College for allowing me this opportunity by accepting me into their program.

INTRODUCTION

When it comes to humans, adipose tissue is often flagged for being undesirable due to the growing concern of obesity. Recently, adiponectin has been regarded as a major component of the secretory portion of the mammalian secretory system (Trayhurn et. al, 2001). Research shows that adipose tissue plays a major role in normal glucose homeostatsis, energy storage, and may even have a role in the inflammatory process of the body (Trayhurn et. al., 2001). In addition, adipose tissue secretes many regulatory proteins that include, but are not limited to, adiponectin, leptin, adipsin, resistin, and visfatin, several cytokines, plasminogen activator inhibitor-1 (PAI-1) and angiotensinogen (Guerre-Millo, 2002) among other products.

As the concern about obesity rises and research reveals the connection between adipose tissue levels, diabetes and hypertension, further research has gone into uncovering the functions of adipocytes. After the discovery of leptin, an adipocyte with a direct correlation between obesity in mice (Frederich, 1995), interest has shifted to adiponectin which has been found in low levels in obese patients and, in particular, insulin-insensitive ones (Koerner, 2005).

Adiponectin

Adiponectin was discovered roughly around the time of leptin, but did not receive any special attention until some years after its discovery (Koerner, 2005). It is a 30kDa adipocytokine hormone (Scherer et. al., 1995; Hu et., al., 1996), and is composed of 244-amino acids (Kishore and Reid, 1999, 2000). In mouse and human plasma, adiponectin circulates throughout the blood as a trimer, hexamer, heavy molecular weight forms, and as small proteolytic cleavage products

(Fruebis et. al, 2001; Kishida et. al., 2003). Structurally, adiponectin belongs to the collagen superfamily and consists of four distinct domains: a collagen-like domain, a C-terminal globular domain, a variable region, and a signal peptide at the N-terminus (Koerner et. al., 2005; Shapiro and Scherer, 1998).

In human plasma, the low molecular weight (LMW) hexamer of adiponectin (180 kDa) and the high molecular weight (HMW) hexamer of adiponectin (400-600 kDa) are the predominant forms of adiponectin molecules (Koerner et. al., 2005), even though there exists three different molecular mass species in both mouse and human plasma: LMW, medium molecular weight (MMW) (180-400 kDa), and HMW (Waki et. al., 2003). It was also found in 2013 by Ramachandran et. al. that chicken adiponectin is predominantly a HMW isoform of mass that may be greater than or equal to 669 kDa (Ramachandran et. al., 2013; Hendricks et. al., 2009).

Adiponectin has been found to be exclusive to mature adipocytes, with higher levels secreted by human adipose tissue (Koerner et. al., 2005). It has been found in both humans and mice that adiponectin levels are decreased in situations of obesity, insulin insensitivity, diabetes and cardiovascular disease (Matsuzawa et. al., 2004). Weight loss results in increased levels of circulating adiponectin and improvements in insulin sensitivity (Matsuzawa et. al., 2004) leading us to believe that adiponectin plays a major role in controlling weight and insulin sensitivity. Adiponectin shows an apparent importance in the body of mammalian species, however, there have been very few studies done on the effect of adiponectin in the body of avian species (Ramachandran et. al., 2013).

Adiponectin Receptors

There are two receptors through which adiponectin signals through: AdipoR1 and AdipoR2 (Yamauchi et. al., 2003). The first receptor to be discovered was AdipoR1 (Yamauchi et. al., 2003). After this discovery, there was a search through the human and mouse databases for any AdipoR1-homologous genes, leading to the discovery of AdipoR2, which had a 68% identity to AdipoR1 (Yamauchi et. al., 2003). It was found that in chickens, chicken AdipoR1 cDNA was 91% homologous to mammalian AdipoR1 (Ramachandran et. al., 2013) and chicken AdipoR2 cDNA was 82% homologous to mammalian AdipoR2 (Ramachandran et. al., 2007). Yamauchi et. al. also discovered that AdipoR1 has a greater binding affinity to the globular domain of adiponectin, while AdipoR2 has a greater binding affinity to both the globular domain and full length adiponectin (Yamauchi et. al., 2003).

Initially, these receptors have been identified to be predominantly located on muscle cells and liver cells in the mouse (Yamauchi et. al., 2003), but also appear to exist throughout the human body (Koerner et. al., 2005). In the chicken, it was found that the AdipoR1 and AdipoR2 genes are expressed in multiple tissues, but AdipoR1 was principally found in the skeletal muscle, adipose tissue, and diencephalon, while AdipoR2 was found mostly in adipose tissue (Ramachandran et. al., 2013).

Adiponectin and the Brain

The signaling molecules activated by adiponectin include AMPK, p38-MAPK and PPARα (Matsuzawa, 2005). AMPK acts more as a downstream component of adiponectin signaling (Thundyil et. al., 2011) and may be involved in vascular endothelial cells and the heart, in a manner that is beneficial in protecting against cardiovascular diseases (Kobayashi et. al.,

2004). Still, very little is known about the effects of adiponectin in the central nervous system. It has been suggested that adiponectin is not present in the brain because it was unable to cross the blood-brain barrier, but this was based on results from studies that could not locate adiponectin (Thundyil et. al., 2011). This was disputed in 2004 when Y. Qi and his colleagues detected adiponectin in the central nervous system (CNS) after an i.v. injection of full-length adiponectin in C57BL/6J mice caused them to lose body weight (Qi et. al., 2004).

There is also evidence that suggests AdipoR1 and AdipoR2 are expressed throughout the brain (Thundyil et. al., 2011). Yamauchi et. al. found in their extensive study that mouse AdipoR1 and AdipoR2 are found in regions of the hypothalamus, brainstem, and endothelial cells, as well as the brain in its entirety and in the pituitary gland (Yamauchi et. al, 2003). Moderate to strong expression of AdipoR1 and AdipoR2 have also been found in the pituitary gland of the rat and in the human; AdipoR1 expression was localized in the lateral hypothalamic area (Psilopanagioti et. al., 2009).

Adiponectin and the White-Throated Sparrow

The white - throated sparrow, as well as other species of song birds, relies heavily on circadian clocks to regulate the components of migration (Bartell and Gwinner, 2005). One such component of migration is pre-migratory fattening, a process that allows the sparrow to continue its flight without having to stop continually for nourishment. This, too, is regulated by biological clocks (Stuber et. al, 2013).

It is understood, controversially, that birds do not produce leptin and therefore need an alternative method to stimulate adiposity and energy sufficiency (Ohkubo and Adachi, 2008). Leptin is a well-characterized hormone best known for its role in lipid metabolism and appetite control in mammals (Prokop, 2014). Leptin in non-mammals, such as birds, may not affect food

intake, or may even not be released from adipose tissue (Huising, 2006; Rajan and Perrimon, 2012; Liu, 2012). It has been shown, however, that birds have a high capacity for lipid biosynthesis and their livers contain four times the amount of lipogenic enzymes than a mammal's liver (Long, 2007). Interestingly, many research groups have attempted to identify the chicken leptin gene and have failed, but many of these groups did find that the chicken leptin receptor is expressed (Ohkubo and Adachi, 2008). As mentioned before, this implies that there must be an alternate mechanism used to maintain fat stores and initiate lipid biosynthesis.

Stuber et. al. found a positive correlation linking the amount of adipose tissue to the level of HMW adiponectin (Stuber et. al., 2013) showing that adiponectin has a close relationship with body fat levels. Stuber et. al. also found that the plasma concentration of adiponectin changed between the non-migratory and migratory periods, suggesting that adiponectin (with other adipokines) may have a role in regulating the changes leading up to migration in the white-throated sparrow (Stuber et. al., 2013).

While the HMW isoform of adiponectin was predominantly detected in sparrow plasma, other isoforms of adiponectin were also detected that have not been observed in the chicken (Stuber et. al., 2013).

METHODS

Preparing Tissue Specimens

The sparrows used in this experiment were housed at the Pennsylvania State Poultry Education Center and were wild-caught. Three sparrow brains were harvested for purpose of this experiment, but only one sparrow's fat score, sparrow number 1 (fat score of 5), was noted according to the subcutaneous fat score classification system developed by Andreas Kiaser (Kaiser, 1993).

The sparrows were overdosed with a ketamine/xylazine mixture and cardiac perfused with .01 M saline-heparine solution (Appendix 1.4). The solution was injected using the MINIPULS 3 (Gilson Inc.). When the sparrow's liver visibly underwent a color change (dark pink to yellow pink) 4% paraformaldehyde (Appendix 1.4) was drawn into the MINIPULS 3 to be circulated throughout the body. A post-flush with PBS (Appendix 1.4) was used to wash out the MINIPULS 3 system. The sparrow's head was then removed for further study.

To remove the brain from the skull, blunt-tipped, curved scissors were used to disconnect muscle tissue from the skull and the optic nerves from the brain. Forceps were then used to carefully chip the skull away from the brain until there was enough room to gently pop the brain from its cavity. Care was taken in ensuring the pineal gland was not lost with the parietal plates during brain's extraction.

The brain tissue, pineal gland, and both eyes were placed in 4% paraformaldehyde (Appendix 1.4) over a 48 hour period to post-fix. The tissue was then removed and placed in PBS buffer for an additional 24 hours before being submerged in a sucrose gradient (10% and 20%, Appendix 1.4) until it was fully saturated. The gradient of sucrose was used to prevent the formation of large ice crystals, which would destroy the delicate brain tissue.

Preparation of Slides

Superfrost microscope slides (Fisherbrand) were subbed with a solution of chromium potassium and gelatin (Appendix 1.4) to provide a better surface for the attachment of brain tissue sections. The slides were dipped three times and left to dry at room temperature over the course of two days.

Prior to sectioning, the tissue was flash-frozen, in a beaker filled with isopentane, over dry ice. It was then carefully wrapped in aluminum foil, labeled, and stored in a -79° C freezer indefinitely.

When all preparations were made, the brain tissue was sectioned at 20 μm thick using a cryostat (Thermo Scientific, Chandlee Laboratory, The Pennsylvania State University). The brain was mounted at a 90° angle closest to the cerebellum and protected by Tissue-Tek CRYO-OCT Compound (Fisher Scientific) before sectioning into coronal sections. Previously subbed slides were pre-warmed at 40° C on a slide warmer to help prevent the tissue from peeling away from the slides after sectioning. An average of six sections fit per slide. Slides were labeled with bird number, slide number and bin number in pencil. Finished slides were placed in a slide box labeled with name, date, and bird number, and stored at 4° C until the staining process.

Immunohistochemistry

The history of immunostaining methods began when J. Marrack created reagents to stain various microorganisms with the use of a red stain conjugated to benzidin tetraedro (Marrack, 1934). Following this milestone in immunohistochemistry was the introduction of enzymes used as marked antibodies, allowing the researcher to view biological reactions through optical microscopy (Nakane et. al., 1966). Discovered in the same period as the unlabeled antibody peroxidase-antiperoxidase (PAP) method by L. Sternberger and his colleagues (Sternberger et. al, 1970) and the alkaline phosphatase-antialkaline phosphatase (APAAP) method by D. Mason and his colleagues (Mason et. al, 1978; Mason et. al, 1978), the diaminobenzidine molecule (DAB) was conjugated to antibodies (Singer, 1959), making it the most popular dye for immunohistological stainings (Luongo de Matos, 2010).

As the research behind immunohistochemistry began to grow, researchers began searching for ways that would allow their target, whether it is a protein structure or infectious agent, to be accurately stained and amplified. Between the years of 1978 and 1981, S Huang and his colleagues discovered various antigen retrieval methods (Huang et. al, 1978) and S. Hsu and his colleagues discovered bioatinylated antibodies (Hsu et. al., 1981) allowing for this improvement to the procedure. As discovered later, however, proper immunoperoxidase treatments were required for the staining of fixed tissues by these methods before the experiments could be successful (Luongo de Matos, 2010).

The use of immunohistochemistry in staining neurological tissue dates back to 1978 (Cuello, 1983). In this experiment, a primary antibody incubated over the course of 46 hours will be marked with a secondary, bioatinylated antibody before stained (Appendix 1.1; Appendix 1.3) The primary antibodies (AdipoR1, AdipoR2, and α-Adiponectin) are custom-made by the Ramachandran lab (Ramachandran et. al., 2013) and were used to pinpoint both adiponectin

receptors and adiponectin itself. The secondary antibody, bioatinylated α-rabbit (Thermo Scientific), was used to further amplify the signal put out by our primary antibodies. In order to minimize false positives, the primary antibody treated slides in 4°C (Appendix 1.1; Appendix 1.3). After treatment with the secondary antibodies, the slides were stained briefly with a DAB solution using $CoCl_2$ to intensify the staining with DAB (10 minutes). The DAB was activated with hydrogen peroxide (H_2O_2) and then the slides were counterstained with a red counterstain (nuclear fast red B salt/ammonium sulfate, 5 minutes) (Appendix 1.3; Appendix 1.4). $NiCl_2$ was taken into consideration when preparing the DAB stain, but was ultimately replaced with $CoCl_2$.

The data obtained from the stained slides was analyzed qualitatively using an Axioskop compound microscope (Zeiss). Densitometry, based on the location and quantity of the receptors, was also used for analysis and captured using the Axioskop's camera and camera software.

DISCUSSION

The first brain in the series of three included many stained blood vessels. This result was noticed in slides that either never got a peroxidase treatment or in the case that the peroxidase treatment did not have the correct hydrogen peroxide to ddH_2O ratio. The brain segments made from brain number two were sliced too thick due to an issue with the cryostat and were removed from the experiment. After examining the slides from brain number three, it was discovered that the staining was either nonexistent or any stained portions appeared to be indistinguishable masses unlike the structures that we were searching for. This could be due to several different factors including immunohistochemistry as a method, the choice of primary antibodies, and choice of antibody retrieval.

One of the major limitations of this study, and of immunohistochemistry in general, involves the researcher conducting the study. The usefulness of solving problems pertaining to the immunostaining of the neuro-anatomy is directly proportionate to the eyes and experience of the hands that run the protocol and interpret the results (Luongo de Matos et. al., 2010). Because of this, the result may appear positive when it may actually be a false-positive. Treatment in a 4°C freezer (Appendix 1.1- Appendix 1.3) is meant to prevent the chances of this being the case, but this is still a possibility. A positive in these tissues is, really, any visual of brown staining (Luongo de Matos et. al., 2010). This was one of the errors in this experiment and required a double and triple look at the slides just to make sure observations were correct. The procedure also becomes more of a trial-and-error when considering the correct antibody dilutions, incubation time, and antigen retrieval method. There were multiple alterations to the current protocol (Appendix 1.3) from the very first protocol (Appendix 1.1) with the outcome still being

inconclusive. Therefore, this experiment is still a work in progress and will still need more adjustments until conclusive data can be obtained and the experiment can be repeated without fail.

The primary antibodies used in this experiment were anti-chicken AdipoR1 and AdipoR2 custom-made by the Ramachandran Laboratory at The Pennsylvania University. While the chicken and sparrow epitopes were similar, there appeared to be enough dissimilarity that prevented staining of the correct structures in the brain tissue. This may be why the staining of the sparrow brain was incredibly weak prior to and even after the antigen retrieval methods (Appendix 1.3). In order to successfully conduct this experiment in the future with the proper staining of the adiponectin receptors, it may be more beneficial to obtain sparrow primary antibodies for adiponectin, AdipoR1 and AdipoR2. In addition, incubation times of both the primary and secondary antibodies may have to be shortened or lengthened until the proper incubation period is discovered.

The antibody retrieval methods were incorporated into the experiment more toward the end. They substantially improved the signal from what it was originally, but it was not enough with all other factors aside to produce the necessary results. A sodium citrate buffer was utilized (provided by the Ramachandran Laboratory, The Pennsylvania State University, Appendix 1.4) as a replacement to a formic acid buffer presented by Kitamoto et. al. (Kitamoto et. al., 1987; Appendix 1.4). Out of the many methods listed for paraffin-embedded tissues, theses were some of the few that were documented for fresh-frozen tissue. Brain tissue in and of itself proved to be very delicate and required a retrieval method that did not use heat. When the sodium citrate buffer retrieval method was used in high heat, the brain tissue melted into the buffer solution. A more effective antigen retrieval method would be needed, specifically for brain tissue, in order to be most successful.

Moving forward with the current protocol (Appendix 1.3) in mind, different experiments can be tested in order to make the proper adjustments. Even though fresh-frozen tissue works better with this experiment, it would be beneficial to attempt the procedure using paraffin-embedded sparrow brain. These results can be compared to previous results using fresh-frozen tissue making it possible to conclude which method is better for this particular experiment. Without changing the way the tissue was collected and prepared, the same experiment can be completed with anti-sparrow primary antibodies instead of anti-chicken antibodies in order to obtain a stronger staining of fixed tissue. It would also be interesting to conduct a western blot on the brain tissue to detect the receptors in the brain before using the immunohistochemistry protocol.

When this experiment is successful, it will be the first of its kind and will show localities of adiponectin routes to specific brain regions. To date, there has been more research done with adiponectin in chickens than in white-throated sparrows and other species of birds. Chabrolle et. al. found that adiponectin could exert an autocrine effect on ovarian steroidogenesis (Chabrolle et. al, 2007) and Ramachandran et. al. found that AdipoR1 and AdipoR2 gene expression in the anterior pituitary of the chicken and adipose tissue are significantly altered by food deprivation (Ramachandran et. al., 2007). It has also been hypothesized that adiponectin in the liver of the chicken may play a regulatory role in lipid metabolism and gluconeogenesis (Ghazanfari et. al., 2010) because the liver is the primary site of lipid synthesis in birds instead of in the adipose tissue, which is where lipid synthesis takes place in mammals (Leveille et. al., 1975). It has also been found that the AdipoR1 gene has been found primarily in the diencephalon of the chicken (Ramachandran et. al, 2013) suggesting that perhaps adiponectin gene expression in the chicken may affect energy homeostasis just as it was demonstrated in mice (Qi et. al., 2004).

What is the most interesting aspect of all of these studies was perhaps the fact that none of them explained how adiponectin travels across the blood-brain barrier even though it has been

implied that adiponectin in the diencephalon portion of the brain has an influence on the body of the chicken. In fact, in 2006, Spranger et. al. found that adiponectin did not cross the blood-brain barrier of the mouse nor was found in the cerebral spinal fluid in humans (Spranger et. al., 2006). What they did find was that adiponectin modifies the release of cytokines from the brain endothelium in both the mouse and human, playing an indirect role on energy balance (Spranger et. al., 2006).

In conclusion, it is not well understood how and if adiponectin regulates lipid metabolism, energy expenditure as well as other biological mechanisms in the avian species. There has been a substantial amount of research regarding chicken adiponectin that may help us hypothesize generalities about all avian species. In mapping the adiponectin receptors in the brain of the white-throated sparrow, we would have a greater understanding of the density and influence that adiponectin plays in the brain of these birds, helping us push forward in the quest to decipher all of adiponectin's functions in the bodies of both migratory and non-migratory birds alike.

APPENDIX

Appendix 1.1: EARLY PROTOCOL (MAY 2013)

Day 1:

1. Treat slides in 4% Paraformaldehyde for 10 minutes on orbital shaker
2. Treat slides in Millipore water (ddH_2O) for 10 minutes on orbital shaker
3. Treat slides in 1x TBS for 10 minutes on orbital shaker
4. Treat slides in TBSX for 10 minutes on orbital shaker
5. Treat slides in 3% goat serum (blocking solution) for 30 minutes at room temperature in humidified chamber
    a. Apply 75 µl of serum per slide
    b. Cover with custom parafilm slide covers
6. Remove slides and apply primary antibodies (anti-chicken ADN, AdipoR1, AdipoR2) to one slide each, not including control
    a. Ratio of antibody to 3% goat serum should be 1µl : 250 µl
    b. Each slide should be treated with 75 µl of antibody solution and cover-slipped with parafilm
    c. Slides should be placed in humidified chamber
7. Humidified chamber containing slides must be placed in 4°C overnight and must remain here for at least 36 hours

Day 2:

8. Wash slides in 1x TBS three times for 10 minutes each on orbital shaker
9. When slides are done, remove parafilm and treat slides with 150 µl 1:300 biotinylated anti-rabbit IgG made in 3% goat serum
    a. Place slides in humidified chamber and let them sit at room temperature for 2 hours with parafilm slide covers
10. Remove slides and wash in 1x TBS three times for 10 minutes each on orbital shaker
    a. Add 10 µl of solution A and B (1:200) from Vector ABC kit to 2 ml TBSX. This must be prepared during the first 1x TBS wash
11. Apply 150 µl of ABC/TBSX solution to slides and place in humidified chamber to sit for 1 hour at room temperature with parafilm slide covers
12. Remove slides and wash them in 1x TBS three times for 10 minutes each on orbital shaker
13. Remove the parafilm and wash slides in 0.1M acetate buffer solution (pH 6) for 30 seconds

{DAB PREPARATION}

80 ml 1x TBS
280 µl DAB
2 ml $NiCl_2$
8 µl H_2O_2 (the very last step)

14. Place slides in the stained coplin jar filled with DAB solution for 10 minutes on orbital shaker
    a. Must be in the dark (DAB is light sensitive)
15. Wash slides in ddH_2O quickly twice and a third time for 10 minutes on the orbital shaker
16. Begin dehydration process of slides
    a. Wash in:
        i. 20% EtOH for 30 seconds
        ii. 50% EtOH for 30 seconds
        iii. 70% EtOH for 30 seconds
        iv. 80% EtOH for 30 seconds
        v. 90% EtOH for 30 seconds
        vi. 95% EtOH for 30 seconds
        vii. 100% EtOH twice for 30 seconds each
        viii. Xylene for 5 minutes, 3 times
17. Coverslip using permount in hood and let dry for at least 48 hours

Appendix 1.2: FLUORESCENCE PROTOCOL (LATE MAY 2013)

Day 1:

1. Wash slides in Millipore water (ddH_2O) for 10 minutes on orbital shaker
2. Wash slides in 1x TBS for 10 minutes on orbital shaker
3. Wash slides in TBSX for 10 minutes on orbital shaker
4. Treat slides with 3% goat serum (blocking solution)
    a. Apply 75 µl to each slide and coverslip with custom parafilm coverslips
    b. Place in humidified chamber for 30 minutes
5. Remove slides and apply primary antibodies (anti-chicken ADN, AdipoR1, AdipoR2) to one slide each, not including control
    a. Ratio of antibody to 3% goat serum should be 1µl : 250 µl
    b. Each slide should be treated with 75 µl of antibody solution and cover-slipped with parafilm

   c. Slides should be placed in humidified chamber
6. Humidified chamber containing slides must be placed in 4°C overnight and must remain here for at least 36 hours

Day 2:

7. Wash slides in 1x TBS three times for 10 minutes each on orbital shaker
8. Apply each slide with 35µl of 1:200 µl Alexa Fluor 568 anti-rabbit IgG mixed with 3% goat serum and cover with parafilm coverslip (in DARK)
   a. Let sit for 2 hours at room temperature (humidified chamber) in the DARK
9. Wash slides in 1x TBS three times for 5 minutes each on orbital shaker (in DARK)
10. Coverslip with Prolong Gold Antifade Reagent with DAPI (in DARK)
    a. Allow slides to dry overnight in flat slide box (important to keep them out of the light)

Appendix 1.3: ADJUSTED/CURRENT PROTOCOL (JUNE 2013—MARCH 2014)

Day 1:

18. Treat slides in 1x TBS for 10 minutes on orbital shaker
19. Perform antigen retrieval on slides in sodium citrate buffer (pH 6.0)
    a. Heat up sodium citrate to 50° C in microwave prior to immersing slides
    b. Submerge slides in buffer and allow them to incubate for 20 minutes
20. Submerge slides in peroxidase treatment (.1% H_2O_2) for 20 minutes in the DARK on orbital shaker
21. Wash slides in ddH_2O for 10 minutes on orbital shaker
22. Wash slides in TBSX for 10 minutes on orbital shaker
23. Treat slides in 8% goat serum (blocking solution) for 30 minutes at room temperature in humidified chamber
    a. Apply 150 µl of serum per slide
    b. Cover with custom parafilm slide covers
24. Remove slides and apply primary antibodies (anti-chicken ADN, AdipoR1, AdipoR2) to one slide each, not including control slide
    a. Ratio of antibody to 3% goat serum should be 1µl : 250 µl
    b. Each slide should be treated with 75 µl of antibody solution and cover-slipped with parafilm

    c. Slides should be placed in humidified chamber
25. Humidified chamber containing slides must be placed in 4°C overnight and must remain here for at least 36 hours

Day 2:

26. Wash slides in 1x TBS three times for 10 minutes each on orbital shaker
27. When slides are done, remove parafilm and treat slides with 150 µl 1:200 biotinylated anti-rabbit IgG made in 3% goat serum
    a. Place slides in humidified chamber and let them sit at room temperature for 2.5 hours with parafilm slide covers
28. Remove slides and wash in 1x TBS three times for 10 minutes each on orbital shaker
    a. Add 10 µl of solution A and B (1:200) from Vector ABC kit to 2 ml TBSX. This must be prepared during the first 1x TBS wash
29. Apply 150 µl of ABC/TBSX solution to slides and place in humidified chamber to sit for 1 hour at room temperature with parafilm slide covers
30. Remove slides and wash them in 1x TBS three times for 10 minutes each on orbital shaker
31. Remove the parafilm and wash slides in 0.1M acetate buffer solution (pH 6) for 30 seconds

    {DAB PREPARATION}

    40 ml 1x TBS
    200 µl DAB
    100 µl $CoCl_2$
    60 µl H_2O_2 (the very last step)

32. Place slides in the stained coplin jar filled with DAB solution for 10 minutes on orbital shaker
    a. Must be in the dark (DAB is light sensitive)
33. Wash slides in red counterstain for 5 minutes on orbital shaker
34. Wash slides in ddH_2O quickly twice and a third time for 10 minutes on the orbital shaker
35. Begin dehydration process of slides
    a. Wash in:
        i. 20% EtOH for 30 seconds
        ii. 50% EtOH for 30 seconds
        iii. 70% EtOH for 30 seconds
        iv. 80% EtOH for 30 seconds
        v. 90% EtOH for 30 seconds
        vi. 95% EtOH for 30 seconds
        vii. 100% EtOH twice for 30 seconds each
        viii. Xylene for 5 minutes, 3 times

36. Coverslip using permount in hood and let dry for at least 48 hours

Appendix 1.4: MATERIALS USED (2013-2014)

10% Sucrose, 300 ml

- Add 30g sucrose to 300 ml ddH_2O
- Apply low heat (5° C) to assist in dissolving

20% Sucrose, 300 ml

- Add 60g sucrose to 300 ml ddH_2O
- Apply low heat (5° C) to assist in dissolving

.01 M Saline-Heparine, 200 ml

- Make the saline solution first by adding .1186g NaCl to 200 ml ddH_2O
- Add .1111 mg heparine to .01M saline and stir until dissolved

4% Paraformaldehyde

- Pour 500 ml ddH_2O into a glass container and add 40g paraformaldehyde
- Place in the hood on a hot plate, with the cap open, and heat to 60° C (check solution periodically)
- When most of the paraformaldehyde dissolves into solution, filter off the remainder and add 100ml PBS buffer
- Test pH and adjust to pH 7.5

Acetate Buffer

- 5.72 ml acetic acid added to 994.28 ml ddH_2O
- Test pH and adjust to pH 6.2

TBS Stock Solution (10x)

- Measure out 14.184 g Tris-HCl and 78.894 g NaCl
- Add both to 1000 ml ddH_2O and dissolve using heat and a stir bar
- Test pH and adjust to pH 7.4

TBSX

- Take 100 ml of 10x TBS and mix it with 900 ml ddH_2O to make 1x TBS
- Pour 500 ml of 1x TBS into a separate container and add 500 µl Triton-X100

TBSX + 3% Goat Serum (diluent)

- Pour 15 ml of TBSX into an empty centrifuge tube
- Add 450 µl of goat serum

TBSX + 8% Goat Serum (blocker)

- Pour 15 ml of TBSX into an empty centrifuge tube
- Add 1.2 ml of goat serum

PBS Buffer (1x)

- Measure out 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g KH_2PO_4
- All parts must be added in series or they won't go into solution
- Fill a glass container with 800 ml ddH_2O and place on a burner
- First add the NaCl and stir until all is dissolved
- Then add the KCl, mix until dissolved, and follow this pattern with the last 2 additives
- Test the pH and adjust to pH 7.4
- Adjust the volume to 1000 ml with ddH_2O

Red Counterstain (nuclear fast red B salt/ammonium sulfate)

- Measure out .1g Nuclear Fast Red B Salt and 5g of ammonium sulfate
- Heat 100 ml ddH_2O on a hot plate and add the ammonium sulfate
- Allow to dissolve
- When the ammonium sulfate dissolves, add the red B salt
- Heat the mixture to a boil before allowing it to cool
- Store covered because light damages the dye

Peroxidase Solution

- Add 10ml .1% H_2O_2 (100 ml ddH_2O and 333 µl H_2O_2) to 50 ml methanol

Chromium Potassium Sulfate and Gelatin (.05% and .5% respecitively)

- Measure out 2.5 g gelatin, .25 g CPS (chromium potassium sulfate), and .375 g sodium azide
- Heat 250 ml ddH_2O to around 55° C before adding gelatin
- Add gelatin to heated ddH_2O
- After gelatin dissolves, add CPS and sodium azide to solution
- When everything is dissolved, filter the solution

- Add another 250 ml of ddH_2O

Sodium Citrate Buffer (10mM Sodium Citrate)

- Measure out 2.94g Trisodium citrate (dehydrate)
- Add this to 1000 ml ddH_2O and mix to dissolve
- Test pH and adjust to pH 6.0
- Add 0.5 ml of Tween 20 and mix well

Formic Acid (for antigen retrieval)

- Add 20 ml 88% formic acid to 80 ml ddH_2O
- Mix well
- Test pH and adjust to pH 1.6
- Store at room temperature

PBS Tween (formic acid retrieval)

- Add 250 µl polysorbate 20 (Tween 20) to 250 ml PBS
- Mix well

REFERENCES

Bartell, P.A., Gwinner, E. (2005) A separate circadian oscillator controls nocturnal migratory restlessness in the songbird *Sylvia borin*. Journal of Biological Rhythms; 20: 538-549.

Chabrolle, C., Tosca, L., Crochet, S., Tesseraud, S., Dupont, J. (2007) Expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in chicken ovary: potential role in ovarian steroidogenesis. Domestic Animal Endocrinology; 4: 480-487.

Cuello, A.C. *Immunohistochemistry.* Great Britain: The International Brain Research Organization, 1983. Print.

Frederich R.C., Hamann A., Anderson, S., Lollmann, B., Flier J.S. (1995) Leptin levels reflect body lipid content in mice: evidence for diet induced resistance to leptin action. Nature Medicine; 12: 1311-4.

Fruebis, J., Tsao, T.S., Javorschi, S., Ebbets-Reed, D., Erickson, M.R., Yen, F.T., Bihain, B.E., Lodish, H.F. (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proceedings of the National Academy of Sciences of the United States of America; 98: 2005-2010.

Ghazanfari, S., Nobari, K., Yamauchi, T. (2010) Adiponectin: A Novel Hormone in Birds. Asian Journal of Animal and Veterinary Advances; 6: 429-439.

Guerre-Millo, M. (2002) Adipose Tissue Hormones. Journal of Edocrinological Investigation; 10: 855-861.

Hendricks 3rd, G.L., Hadley, J.A., S.M. Krzysik-Walker, S.M., Prabhu, K.S. Vasilatos-Younken, R., Ramachandran, R. (2009) Unique profile of chicken adiponectin, a predominantly heavy molecular weight multimer, and relationship to visceral adiposity. Endocrinology; 150: 3092–3100.

Hsu S.M., Raine L, Fanger H. (1981) A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. American Journal of Clinical Pathology; 75: 734–738.

Huang SN, Minassian H, More JD. (1976) Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. Oxford Handbook of Clinical and Laboratory Investigation; 35: 383–390.

Huising, M.O., Geven, E.J., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H. (2006) Increased leptin expression in common Carp (Cyprinus carpio) after food intake but not after fasting or feeding to satiation. Endocrinology; 147: 5786–5797.

Hu, E., Liang, P., Spiegelman, B.M. (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. Journal of Biological Chemistry; 271: 10697-10703.

Kaiser, A. (1993) A New Multi-Category Classification of Subcutaneous Fat Deposits of Songbirds. Journal of Field Ornithology; 64: 246—255.

Kiamoto, T., Ogomori, K., Tateishi, J., Prusiner, S.B. (1987) Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Oxford Handbook of Clinical and Laboratory Investigation; 2: 230-236.

Kishida, K., Nagaretani, H., Kondo, H., Kobayashi, H., Tanaka, S., Maeda, N., Nagasawa, A., Hibuse, T., Ohashi, K., Kumada, M., Nishizawa, H., Okamoto, Y., Ouchi, N., Maeda, K., Kihara, S., Funahashi, T, Matsuzawa, Y. (2003) Disturbed secretion of mutant adiponectin associated with the metabolic syndrome. Biochemical and Biophysical Research Communications; 306: 286–292.

Kishore, U., Reid. K.B. (1999) Modular organization of proteins containing C1q-like globular domain. Immunopharmacology; 42: 15–21.

Kishore, U., Reid. K.B. (2000) C1q: structure, function, and receptors. Immunopharmacology;

49: 159–170.

Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y et al. (2004). Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. Circulation Research; 94: E27–E31.

Koerner, A., Kratzsch, J., Kiess, W. (2005) Adipocytokines: leptin—the classical, resistin—the controversial, adiponectin—the promising, and more to come. Best Practice & Research Clinical Endocrinology & Metabolism; 19: 525-546.

Leveille, G.A., Romsos, D.R., Yeh, Y., O'Hea, E. K. (1975) Lipid biosynthesis in the chick. A consideration of site of synthesis, influence of diet and possible regulatory mechanisms. Poultry Science; 54: 1075-1093.

Liu, Q., Dalman, M., Chen, Y., Akhter, M., Brahmandam, S. (2012) Knockdown of leptin A expression dramatically alters zebrafish development. General Comparative Endocrinology; 178: 562–572.

Long, J.A. (2007) Hormonal and cellular mechanisms of fattening in migratory songbirds. PhD. Thesis. The University of Maine: U.S.A.

Luongo de Matos, L., Trufelli, D.C., Luongo de Matos, M.G., Aparecida da Silva Pinhal, M. (2010) Immunohistochemistry as an important tool in biomarkers Detection and Clinical Practice. Biomark Insights; 5: 9-20.

Marrack J. (1934). Nature of antibodies. Nature; 133: 292–293.

Mason DY, Sammons R. (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. Journal of Clinical Pathology; 31:454–460.

Mason DY, Sammons R. (1978) Rapid preparation of peroxidase: anti-peroxidase complexes for immunocytochemical use. Journal of Immunological Methods; 20:317–324.

Matsuzawa Y, Funahashi T, Kihara S. (2004) Adiponectin and metabolic syndrome.

Arteriosclerosis, Thrombosis and Vascular Biology; 24: 29–33.

Matsuzawa Y (2005). Adiponectin: identification, physiology and clinical relevance in metabolic and vascular disease. Atherosclerosis; 6: 7–14.

Nakane PK, Pierce GB., Jr (1966) Enzyme-labeled antibodies: preparation and application for the localization of antigens. Journal of Histochemistry and Cytochemistry; 14: 929–931.

Ohkubo, T., Adachi, H. (2008) Leptin signaling and action in birds. The Journal of Poultry Science; 45: 233-240.

Prokop, J.W., Schmidt, C., Gasper, D., Duff, R.J., Milsted, A., Ohkubo, T., Ball, H.C., Shawkey, M.D., Mays, H.L., Jr., Cogburn, L.A., Londraville, R.L. (2014) Discovery of the elusive leptin in birds: identification of several 'missing links' in the evolution of leptin and its receptor. PLOS One; http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0092751.

Psilopanagioti A, Papadaki H, Kranioti EF, Alexandrides TK, Varakis JN (2009). Expression of adiponectin and adiponectin receptors in human pituitary gland and brain. Neuroendocrinology; 89: 38–47.

Qi, Y., Takahaski N., Hileman, S.M., Patel, H.R., Berg, A.H. Pajvani, U.B. (2004). Adiponectin acts in the brain to decrease body weight. Nature Medicine; 10: 524-529.

Rajan, A., Perrimon, N. (2012) Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. Cell; 151: 123–137.

Ramachandran, R., Ocon-Grove, O.,.M., Metzger., S.L. (2007) Molecular cloning and tissue expression of chicken AdipoR1 and AdipoR2 complementary deoxyribonucleic acids. Domestic Animal Endocrinololgy; 33: 19–31.

Ramachandran, R., Maddineni, S., Ocon-Grove, O., Hendricks, G. 3rd, Vasilatos-Younken, R., Hadley, J.A. (2013) Expression of Adiponectin and its Receptors in Avian Species. General and Comparative Endocrinology; 190: 88-95.

Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., Lodish, H.F., (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. Journal of Biological Chemistry; 270: 26746-26749.

Shapiro, L., Scherer, P.E. (1998). The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. Current Biology; 8: 335–338.

Singer, S.J. (1959) Preparation of an electron-dense antibody conjugate. Nature; 183: 1523–1524.

Spranger, J., Verma, S., Gohring, I., Bobbert, T., Seifert, J., Sindler, A.L., Pfeiffer, A., Hileman, S.M., Tschop, M., Banks, W.A. (2006) Adiponectin does not cross the blood-brain barrier but modified cytokine expression of brain endothelial cells. Diabetes; 55: 141-147.

Sternberger, L.A., Hardy, P.H., Jr, Cuculis, J.J. (1970) The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. Journal of Histochemistry and Cytochemistry; 18: 315–333.

Stuber, E.F., Verpeut, J., Horvat-Gordon, M., Ramachandran, R., Bartell, P.A. (2013). Differential regulation of adipokines may influence migratory behavior in the white-throated sparrow (*Zonotrichia albicollis*). PLOS One; 8: 1-10.

Thundyil, J., Pavlovski, D., Sobey, C.G., Arumugam, T.V. (2011) Adiponectin receptor signaling in the brain. British Journal of Pharmacology; 165: 313-327.

Trayhurn, P., Beattie, J.H. (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proceedings of the Nutrition Society; 60: 329-339.

Yamauchi T., Kamon J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature; 423: 762-769.

ACADEMIC VITA

Kelly Pandolpho
201 Vairo Blvd, Apt 306B
State College, PA 16803
kep5239@psu.edu

Education:

- **The Pennsylvania State University, University Park**
  - B.S. in Veterinary and Biomedical Sciences with Honors in Animal Sciences
  - Expected date of graduation: May 2014

Extracurricular Activities:

- Member of THON (2010—2012)
- Society of the Arts (2010—2012)
- Lion Ambassadors (2011-2012)
- Pre-Vet Club (2013—Present)

Experience:

- **Marsh Animal Hospital, Verona, NJ, STUDENT INTERN (with Dr. Milwicki)**
  - Shadowed from 9 AM – 6 PM (Summer 2009)
    - Learned to act professionally in the workplace
    - Oversaw spaying, neutering and teeth cleaning
    - Learned surgery protocol
- **The Raptor Trust, STUDENT INTERN**
  - Every Friday, 8 AM—12 NOON (Summer 2011/2012)
    - Cleaned the cages of various bird species
    - Prepared diet-specific meals weekly
    - Worked outside in all weather conditions
    - Mastered handling small birds and taught to respect an animal's space
- **Holistic Pet Care, Little Falls, NJ, STUDENT INTERN (with Dr. Buchoff)**
  - Summer (2011), 2 PM—6 PM
    - Veterinary technician
    - Learned how to use a centrifuge
- **PAWS Animal Shelter, STUDENT INTERN**

- Fall 2012-Present
  - Volunteered in the Dog Wing
    - Administered food and water
    - Took dogs on walks and socialized with them

- **Laboratory Tech, Research at Penn State, Bartell Lab**
  - March 2013-Present
    - Trained with pipettes
    - Learned how to prepare tissue on slides for view on the microscope
    - Worked with tissue samples from the white-throated sparrow; studied adiponectin

- **Central Pennsylvania Veterinary Emergency Treatment Services, STUDENT INTERN (with Dr. Allgeier)**
  - Summer 2013 (Started May 11th)—Present
    - Observed treatment of different species in an emergency setting

Honors:

- Chimera Research, Penn State Hazleton Research Fair, 2011
- Recipient:
  - *Most Outstanding Female Student Athlete Award* (High School, 2010);
  - *Academic Achievement Award* (2011)
- Scholarship Recipient:
  - *Chancellor's Award* (2010-2011),
  - *Schreyer Honors College Scholarship* (2013-2014);
  - *Rosie and Stuart Kahan Scholarship in Animal Health in Memory of Lawrence Kahan* (2013-2014)