ABSTRACT

Estrogen protects females from hypertension. The nucleus tractus solitarius (NTS) is a hindbrain site involved in the regulation of blood pressure, however little is known about estrogen receptors within the NTS. The purpose of these studies was to determine the phenotype of the cells expressing estrogen alpha receptors in the nucleus tractus solitarius.

Four female Sprague-Dawley rats were transcardially perfused with 4% paraformaldehyde and hindbrains harvested. In coronal sections containing the NTS (40μm thick), immunohistochemistry was performed to determine which type of cells were expressed with estrogen receptor alpha (ERα) expressing cells. We used the anti-ERα antibody with an antibody for each protein of interest: anti-tyrosine hydroxylase (TH), anti-glial fibrillary acidic protein (GFAP), anti-NeuN, and anti-Iba-1. Sections were captured using an Olympus BX41 Fluorescence Microscope and analyzed using ImageJ. The NTS was divided into 2 regions: sections caudal to the area postrema (caudal) and sections lying below the area postrema (sub-postrema, SP) and the number of immunoreactive neurons in each region counted and expressed as an average number of labeled neurons per section±SEM. The number of sections analyzed ranged from 5-10 per individual in caudal and 2-4 per individual in SP. At sacrifice, females were in estrus (1), diestrus (2) or proestrus (3).

NeuN in SP NTS (n=4) was observed in 151±53 and ERα in 50±21 neurons per section. Colocalization of ERα and NeuN in SP NTS was observed in 11±6 neurons per section (about 7%). NeuN in caudal NTS was observed in 59±7 and ERα 27±3 neurons per section. Colocalization of ERα and NeuN in caudal NTS was observed in 4±1 neurons per section (about 7%).

TH in SP NTS (n=6) was observed in 49±8 and ERα in 51±12 neurons per section. Colocalization of ERα and TH in SP NTS was observed in 26±4 neurons per section (about 53%). TH in caudal NTS was observed in 26±6 and ERα 29±7 neurons per section. Colocalization of ERα and TH in caudal NTS was observed in 17±4 neurons per section (about 51%).

Due to the quantity and shape of GFAP immunoreactive cells in the NTS (n=4), we were not able to count the number cells. Colocalization of ERα and GFAP expressing cells were not observed in our study. Cells expressing Iba1 were not observed in the later trials of our study (n=4).

ERα is expressed on a subset of catecholaminergic NTS neurons, as well as non-catecholaminergic neurons. Since the NTS catecholaminergic neurons contribute to responses to stress (e.g., hypoxia), this finding could provide a substrate for estrogen-mediated cardiovascular protection in females.

THE PHENOTYPE OF CELLS EXPRESSING

ESTROGEN RECEPTOR ALPHA

IN THE NUCLEUS OF THE

SOLITARY TRACT

Christopher L. Horn, B.S.

APPROVED:

_____
Steve Mifflin, Ph.D., Major Professor

_____
Ann Schreihofer, Ph.D., Committee Member

_____
Tom Cunningham, Ph.D., Committee Member

_____
Nicole Phillips, Ph.D., University Member

_____
Steve Mifflin, Ph.D., Department Chair

_____
Johnny He, Ph.D., Interim Dean
Graduate School of Biomedical Sciences

A PRACTICUM REPORT

THE PHENOTYPE OF CELLS EXPRESSING ESTROGEN RECEPTOR ALPHA IN THE NUCLEUS OF THE SOLITARY TRACT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial fulfillment of the requirements

For the degree of

Master of Science

By

Christopher L. Horn

Fort Worth, Texas

April 9, 2018

ACKNOWLEDGEMENTS

This work was supported by National Institute of Health P01 HL-088052.

Special thanks to Dr. Steve Mifflin, Dr. Lei Wang, Joel Little, Dianna Nguyen

Committee Members:

Dr. Steve Mifflin

Dr. Tom Cunningham

Dr. Ann Schreihofer

Dr. Nicole Phillips

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 15. Bar graph comparing the ERα expression between SP and caudal sections in sections that were stained for ERα and Iba1.

CHAPTER I. BACKGROUND AND LITERATURE

Function of the NTS

The nucleus of the solitary tract is located in the hindbrain and is the integrating center for multiple viscerosensory processes and mechanisms of control for cardiovascular, respiratory, gustatory, hepatic and renal systems. The circuitry of the NTS has been the focus of various research, yet still remains a complex part of the brain. It is a relay center for the autonomic nervous system that plays a large part in the regulation of blood pressure through the baroreflex. The NTS has been described as the first central relay for visceral afferents (Lawrence & Jarrott, 1996) and is the focal point of much research for cardiovascular reflex control. The NTS receives many different projections from different nuclei all over the brain that all could play part in the mechanisms of cardiovascular control. Many studies have demonstrated the abilities of such projections to affect the reactivity of neurons in the NTS.

Within the NTS there are different cell types, and several subtypes among those. In addition to receiving a variety of afferent inputs, NTS neurons can also release a variety of neurotransmitters, both excitatory and inhibitory. We know that the NTS has catecholaminergic, GABAergic, glutamatergic, cholinergic, serotonergic, vasopressinergic, as well as opioidergic neurons and many more (Lawrence & Jarrott, 1996). The type of neuron that expresses estrogen receptors observed in our study could help give insight on which proteins or mechanism is part of the protective effect seen with estrogen. Estrogen has been shown to have different effects on neurons. Estrogen activates signaling cascades through modulations of L-type calcium channels (Vega-Vela et al., 2017). Estradiol (E2) also acutely potentiates glutamatergic synaptic transmission in the hippocampus through both presynaptic and postsynaptic mechanisms. E2 increases the amplitude and frequency of spontaneous excitatory post-synaptic potential currents

in hippocampal neurons in both males and females (Oberlander & Woolley, 2016). Ovarectomized females were given estradiol treatment and were observed to have modulated spontaneous and evoked neuronal responses (Woolley, 1999).

The other cell type of interest is glia. Glia have multiple functions in the brain and also consists of several subtypes. They play a variety of support roles and help protect neurons. Estrogens have been shown to act on glia and activate astrocytic extension in the hypothalamus. The expression of estrogen as well as other androgen receptors on glial cells, astrocytes, has been observed in the hippocampus in lesioned brains (Jäkel & Dimou, 2017). Estradiol also increases the production of GFAP and promotes astrocyte differentiation in both the hypothalamus and hippocampus (García-Ovejero, Veiga, García-Segura, & Doncarlos, 2002). It is not known if receptors are expressed in glial cells in the NTS.

Microglia also helps protect the brain by surveying brain tissue and removing damaged and dying cells through phagocytosis. They are responsible for synaptic pruning during development and help with synaptic transmission (Sousa, Biber, & Michelucci, 2017). Initially it was assumed that estrogen-microglia and estrogen-astrocyte activation was not direct since researched suggested estrogen receptors were predominantly located on neurons. However, recent evidence suggests that there is a direct relationship between estrogen and glial cells, specifically, astrocytes and microglia. Estrogen receptors have been observed in microglia and has shown to have direct effects through activation and inhibition (Drew & Chavis, 2000).

Phenotyping cells in the NTS which have ERs could help indicate the functions of those cells which are modulated by estrogen.

Estrogens and the Estrous Cycle

Estrogens play several important roles in the body and especially areas of the brain. Estrogen also has effects on the cardiovascular system that are mediated through estrogen receptors (Mendelsohn, 2002). Similar to other mammals, rats undergo sex hormone (estrogen, progesterone and luteinizing hormone) cycles accompanied by changes in their vaginal epithelial cell structure. The estrous cycle for rats lasts about 4 to 5 days in length and begins about 32 to 36 days postnatal. A vaginal smear is used to characterize the cell structures to correlate what phase of the estrous cycle the rat is in. The cellular alterations are linked to changes in circulating concentrations of sex steroids and gonadotropins. In a 4-day cycle, the estrous cycle begins with proestrus for 1 day, 2 days in a 5-day cycle, followed by vaginal estrus. The estrus phase is distinct and most identifiable by the presence of many needle-like cornified (or keratinized) cells (Goldman, Murr, & Cooper, 2007). Metestrus is the next phase that is described as the transitional period during the first few hours of the first day of diestrus and is therefore also described as diestrus 1. The second day, day 4, of diestrus is known as dietrus 2 which is the last phase of the estrous cycle before it repeats. It is rising levels of circulating estradiol that initiates the keratinization of the vaginal epithelial cells that are seen during the estrus phase. This increase in estradiol up-regulates hypothalamic mechanisms that eventually lead to the surge in Luteinizing Hormone. This leads to assume there could also be an upregulation in estrogen receptors in other areas of the brain that express estrogen receptors.

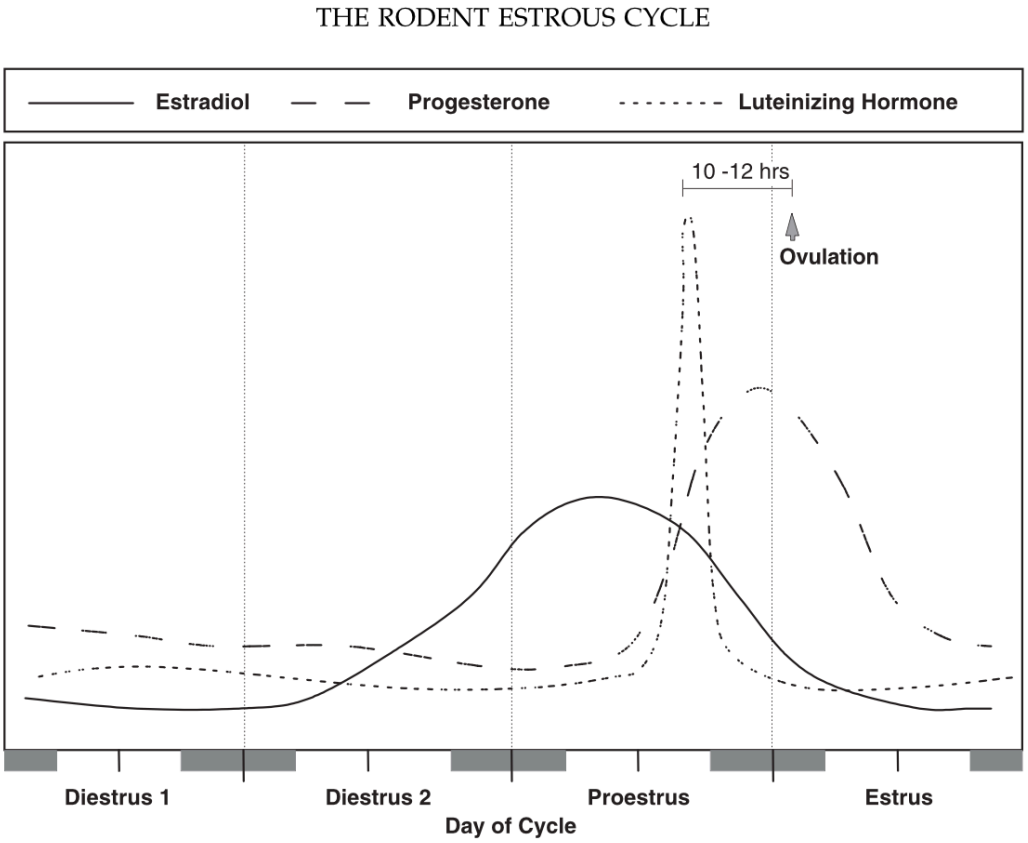


Figure 1. Estradiol, progesterone, and Luteinizing Hormone cycle as it relates to the Estrous Cycle and its phases.

Estrogen receptors

There are at least two types of estrogen receptors, ERα and ERβ, and there have been studies documenting the different areas of the brain expressing these receptors. For example, Mendoza-Garcés et al. 2011 compared the differential expression of those two estrogen receptors and how their expression was altered during the estrous cycle. They examined areas of the hippocampus using immunohistochemistry and found that the highest number of ERα expressing cells in the CA1 region was observed during the estrous phase. That expression pattern was different in the CA3 regions where the highest number of ERα expressing cells was observed during the metestrus phase.

Protective effects of estrogen in HT

Estrogen's beneficial effects have been demonstrated by numerous studies, many of which described how the incidence of hypertension and cardiovascular disease is much lower in premenopausal females compared to age matched males (Spary, Maqbool, & Batten, 2009). We know that there are sex differences in mean arterial pressure and heart rate responses to intermittent hypoxia in rats and that it may be due to the role sex hormones play in stressor response. It has been shown that in an intermittent hypoxia hypertension model, ovary intact female rats are protected from the hypertensive and tachycardia responses of the intermittent hypoxia (Hinojosa-Laborde & Mifflin, 2005). Once the females were ovarectomized this protection was removed. Estradiol can inhibit excitatory amino acid induced neuronal activity in the nucleus tractus solitaries (Xue & Hay, 2003), which may be an explanation towards how the decreased incidence of increased BP in female rats. Estrogen also promotes vasodilation of vascular smooth muscle cells through the activation of ERα on smooth muscle cells, as well as endothelial cells, which stimulates the production of nitric oxide. Estrogen also protects against hypertension by preventing atherosclerotic plaque formations. E2 inhibits the activation of neutrophils and monocytes so there is no adhesion onto the vascular endothelium to elicit an inflammatory response (Boese, Kim, Yin, Lee, & Hamblin, 2017). The question remains, if estrogen has protective effects in premenopausal women, and the NTS is the first center in the brain to integrate blood pressure reflexes, on what cells within the NTS are estrogen receptors expressed? Describing of the phenotype of the NTS cells expressing estrogen receptors might provide an anatomical substrate for the cardiovascular protective effects of estrogen.

CHAPTER II. RESEARCH PROJECT

The goal of my project was to phenotype estrogen receptor expressing cells in the nucleus tractus solitarius. This was done by performing immunohistochemical labeling experiments on the brains of 6 different female rats. The phase of estrous cycle for each female rat was determined before harvesting the brains. After slicing the brains into four different sets of sections, each underwent a different IHC experiment to double label the cells for ERα and: NeuN, TH, GFAP, and Iba1.

SPECIFIC AIMS

The aim of this study was to determine the phenotype of the cells expressing estrogen alpha receptors in the nucleus tractus solitarius.

SIGNIFICANCE & INNOVATION

There is little to no description of neurons expressing estrogen receptors in the NTS. Hopefully this information will provide a basis for future studies on the mechanism by which estrogen acts to help protect premenopausal women from hypertension and related cardiovascular diseases.

MATERIALS AND METHODS

General

The brains of 6 adult female Sprague-Dawley rats were harvested for analysis for this experiment. The rats were anesthetized with 0.1ml/100g of Inactin and their state of estrous was determined before sacrifice with a vaginal smear by lavage. Rats were then tanscardially

perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Brains were immediately fixed in 4% paraformaldehyde for 24 hr at 4 ºC. After the brains were fixed they were transferred to a 30% sucrose solution for three days. Four sets of coronal 40 micrometer sections were collected in cryoprotectant fluid and stored at -20 ºC until undergoing immunohistochemistry. In order to phenotype the cells expressing estrogen receptor alpha (ERα), the polyclonal anti-ERα antibody (1:5000, Millipore) was used with either monoclonal anti-tyrosine hydroxylase (TH) antibody (1:1000, Millipore), monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Ms-1:500, Sigma), monoclonal anti-NeuN (1:1000, Millipore), or monoclonal anti-Iba-1 antibody (1:400, Abcam). Primary antibodies were incubated in the tissue overnight at -4 degrees C. The secondary antibodies used for all sets was a cocktail of secondary antibody Alexa Fluor 488 donkey anti-mouse (1:2000, Jackson Lab) to label NeuN, TH, GFAP, and Iba1 proteins. The secondary antibody Cy3 donkey anti-rabbit (1:2000, Jackson Lab) was used to label the ERα labelled sections. The secondary antibodies incubated with the tissue sections for 1 hour before the slices were mounted and cover slipped to be imaged. All antibodies were used and verified in previous studies in the lab (Bathina et al., 2013) (Lopez, 2017).

Imaging and cell counts

Imaging of the double labelled cells in the NTS was performed using an Olympus BX41 Fluorescence Microscope. Image J software was used to count the number of ERα, TH, GFAP, NeuN, and Iba-1 immunoreactive cells in 2 to 10 sections for the NTS and the cells that co-localized with ERα and the protein of interest. The NTS was divided into two regions: sections that were caudal to the area postrema and sections lying below the area postrema (sub-postrema

SP). The number of sections analyzed ranged from 5-10 per individual in the caudal region and 2-4 per individual in SP.

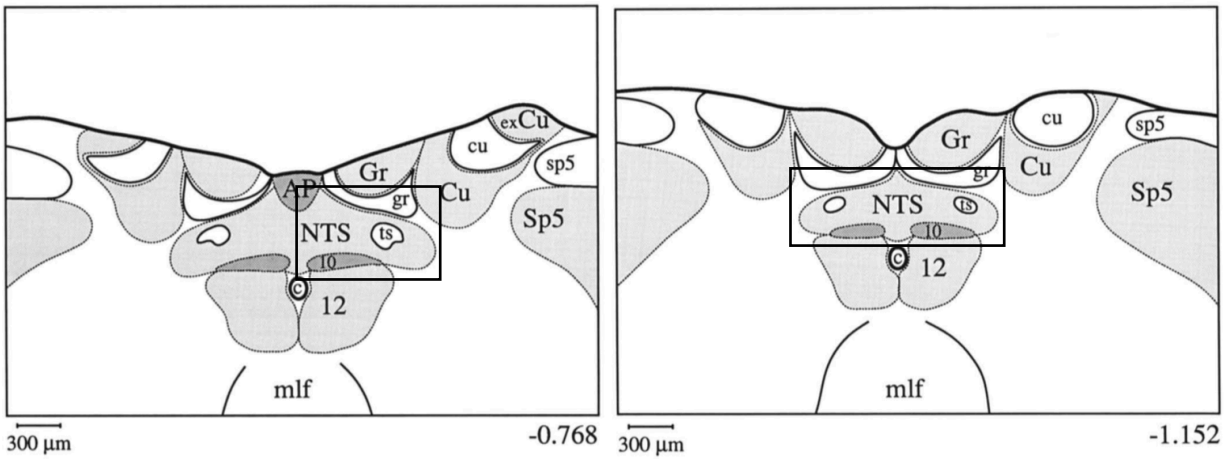


Figure 3. Atlas schematic of a subpostremal NTS section (left) and a caudal NTS section (Barraco, el-Ridi, Ergene, Parizon, & Bradley, 1992).

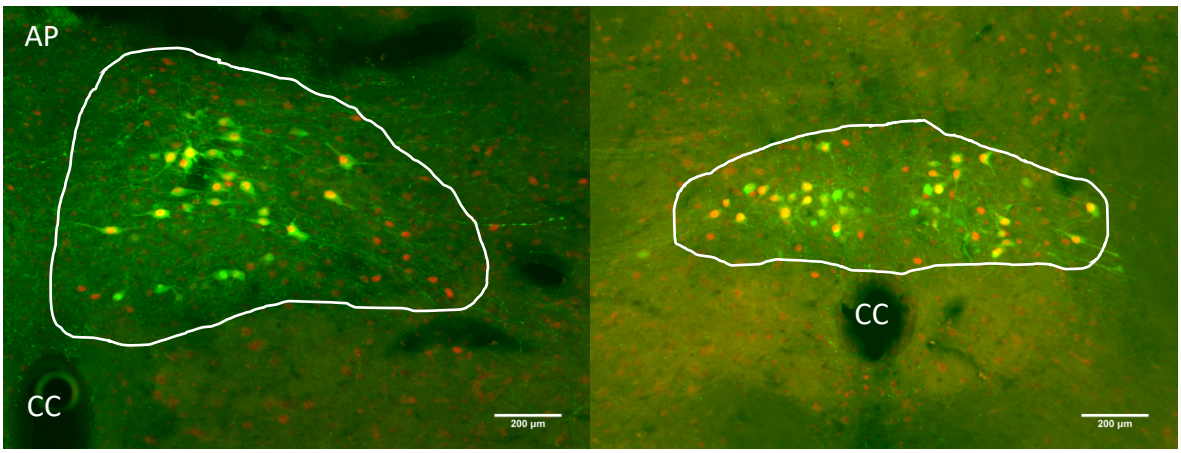


Figure 3. An outline depicting the counted area of a right portion SP (left) and caudal (right) section.

RESULTS

ERα – caudal v SP

In most of the sections, ERα was expressed in higher numbers in SP regions than caudal regions of the NTS. The expression of ERα could have different effect since there are functional differences in cells and neurons in different regions of the NTS. There seemed to be a higher number of ERα expressing cells in the SP regions, however the cells ERα immunoreactive cells in the caudal regions seems to be brighter.

ERα + NeuN

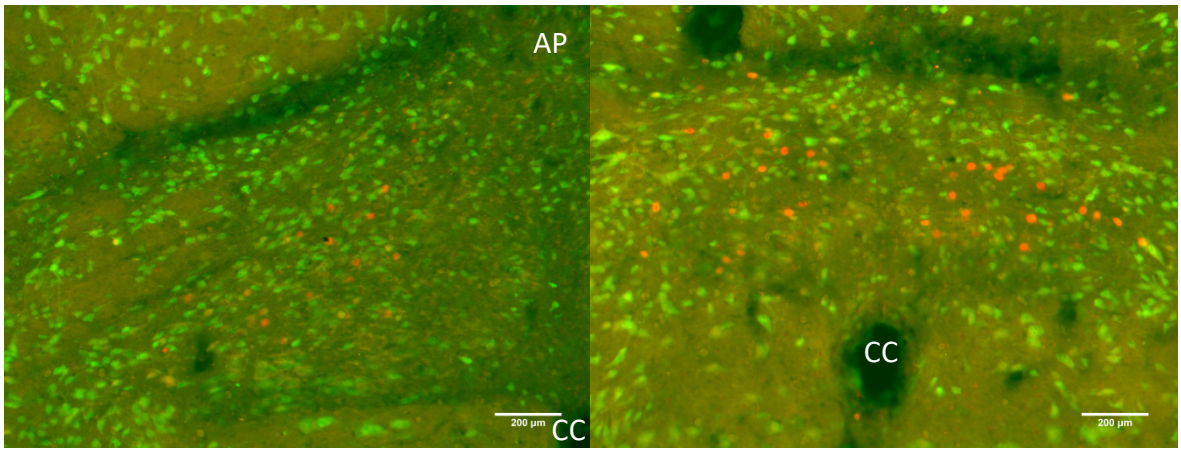


Figure 4. IHC staining of ERα (red) & NeuN (green) in a SP section (left) and a caudal section (right).

NeuN in SP NTS (n=4) was observed in 151±53 and ERα in 50±21 neurons per section. Colocalization of ERα and NeuN in SP NTS was observed in 11±6 neurons per section. NeuN in caudal NTS was observed in 59±7 and ERα 27±3 neurons per section. Colocalization of ERα and NeuN in caudal NTS was observed in 4±1 neurons per section. Of the NeuN

immunoreactive cells about 7% of them colocalized with ERα expressing cells in both SP and caudal regions. Of the ERα immunoreactive cells about 21% colocalized with NeuN expressing cells in the SP regions and about 15% in caudal regions.

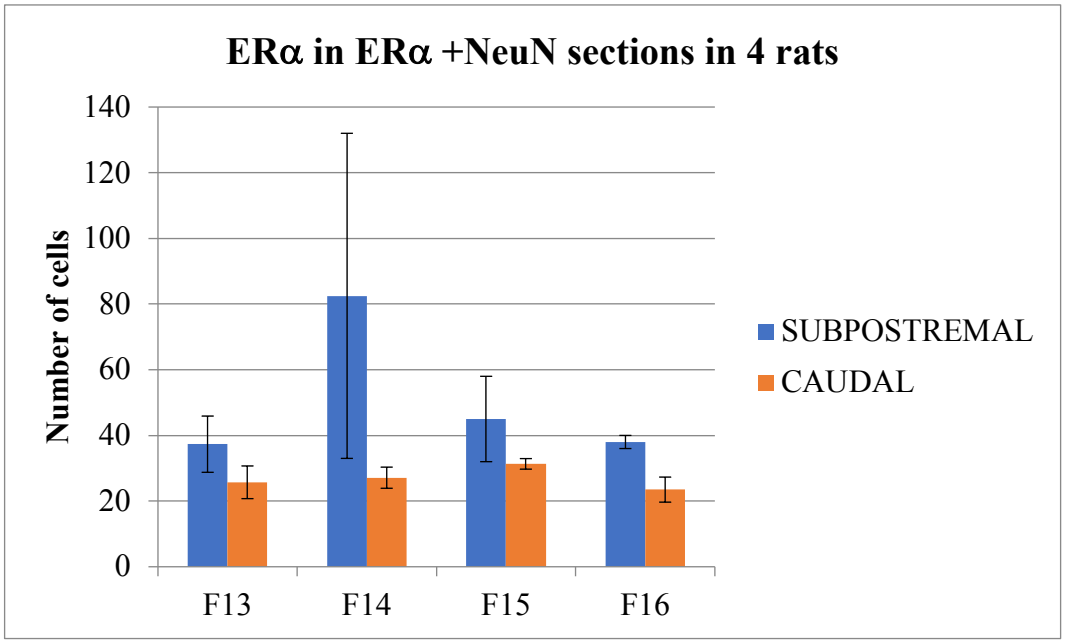
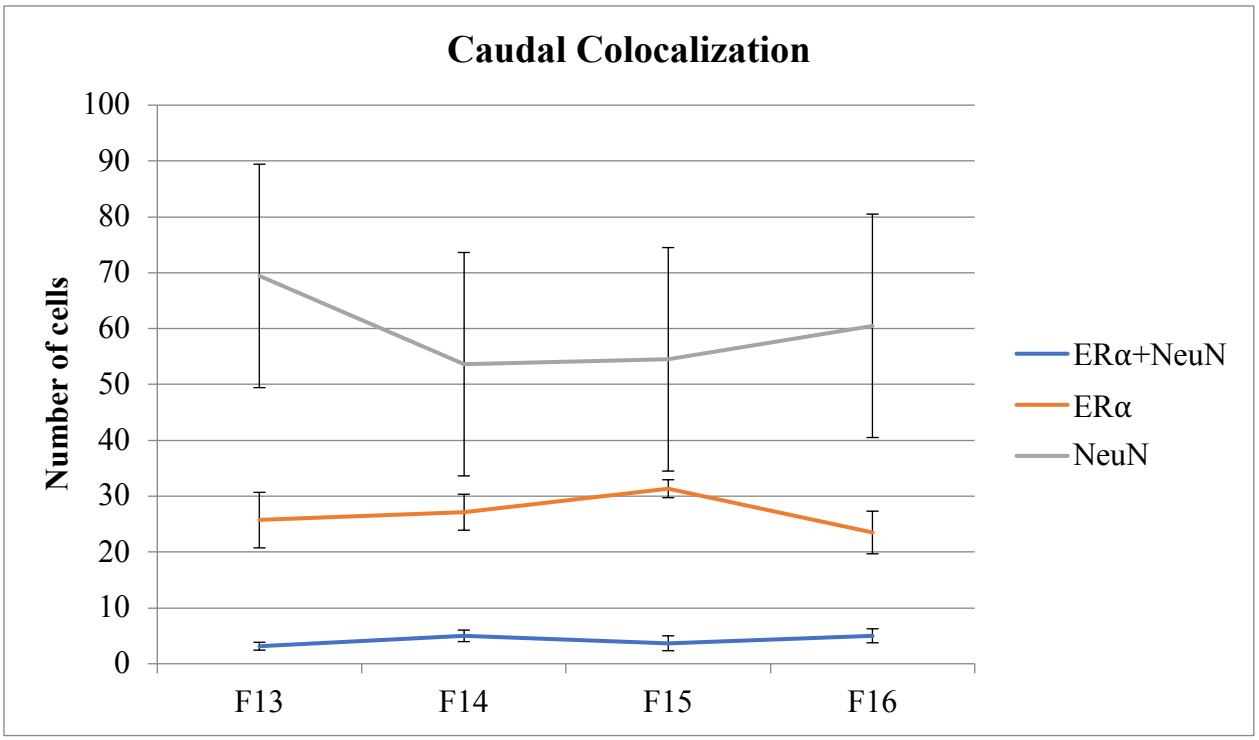


Figure 5. Bar graph comparing the ERα expression between SP and caudal sections in sections that were stained for ERα and NueN. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus.


Caudal Colocalization
Number of cells
100
90
80
70
60
50
40
30
20
10
0
F13
F14
F15
F16
ERα+NeuN
ERα
NeuN

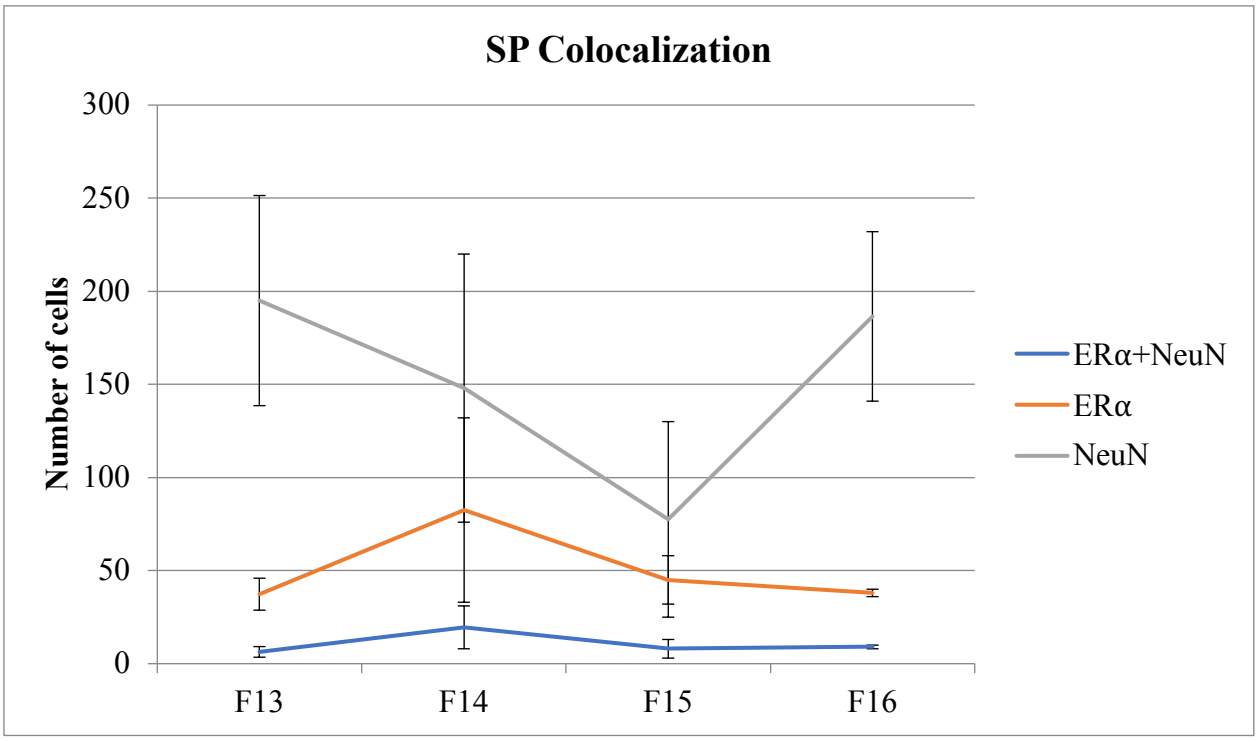

SP Colocalization
Number of cells
300
250
200
150
100
50
0
F13
F14
F15
F16
ERα+NeuN
ERα
NeuN

Figure 6&7. Line graphs illustrating the colocalization of ERα and NueN in SP and Caudal sections. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus.

ERα + TH

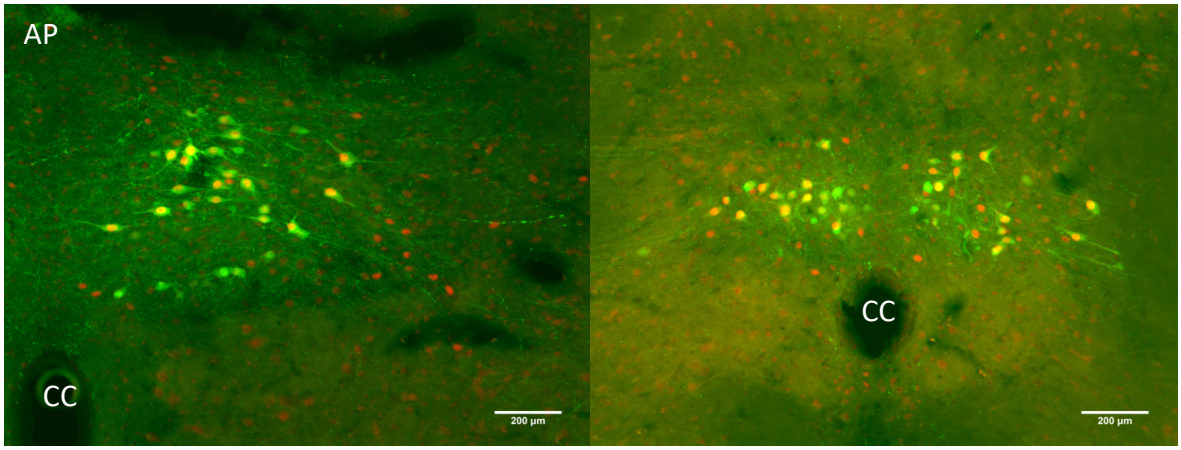


Figure 8. IHC staining of ERα (red) & TH (green) in a SP section (left) and a caudal section (right).

TH in SP NTS (n=6) was observed in 49±8 and ERα in 51±12 neurons per section. Colocalization of ERα and TH in SP NTS was observed in 26±4 neurons per section. TH in caudal NTS was observed in 26±6 and ERα 29±7 neurons per section. Colocalization of ERα and TH in caudal NTS was observed in 17±4 neurons per section. Of the TH immunoreactive cells in caudal NTS about 63% colocalized with ERα expressing cells. Of the TH immunoreactive cells in SP NTS 53% colocalized. Of the ERα immunoreactive cells in caudal NTS 57% colocalized with TH expressing cells and of the ERα immunoreactive cells in SP NTS 51% colocalized. Table 1 shows the statistical analysis comparing ERα and TH labelling, as well as ERα + TH colabeling between SP and caudal sections.

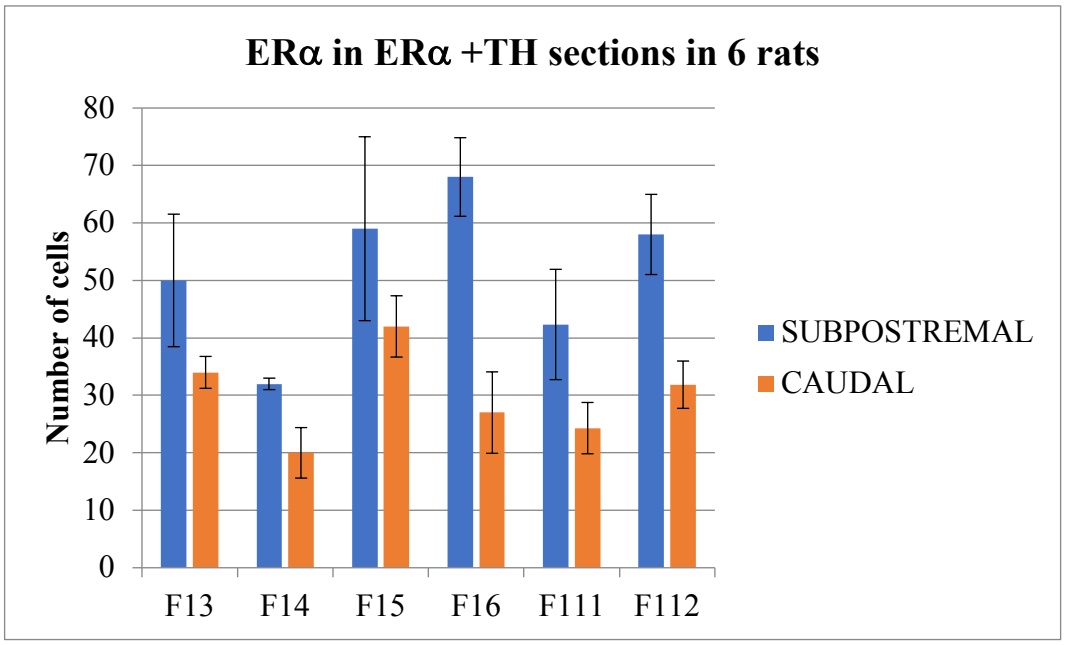
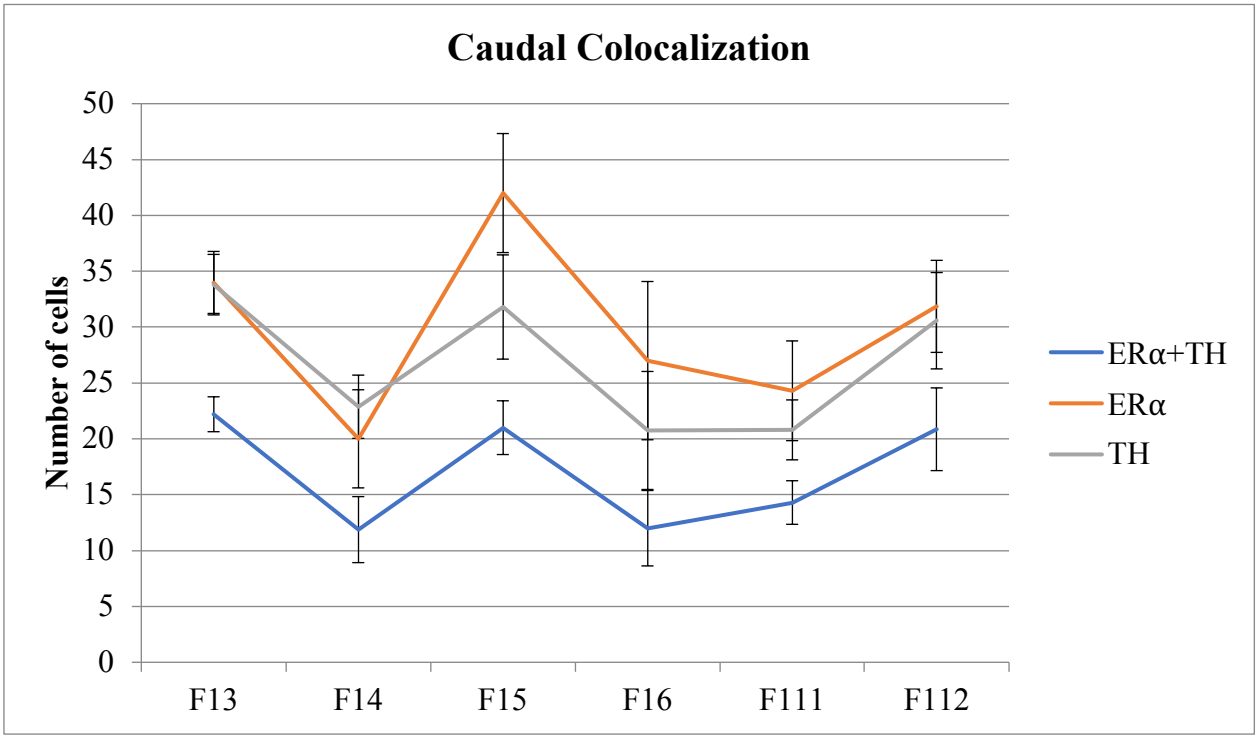


Figure 9. Bar graph comparing the ERα expression between SP and caudal sections in sections that were stained for ERα and TH. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus. F111 – proestrus, F112 – diestrus.



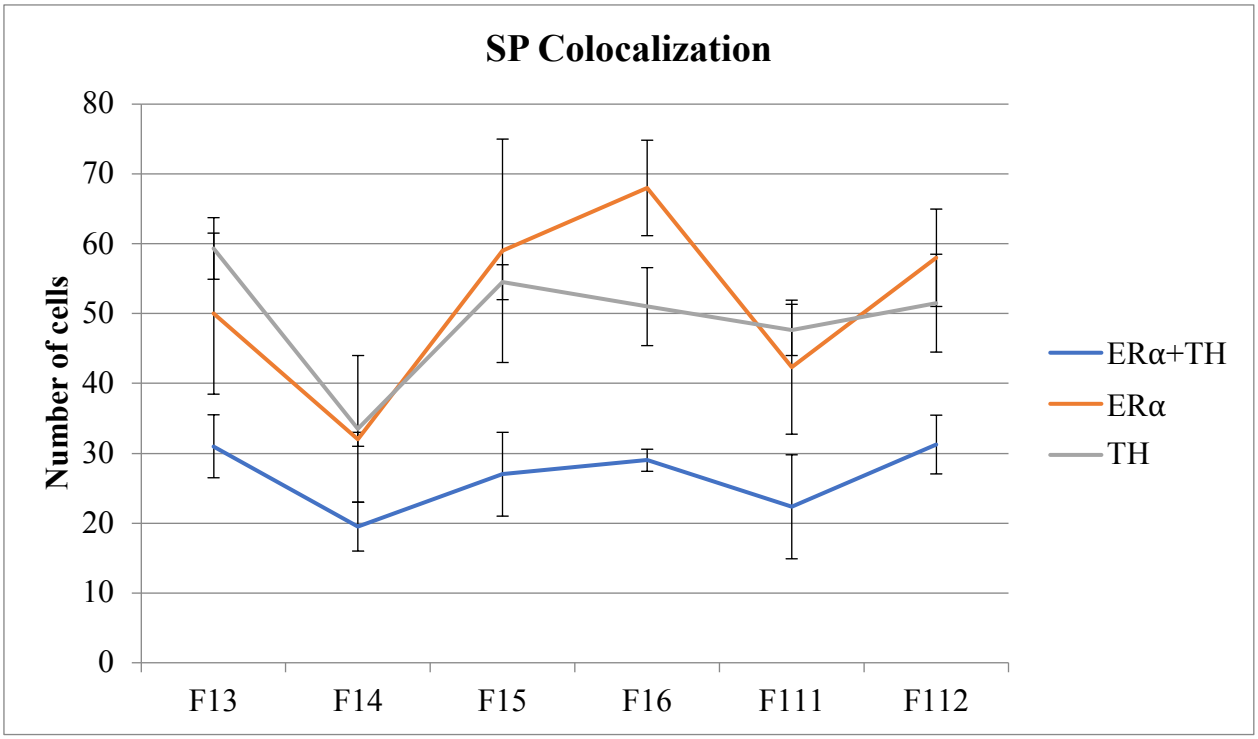


Figure 10&11. Line graphs illustrating the colocalization of ERα and NueN in SP and Caudal sections. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus. F111 – proestrus, F112 – diestrus.

Table1. Statistical analysis of ERα and TH comparing SP to caudal NTS in 6 female rats.

	SP	**Caudal**	**p = (t-test)**
ERα	51±12	29±7	0.144
TH	49±8	26±6	0.044
ERα + TH	26±4	17±4	0.143

A t-test was used to compare ERα labeling in SP versus caudal sections, TH labeling in SP versus caudal sections, and colabeling in SP versus caudal sections to see if there was statistical difference.

ERα + GFAP

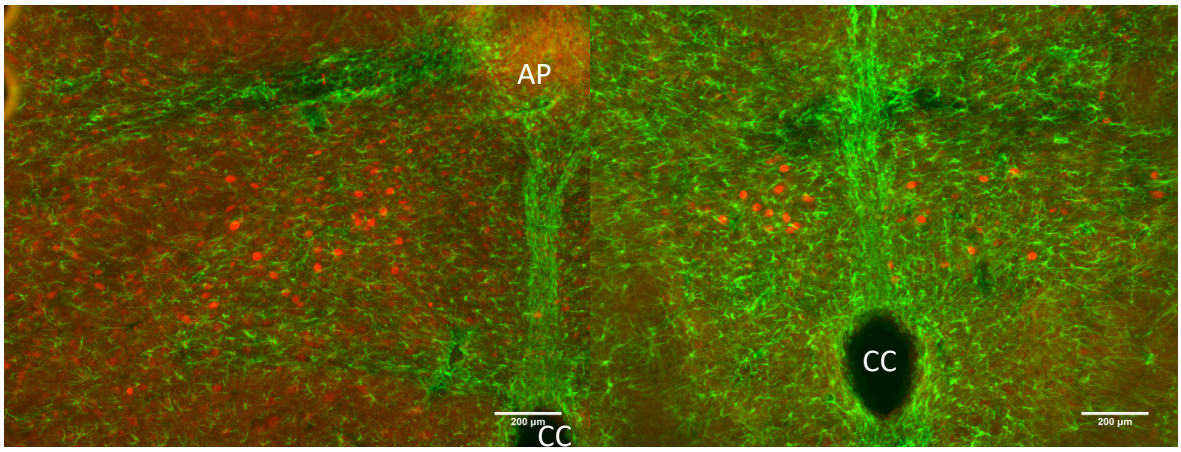


Figure 12. IHC staining of ERα (red) & GFAP (green) in a SP section (left) and a caudal section (right).

Due to the quantity and shape of GFAP immunoreactive cells in the NTS (n=4), we were not able to count the number of cells. Colocalization of ERα and GFAP expressing cells was not observed in our study.

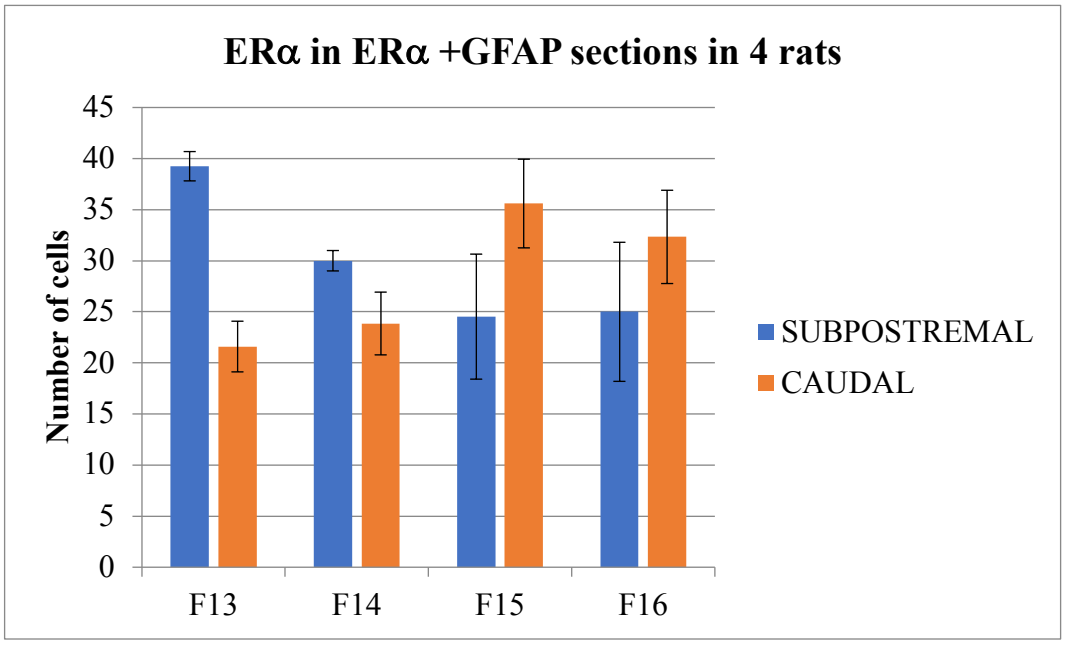


Figure 13. Bar graph comparing the ERα expression between SP and caudal sections in sections that were stained for ERα and GFAP. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus.

ERα + Iba1

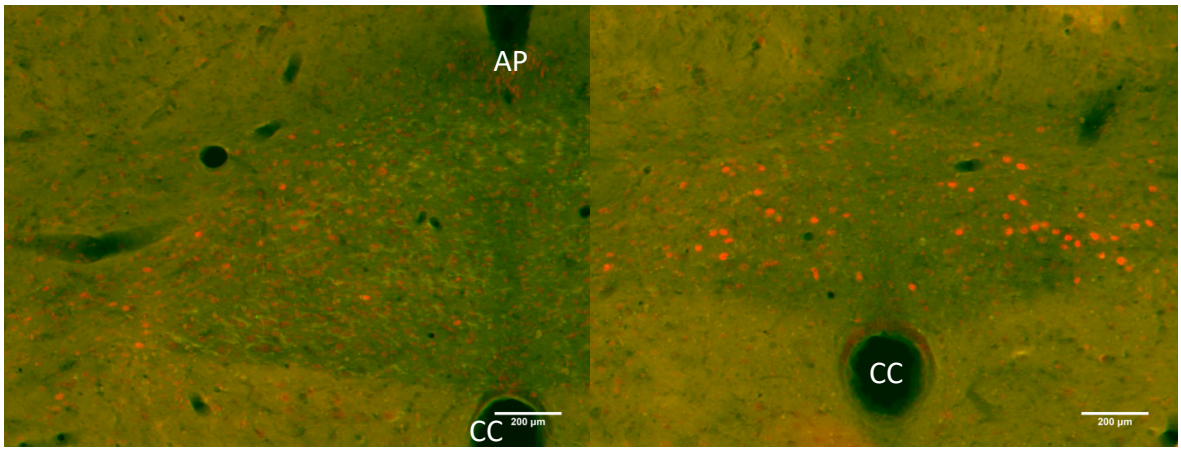


Figure 14. IHC staining of ERα (red) & Iba1 (green/not seen) in a SP section (left) and a caudal section (right).

Cells expressing Iba1 were not observed in the trials of our study (n=4). This could be due to the lack of activation since our results are similar to those seen in other control experiments looking at microglial expression of ERα.

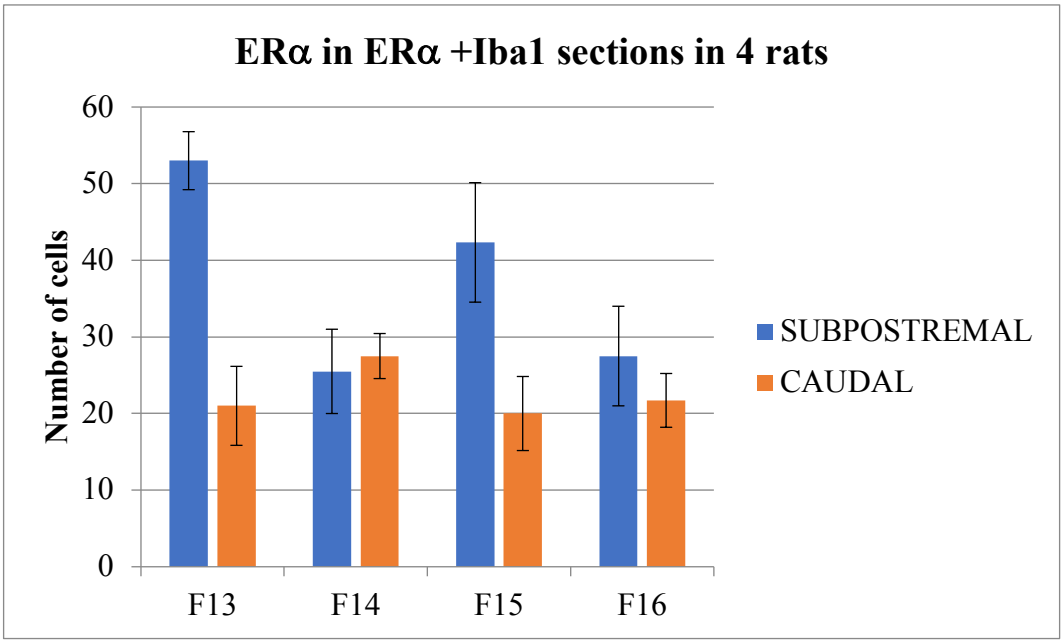


Figure 15. Bar graph comparing the ERα expression between SP and caudal sections in sections that were stained for ERα and Iba1. Estrus phases for each rat was; F13 – estrus, F14 – proestrus, F15 – proestrus, F16 – diestrus.

Estrous cycle

Even though it has been shown that there is a difference in ERα expression between rats in different phases of the estrous cycle (Mendoza-Garcés et al., 2011), we did not see a noticeable difference in the number of ERα between rats in different phases of the estrous cycle. Further research would need to be done to increase the number of rats in each phase to say for certain if the expression of ERα in the NTS changes with the estrous cycle.

DISCUSSION

The highest number of colocalization with ERα expressing cells was with cells that were labelled with the anti-tyrosine hydroxylase (TH) antibody, indicating that the cells are

catecholaminergic. This is especially interesting because we know that knockdown of TH in the NTS reduces the increase in blood pressure during chronic intermittent hypoxia (Bathina et al., 2013).

There was little to no colocalization with cells expressing the GFAP marker or the Iba-1 protein leading us to believe that ERα expressing cells may not be on glia or microglia. García-Ovejero et al., 2002, found that glial ERα expression can be induced through neurotoxin treatment or traumatic brain injury; however, they also observed no ERα immunoreactivity in the glial cells of control animals.

The expression of the neuronal biomarker, NeuN, was observed to be widespread in the NTS and surrounding areas. However, colocalization with ERα expressing cells was not as high observed as expected. Because of the high percentage of TH colocalization, we expected to see a similar percentage of expression in neurons. Even though there was a high number of NeuN positive cells, it was difficult to distinguish the cells that were dual-labelled. The secondary fluorescent antibody for the anti-NeuN was pale compared to the ERα secondary and could probably have used a higher concentration. We used the same concentration of secondary for the other primary substrates, but as I have learned through this experiment, not all antibodies are created equal.

Our sections were also divided into subpostremal (SP) and caudal groups. NTS neurons are not all similar as the NTS projects to several areas of the brain and have different functions. The functionality of NTS neurons change as you in different regions so it would be interesting to look at the expression of ERα in relation to bregma in the rat brain. This would give us a better idea of the type of neurons that have higher expression of ERα.

The results from the ERβ phenotyping experiment were inconclusive due to the unreliability of commercially available ERβ primary antibodies. Further research would need to be done to find

a reliable primary antibody to continue IHC testing or we could use laser capture to look at mRNA in single NTS neurons. Electrophysiology could also be used to test functionally if cells are expressing estrogen receptors.

SUMMARY AND CONCLUSIONS

ERα is expressed on a subset of catecholaminergic NTS neurons, as well as non-catecholaminergic neurons. Since the NTS catecholaminergic neurons contribute to responses to stress (e.g., hypoxia), this finding could provide a substrate for estrogen-mediated cardiovascular protection in females.

REFERENCES

Barraco, R., el-Ridi, M., Ergene, E., Parizon, M., & Bradley, D. (1992). An atlas of the rat subpostremal nucleus tractus solitarius. *Brain Res Bull, 29*(6), 703-765.

Bathina, C. S., Rajulapati, A., Franzke, M., Yamamoto, K., Cunningham, J. T., & Mifflin, S. (2013). Knockdown of tyrosine hydroxylase in the nucleus of the solitary tract reduces elevated blood pressure during chronic intermittent hypoxia. *Am J Physiol Regul Integr Comp Physiol, 305*(9), R1031-1039. doi:10.1152/ajpregu.00260.2013

Boese, A. C., Kim, S. C., Yin, K. J., Lee, J. P., & Hamblin, M. H. (2017). Sex differences in vascular physiology and pathophysiology: estrogen and androgen signaling in health and disease. *Am J Physiol Heart Circ Physiol, 313*(3), H524-H545. doi:10.1152/ajpheart.00217.2016

Drew, P. D., & Chavis, J. A. (2000). Female sex steroids: effects upon microglial cell activation. *J Neuroimmunol, 111*(1-2), 77-85.

García-Ovejero, D., Veiga, S., García-Segura, L. M., & Doncarlos, L. L. (2002). Glial expression of estrogen and androgen receptors after rat brain injury. *J Comp Neurol, 450*(3), 256-271. doi:10.1002/cne.10325

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol, 80*(2), 84-97. doi:10.1002/bdrb.20106

Hinojosa-Laborde, C., & Mifflin, S. W. (2005). Sex differences in blood pressure response to intermittent hypoxia in rats. *Hypertension, 46*(4), 1016-1021. doi:10.1161/01.HYP.0000175477.33816.f3

Jäkel, S., & Dimou, L. (2017). Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation. *Front Cell Neurosci, 11*, 24. doi:10.3389/fncel.2017.00024

Lawrence, A. J., & Jarrott, B. (1996). Neurochemical modulation of cardiovascular control in the nucleus tractus solitarius. *Prog Neurobiol, 48*(1), 21-53.

Lopez, J. (2017). *Phenotype of Neurons in the Nucleus Tractus Solitarius Labeled using an Optogenetic Construct with a CaMKIIa Promoter.* UNTHSC.

Mendelsohn, M. E. (2002). Protective effects of estrogen on the cardiovascular system. *Am J Cardiol, 89*(12A), 12E-17E; discussion 17E-18E.

Mendoza-Garcés, L., Mendoza-Rodríguez, C. A., Jiménez-Trejo, F., Picazo, O., Rodríguez, M. C., & Cerbón, M. (2011). Differential expression of estrogen receptors in two hippocampal regions during the estrous cycle of the rat. *Anat Rec (Hoboken), 294*(11), 1913-1919. doi:10.1002/ar.21247

Oberlander, J. G., & Woolley, C. S. (2016). 17β-Estradiol Acutely Potentiates Glutamatergic Synaptic Transmission in the Hippocampus through Distinct Mechanisms in Males and Females. *J Neurosci, 36*(9), 2677-2690. doi:10.1523/JNEUROSCI.4437-15.2016

Sousa, C., Biber, K., & Michelucci, A. (2017). Cellular and Molecular Characterization of Microglia: A Unique Immune Cell Population. *Front Immunol, 8*, 198. doi:10.3389/fimmu.2017.00198

Spary, E. J., Maqbool, A., & Batten, T. F. (2009). Oestrogen receptors in the central nervous system and evidence for their role in the control of cardiovascular function. *J Chem Neuroanat, 38*(3), 185-196. doi:10.1016/j.jchemneu.2009.05.008

Vega-Vela, N. E., Osorio, D., Avila-Rodriguez, M., Gonzalez, J., García-Segura, L. M., Echeverria, V., & Barreto, G. E. (2017). L-Type Calcium Channels Modulation by Estradiol. *Mol Neurobiol, 54*(7), 4996-5007. doi:10.1007/s12035-016-0045-6

Woolley, C. S. (1999). Electrophysiological and cellular effects of estrogen on neuronal function. *Crit Rev Neurobiol, 13*(1), 1-20.

Xue, B., & Hay, M. (2003). 17beta-estradiol inhibits excitatory amino acid-induced activity of neurons of the nucleus tractus solitarius. *Brain Res, 976*(1), 41-52.