TESTOSTERONE ATTENUATES POST HERPETIC NEURALGIA, IN PART, BY CONVERSION TO ESTRADIOL

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

Herpes zoster (HZ) or Shingles is often followed by Post Herpetic Neuralgia (PHN). PHN is a chronic pain condition usually occurring a few months after the visible symptoms of HZ subside. Although Testosterone can affect pain responses, the role of testosterone in PHNs is unknown. Testosterone is converted into estrogen in certain areas including the thalamus of the brain by the enzyme aromatase. We tested our hypothesis that testosterone conversion to estrogen in thalamus attenuates HZ induced pain.

Male Sprague-Dawley rats were divided into control and virus groups receiving whisker pad injections of either MeWo cells or MeWo cells containing varicella Zoster Virus (VZV), respectively. Virus injections (100 µl) were in the left whisker pad and contained either 60,000 pfu of virus or control. Virus and control groups were further divided in two drug groups receiving the aromatase inhibitor letrozole or the vehicle dimethyl sulfoxide (DMSO). Drug was given either locally or systemically. Guide cannulas were placed in the thalamic area for a portion of the rats using stereotaxic coordinates for local administration of drug. The conversion of testosterone into estrogen is selectively inhibited using Letrozole. The motivational and affective aspect of nociception was measured using Place Escape Avoidance Paradigm (PEAP) assay. Measurements were completed once a week for three weeks.

Data obtained were statistically analyzed and demonstrated that virus injection significantly increased the nociceptive response compared to the control. This nociceptive response was significantly increased after administration of Letrozole. In conclusion, testosterone reduces the VZV associated nociceptive response, in part, due to conversion into estrogen in the thalamic region.

DEDICATION

Firstly, I would like to thank my parents, Padmanabha Rao and Usha Rao for their support and prayers. Special thanks to my sister Priya Rao, who has been a pillar of my strength and support. I would also like to thank my other two sisters Sandhya Rao and Pooja Rao, my brother-in-law Tushar Majithia as well as my other family and friends for their encouragement.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

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Dr. Crystal Stinson helped with the conception of experimental design and data collection for the study.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

1. INTRODUCTION

The varicella zoster virus (VZV) causes Herpes Zoster (HZ). The Virus affects humans in early age causing chicken pox and thereafter it remains latent in sensory nerve ganglia (Mahalingam et al. 1992). The virus gets reactivated mostly in elderly and immunocompromised patients and triggers HZ also known as Shingles (Donahue et al. 1995; Jung et al. 2004). It is one of the common neurological disorders in the USA, with almost a million of reported incidences annually (Donahue et al. 1995; Johnson et al. 2015). The Advisory Committee on Immunization Practices mentioned in their report that one in every three persons develops HZ during their lifetime (Harpaz et al. 2008). In spite of immunity due to vaccination, about 20% of patients develop PHN. The increased load of Medicare costs is another major concern (Brisson et al. 2001; Donahue et al. 1995; Johnson et al. 2015; Johnson et al. 2010). Although HZ incidence increases after 50 years of age, it can affect all ages (Civen et al. 2009). The clinical presentation of VZV infection usually involves neuronal inflammation, blisters and dermatomal rash causing severe itching (Cohen 2013). This outbreak of shingles is also associated with pain which is often observed during an early phase of HZ and then abates. The pain then often returns few months (>3 months) after the episode of HZ, can be chronic and is called post- herpetic neuralgia (PHN). PHN is considered as a potential complication of HZ with inflammation and nerve damage (Friesen et al. 2017). The pain distribution is along one or more sensory nerve roots after the HZ (Soares and Provenzale 2016).

Often, variation of this time scale is noted (Hadley et al. 2016; Johnson and Higa 2012). This pain can severely affect the quality of life of the patient suffering with PHN (Johnson et al. 2010).

The thalamus has an active role in orofacial nociceptive responses (de Leeuw et al. 2005; Sessle 1986; Yen and Lu 2013). Lesions of the thalamus increase nociceptive response (Saade et al. 1999). Orofacial nociception modulation by sex steroids has been an area of interest for quite some time. These steroids are derivatives of the steroid hormone family with cholesterol being its precursor (Amandusson and Blomqvist 2013). Gender differences in pain has been attributed to sex- specific neurobiological mechanisms, differences in reproductive circuitry and behavior (Craft et al. 2004). Modulation of pain sensitivity by steroids, especially estradiol, has been widely studied (Aloisi and Sorda 2011; Amandusson and Blomqvist 2013; Craft 2007; Craft et al. 2004; Palmeira et al. 2011; Pieretti et al. 2016). These neurosteroids influence the excitability of neurons, particularly by acting on neurotransmitter. (Balthazart and Ball 2000; Beyenburg et al. 2001; Mellon 1994). The ovaries are the primary source of estrogen but it can also be found in fat, muscles, testes and the brain.

Aromatase inhibitors are well known for their effectiveness in treatment of breast cancer in postmenopausal women. They act my inhibiting the synthesis of estradiol (Dowsett et al. 1995). Patients undergoing the aromatase inhibitor therapy often discontinued their treatment due development complaint of pain in their joints and/or muscles (Henry et al. 2012; Henry et al. 2008). Robarge et al, suggested estrogen to be negative regulator of pain with references to many studies where estrogen was shown to

have antinociceptive effects (Robarge et al. 2016). In the brain, enzyme aromatase catalyzes the synthesis of estrogen from androgens (Amandusson and Blomqvist 2013). Aromatase is present in different brain regions in different proportions. High levels of aromatase activity are seen in regions with high estradiol levels (Konkle and McCarthy 2011). Although this distribution was similar in males and females; males showed a higher number of aromatase mRNA expressing cells than females (Stoffel-Wagner 2003). In humans, aromatase mRNA levels are found to be highest in the thalamus, pons, hypothalamus and hippocampus (Azcoitia et al. 2011; Sasano et al. 1998). Within the central nervous system, the modulation of nociception occurs in the presence of locally produced estrogen by aromatization of testosterone (Evrard 2006; Evrard and Balthazart 2004; Hau et al. 2004). Genes controlling nociception are increased in the thalamus when estrogen levels are elevated (Umorin et al. 2016).

Blockade of aromatase with the use of aromatase inhibitors increases the nociceptive responses in rats (Ghorbanpoor et al. 2014; Robarge et al. 2016). To study chronic VZV induced orofacial PHN male rats were given Letrozole, a third-generation aromatase inhibitor and nociception was measured. The male Sprague Dawley rats were given letrozole either systemically and or directly into the thalamus using chronically bilaterally placed guide cannulas and then orofacial induced VZV PHN nociception was measured.

2. MATERIAL AND METHODS

2.1 Animal Husbandry

The protocol was approved by Texas A&M University College of Dentistry Institutional Animal Use Committee. Adult male Sprague-Dawley rats (280-300g) from Harlan Industries, Houston, Texas were kept on a 12:12 light/dark cycle. The rats were given food and water ad libitum. Rats in a first experiment were divided so that half received an injection of VZV and half received control. These animals were further divided so that half received a subcutaneous injection of letrozole and half received vehicle thus, the groups were control/vehicle, control/letrozole, VZV/vehicle, VZV/letrozole. Rats in a second experiment underwent surgery to place a bilateral guide cannula into the thalamus and were divided so that half received an injection of VZV and half received control. These animals were further divided so that half received letrozole infusion and half received vehicle thus, the groups were control/vehicle, control/letrozole, VZV/vehicle, VZV/letrozole. After a four-day acclimation period surgeries were performed. After a four-day acclimation period surgeries were performed.

2.2 Surgery

After the acclimation period 28 rats (300-350 grams) were anesthetized with 2% isoflurane with an airflow of 2 liters per minute. Using sterile technique guide cannulas (C313G Plastics One, Roanoke, VA) were placed bilaterally into the thalamus stereotaxically using the coordinates 3.6 mm posterior of Bregma, 2.8 mm from midline at a depth of 5.5mm from dura. The guide cannulas were closed with obturators. Post-

surgery the animals were given nalbuphine (2mg/kg) subcutaneously. This dose of analgesic was repeated once again after 24 hours and the animals were allowed to recover for a week.

2.3 Behavioral Testing

One week after thalamic injections were complete the rats were anesthetized briefly with 2% isoflurane using a 2 liter per minute flow of air and the left whisker pad was injected with 100 µl of MeWo cells infected with VZV or control MeWo cells lacking virus. After a week, post-surgery, animals from both groups were further divided into two groups, Virus (VZV) and Control (MeWo) group. Left whisker pad of VZV animals were injected with 100 µl of high concentration MeWo cells (human skin cell line) infected with VZV (>60,000 pfu/µl) and the control group received MeWo cells lacking virus. The measurement of infectious virus particles is quantified by plaque forming units (pfu).

In the cannulated group one week after whisker pad injections, the VZV injected group and the MeWo injected groups were sub-divided and half received bilateral thalamic infusions of either letrozole dissolved in Dimethyl Sulfoxide (DMSO) or only DMSO (vehicle). At the time of infusions, the obturators were removed and an injection syringe was inserted. Bilateral infusions (0.05 µl/min) were made of letrozole (500 µl at a concentration of 5 mg/ml) or DMSO (500 µl). Bilateral infusions were made to avoid any bias in the results seen in right thalamus because of the use of letrozole. After infusion, the injector cannula was left in place for 5 minutes, then removed and the

obturator replaced. The letrozole infusions were done a day (24 hours) prior and again four hours before behavioral testing. The Systemic group was subdivided and one subgroup was injected subcutaneously with (500 µl to reach a dose of 5 mg/kg) letrozole and the control subgroup with DMSO (500 µl). The injections were given a day (24 hours) prior and again four hours before behavioral testing.

Place Escape/Avoidance Paradigm (PEAP) testing was performed to determine nociception. To accomplish this the rats were placed in a 30 cm X 30 cm X 30 cm acrylic box where half the box was covered in black cloth. This test chamber was modeled from the PEAP test performed by the Fuchs's laboratory (LaBuda and Fuchs 2000). This assay is used to measure the affective aspect of nociception in neuropathic models (Baastrup et al. 2011; LaBuda and Fuchs 2000). The PEAP test is based on the assumption that if animals escape and/or avoid a noxious stimulus, then the stimulus is aversive to the animal (Fuchs and McNabb 2012). PEAP has been used in both inflammatory and neuropathic nociception models with rats of both sexes and with various strains and ages as a means of measuring the affective aspect of nociception. Rodents being nocturnal in nature preferred to stay on the dark side when placed into the test chamber that has a light and dark side. After placing the rat in the test chamber, it was immediately poked with a 60-gram filament every 15 seconds on the injected side if the rat was on the dark side and on the non-injected side if it was on the light side. The target region for the poking was the area below the eye and caudal to the whisker pad. This region is innervated by the second branch of trigeminal nerve (DaSilva and DosSantos 2012), the nerve infected by VZV injection of the whisker pad. Sensitivity of

the face around the eye was expected. Pokes were performed in the region below the eye and caudal to the whisker pad, a region innervated by the second branch of the trigeminal ganglia. The time spent on the dark side of the box was recorded in 5-minute bins and testing was performed for a total of 30 minutes. Testing was performed once a week. Testing was completed for 2 weeks in both groups. Thus, the theory behind the test is that if the rat is experiencing VZV induced nociception when poked in the sensitive area it will not stay on it preferred dark side but will move to the non-preferred light side and stay there is avoid the nociceptive poke.

2.4 Tissue Collection

The cannulated animals were sacrificed in the second week within 1 hour post PEAP testing. 4 animals from each of the four groups VZV-Letrozole, VZV-DMSO, MeWo-Letrozole and MeWo-DMSO were randomly selected and were sacrificed by exposure to CO2 followed by decapitation. The brain was removed using a rongeur and sliced on a slicer with 1 mm increments (Zivic, Pittsburgh, PA). Sections were made to be 2 mm thick by skipping every other slot in the slicer and the sections between Bregma -3 to -5 were placed on glass slides and kept on dry ice. Lateral thalamic tissue was collected with punches 2 mm in diameter centered on the injection site. Punches included the posterior nucleus, ventral posteromedial, ventral posterolateral and reticular thalamic nuclei termed the lateral thalamus. Tissue was stored in liquid nitrogen until RNA could be isolated.

2.5 Real Time PCR

RNA extraction was performed using the RNA Lipid Tissue Kit from Qiagen (Valencia, CA). The RNA concentration in the resulting sample was determined on a Nanodrop2000. A one-step reverse transcription PCR reaction was performed on BioRAD C1000 Thermal Cycler using the SYBR-Green 1-Step RT-PCR kit and primers from Qiagen (Table 1). The thermal protocol was 30 min @ 50 °C for the reverse transcription reaction, 15 min @ 95 °C for DNA pol activation and 40x (15 s @ 94 °C melting, 30 s @ 56 °C annealing, 30 s @ 72 °C extension). A melting curve was obtained thereafter for quality assurance. The sample amount was adjusted according to total RNA concentration to obtain 20 ng of total RNA per well in the final reaction mix. All reactions were run in triplicate. PCR runs that did not exhibit a proper amplification profile were discarded.

Table 1. PCR primer pairs for RT-PCR

Gene	**Qiagen Catalog #**	**Qiagen ID**
GAPDH	QT00199633	Gapd
GPR30	QT00376943	Gper1
Estrogen receptor alpha	QT00369740	ESrra
Estrogen receptor beta	QTOO190113	Esr2
Aromatase receptor	QT01812433	Ar
Aromatase enzyme	QT00186942	Cyp19a1
VGAT	QT00378413	Viaat

2.6 Immuno-fluorescent Staining

Three animals from each of the four groups were randomly selected for immuno- fluorescent staining to see the neuronal activity around the infusion site. After anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine the animals were perfused with 9% sucrose followed by 4% paraformaldehyde. Fixed tissues were stored in 25% sucrose, frozen, cryo-sectioned and the 20 µm sections placed on Histobond slides (VWR international, Radnor, PA). The tissue was then blocked with a PBS solution containing 5% normal goat serum (Sigma-Aldrich, St. Louis, MO) and 0.3% Triton-X 100 for 2 hours at room temperature. The slides were then incubated in a primary antibody solution overnight at 4°C. The primary antibody consisted of a mixture of the mouse monoclonal NeuN antibody (neuron marker) and mouse monoclonal pERK antibody (phosphorylated extracellular signal-regulated kinase) (neuron activity marker) at 1:400

dilution (Millipore, Billerica, MA). The primary antibody was diluted with PBS, 5% BSA and 0.3% Triton X-100. After incubation in primary antibody the slides were then rinsed three times in PBS and Triton-X 100 for a total of 45 min and placed for 2 hours in secondary antibody and PBS and 0.3% Triton X-100. Secondary antibodies included a mixture of goat anti-rabbit 568 or goat anti-mouse 488 (Invitrogen, Carlsbad, CA). After rinsing the slides three times in PBS for a total of 45 min, the slides were mounted with Fluoromount-G mounting medium containing Hoechst 33342 stain (Electron Microscopy Sciences, Hatfield, PA). The fluorescent signal was imaged using a Nikon fluorescent microscope and NIS-Elements imaging software and a Photometrics CoolSnap K4 CCD camera (Roper Scientific, Inc, Duluth, GA).

2.7 Statistics

Data was analyzed with ANOVA because of the repeated measurements and multiple groups and the independent variable was the PEAP data and the dependent variables were virus and drug. When a significant effect was observed Bonferroni post-hoc tests were completed (Prizm 5.04, GraphPad Software, La Jolla, CA, or Abstat, Anderson Bell Corp, Arvada CO).

3. RESULTS

3.1 PEAP Testing

PEAP test was completed beginning a week after whisker pad VZV or control injections. Fewer seconds on the dark side indicates a greater affective nociception response (LaBuda and Fuchs 2000).

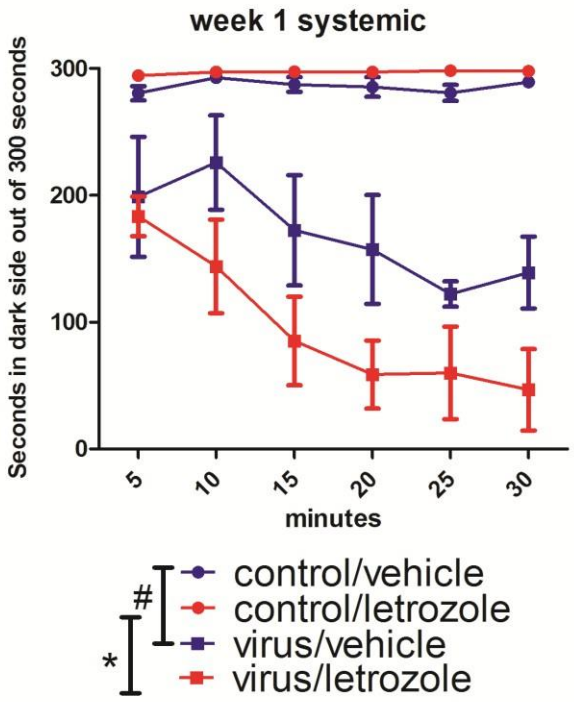

Figure 1. Week 1 PEAP data of systemic group.

3.2 Systemic Injection Groups

PEAP data from the systemic drug treated group (Fig.1) shows that in the 1st week the animals which received virus, whether given letrozole or its vehicle spent much less time on the dark side compared to the controls. Animals in the control group

remained in the dark side almost throughout the testing period. When comparing the virus group injected with vehicle to the virus group injected with letrozole a significant main effect for letrozole was observed ($p<0.001$). No significant difference was calculated when comparing the control/vehicle and control/letrozole groups. Two weeks after injecting the whisker pad (Fig.2) rats followed the same pattern as in week 1. VZV treated groups had a greater level of nociception while the controls showed no nociceptive response ($p<0.001$). The virus group injected with vehicle had less of a nociceptive response compared to the virus group injected with letrozole ($p<0.01$). However, the animals in VZV vehicle group showed a decreased nociception response compared to the first week. The control response in Week 2 was similar to their response in Week 1.

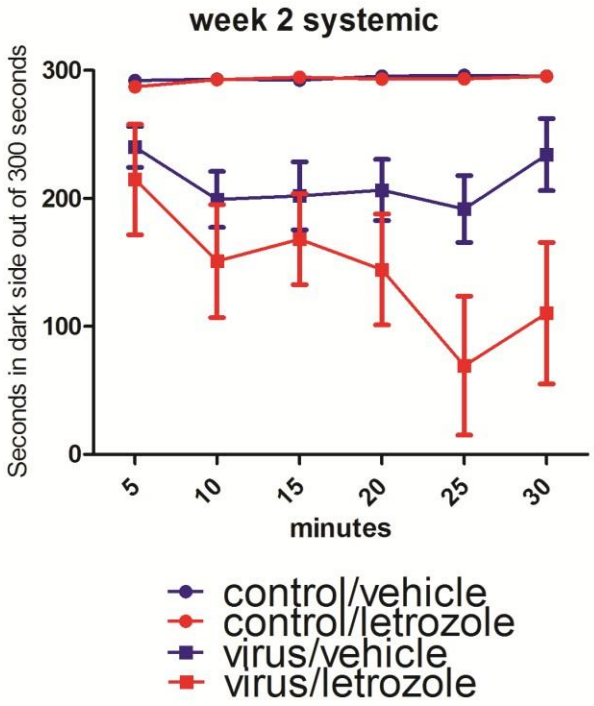

Figure 2. Week 2 PEAP data of systemic group (n=6 per group)

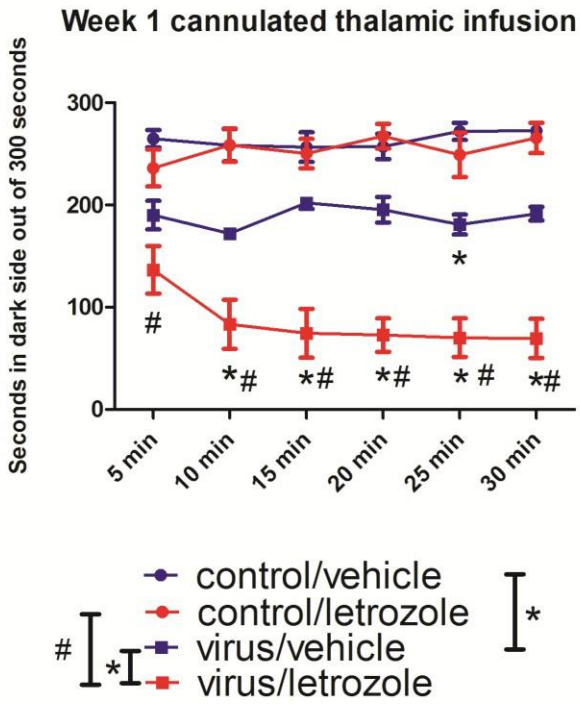

Figure 3. Week 1 PEAP data of cannulated group. The symbols # and * indicate a significant difference between the indicated groups ($p<0.05$), n=8 per group.

During Week 1 animals which received virus, whether given letrozole or vehicle spent significantly less time on the dark side compared to the controls (Fig. 3). The control animals infused with letrozole spent significantly less time on the dark side when compared to the VZV rats infused with letrozole (Fig. 3). This difference was significant at all-time points (5, 10, 15, 20, 25 and 30 minutes). For control and virus groups receiving vehicle, a significant difference was observed in the 25-minute bin (Fig. 3). Within the virus group there was a significant difference observed between animals that received letrozole and those that received vehicle (10,15,20,25 and 30 min). Animals in

the control injected letrozole infused group spent most of their time on the dark side as did the control injected vehicle infused group; there was no significant difference between these groups.

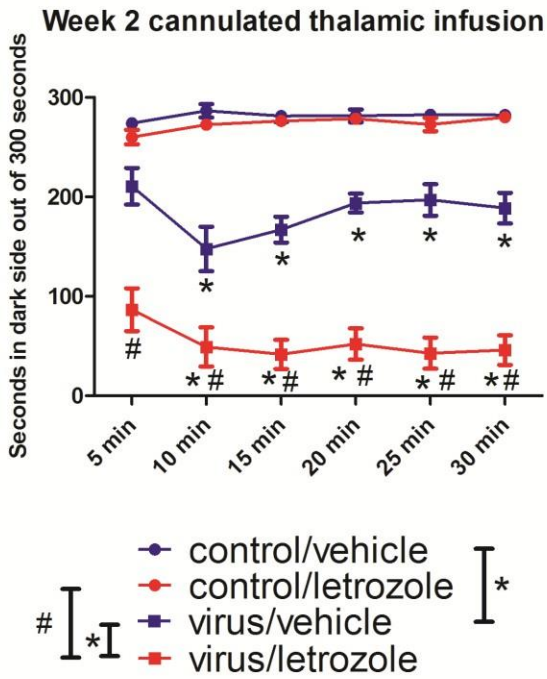

Figure 4. Week 2 PEAP assay data of Cannulated group. The symbols # and * indicate a significant difference between the indicated groups ($p<0.05$), n=8 per group.

In the second week animals in the virus/vehicle and virus/letrozole group continued to show an increased nociceptive response (Fig. 4). The rats in the VZV/letrozole group spent the least amount of time on the dark side. The difference was significant between virus and control group receiving letrozole at all the time points of PEAP testing (Fig. 4). Significant differences were also observed between control and virus group receiving vehicle at all time points of PEAP testing except at first 5-minute

point. Similarly, a significant difference in the nociceptive response was also observed within the virus groups receiving vehicle or letrozole at all time points of PEAP testing except at the first 5-minute mark. The non-virus (control) injected groups spent almost all their on the dark side.

3.3 Thalamus Cannulated Group Summary

The animals which received virus injections showed a greater nociceptive response and spent most of the time on the light side. Non-virus (control) injected animals showed no nociceptive response and stayed on the dark side almost throughout the testing period. Within the virus group the animals that received letrozole showed a significantly greater nociceptive response compared to the animals that received vehicle.

3.4 Thalamus Gene Analysis

Gene transcripts with potential to have a role in testosterone and estradiol regulated pathways were quantitated within the thalamic tissue. These genes included the estradiol receptors ERα and ERβ, as well as, the membrane estradiol receptor GPR30 (Table 1). The aromatase enzyme that converts estradiol to testosterone was analyzed (Table 1). Testosterone can bind to the aromatase receptor to cause changes in pain responses and thus, was analyzed at the transcript level (Fischer et al. 2009; Fischer et al. 2007). VGAT is known to regulate pain responses and has been shown to be regulated by estradiol thus, this gene was analyzed by RT-PCR (Kramer and Bellinger 2009; Kramer et al. 2015). The analysis was completed using RT-PCR and was normalized

against the values of GAPDH to get a expression value (delta-Ct). The delta-Ct values of the genes were plotted on the Y-axis to represent the relative change in expression for each of the transcripts (Fig. 5 and 6). The gene expression levels for the aromatase gene did not show any difference with the use of letrozole (Fig. 5 and 6). Similar to aromatase gene expression levels, androgen and estrogen receptor levels did not show any significant difference with the use of letrozole. The gene expression level of VGAT was significantly ($p<0.05$) elevated with the letrozole treatment in both the right and left thalamus (Fig. 5 and 6).

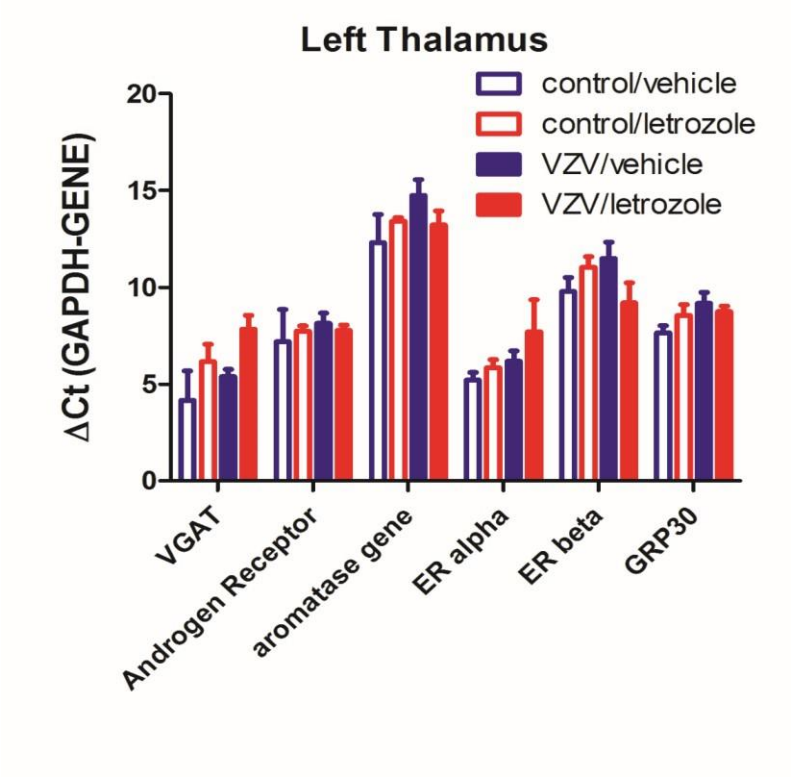

Figure 5. Gene Expression in Left Thalamus

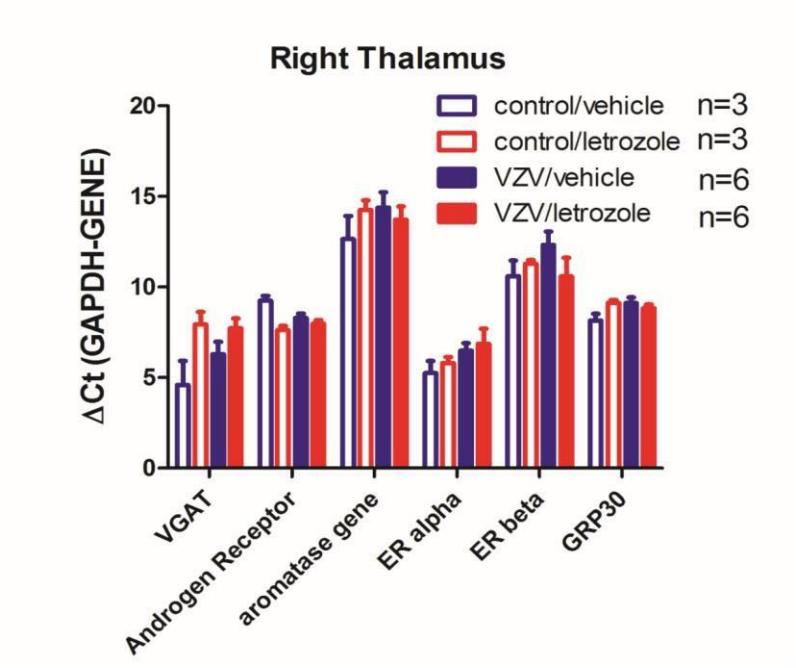

Figure 6. Gene Expression in Right Thalamus

3.5 Immunostaining

Neuronal nuclei (NeuN) positive cells were present in the thalamic area around the infusion site (Fig.7). NeuN is a neuronal marker. Cells in the same area were also positive for phosphorylated extracellular signal-regulated kinase (pERK) (Fig.8). pERK is a good marker for nociception-induced neuronal activation (Gao and Ji 2009). Activated neurons were present adjacent to the cannulation site where infusion of letrozole was performed (Fig. 9). Quantitation of these neurons and correlation of these cell counts to virus and drug treatment would allow for analysis of the role of thalamic neurons in the nociceptive response. For example, if the thalamus has a role in the nociceptive response we would expect an increase in activated neurons in virus infected animals. Moreover, if letrozole increased the number of activated neurons this result

would suggest that the increased nociceptive response due to letrozole included a mechanism localized to the thalamus.

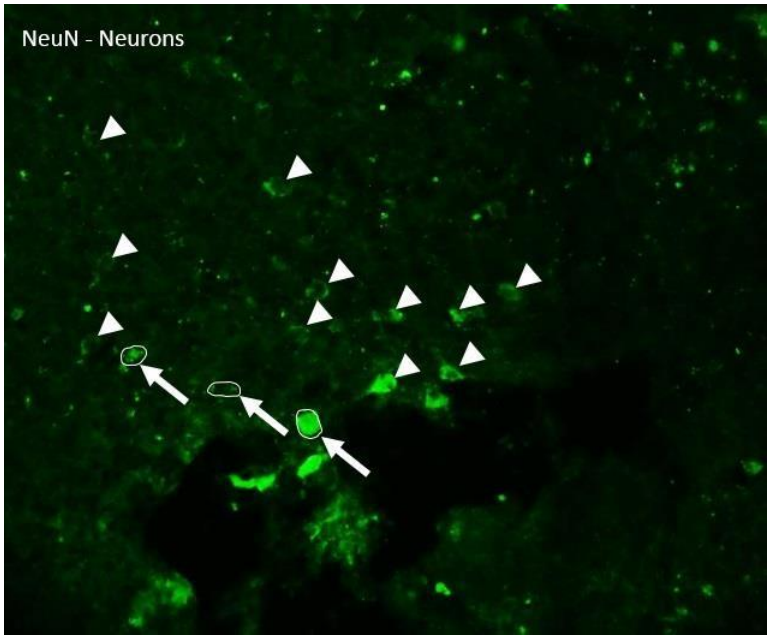

Figure 7. NeuN positive neurons (green)

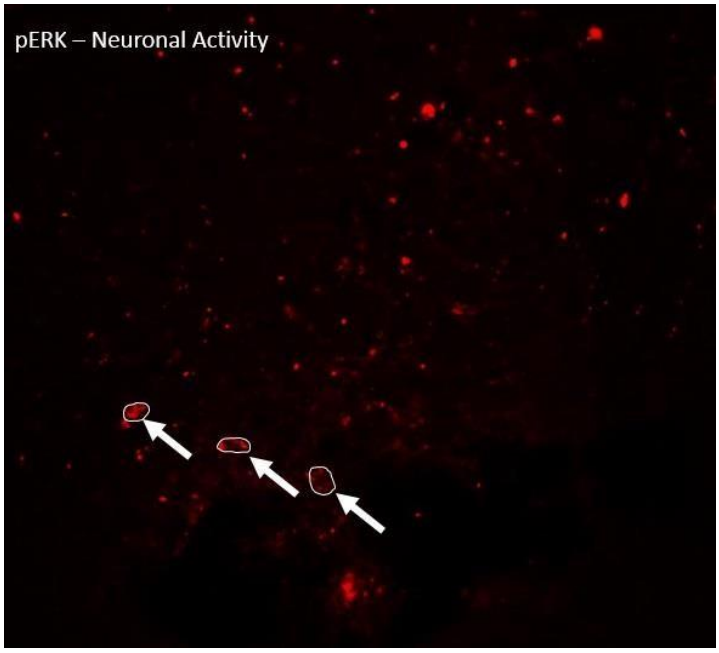

Figure 8. pERK positive neurons (red)

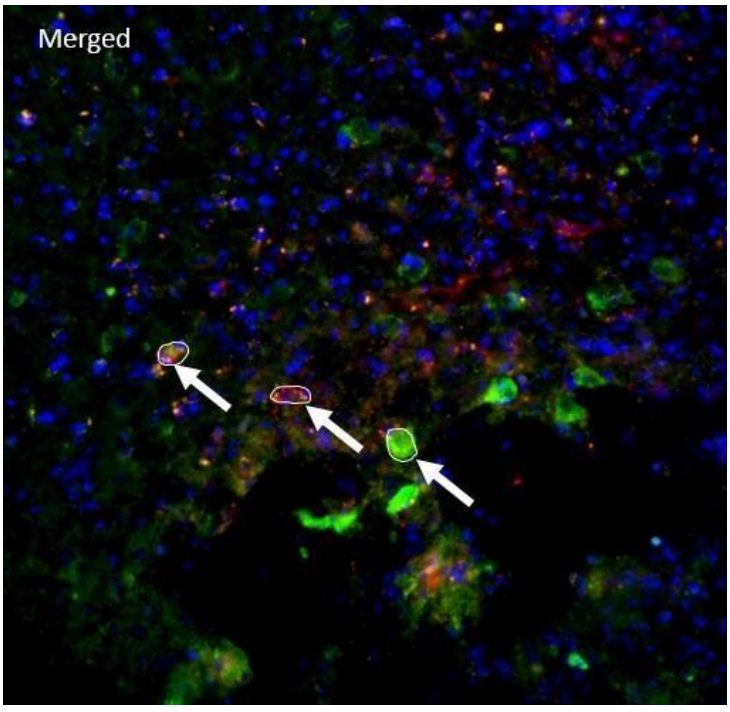

Figure 9. Merged double staining image showing neuronal activity.

4. DISCUSSION

VZV injection into the whisker pad results in a nociceptive response in the facial region. The response for the PEAP assay was significant for the first two weeks. The results from the third week were not significant (data not included). We believe that a reduced response in the third week may be due to the decreasing effect of virus. As seen from the behavioral data letrozole administration increased the VZV associated nociceptive response. The animals in the VZV/letrozole group spent the least amount of time on the dark side. The results from the PEAP assay for both systemic and cannulated groups show that letrozole administration significantly increased the VZV associated nociceptive response compared to the vehicle animals receiving DMSO. This demonstrated that inhibition of the aromatase enzyme does have an effect on the modulation of the nociception, consistent with the findings from previous studies (Evrard 2006; Ghorbanpoor et al. 2014). From the graphs, some difference is seen between the results from the systemic and cannulated groups. We believe the increased response to nociception in the cannulated group when compared to the systemic group might be due to the surgeries done during cannula placements.

Previous studies have suggested that estradiol attenuates nociception. The gene expression for vesicular GABA transporter (VGAT) increases in thalamus when estradiol levels are elevated. Previous studies from our lab suggested thalamic expression of VGAT modulates nociception in the orofacial region (Kramer and Bellinger 2009; Kramer et al. 2015). Letrozole was infused in both the right and left

thalamus and we expected to see a decrease in the thalamic VGAT expression as compared to the vehicle treated groups because letrozole administration would decrease estradiol levels. However, the results from RT-PCR experiments demonstrated that letrozole treatment increased VGAT transcript in both the right and left thalamus as compared to the controls. If the reduced VGAT were affecting the nociceptive response, we should have seen a decrease in the nociceptive response upon letrozole treatment but we saw an increase instead suggesting that modulation of nociceptive response was not likely due to changes in VGAT expression. Further studies looking at protein levels are needed to verify this conclusion. We believe there might be some other factors which might be playing active role in modulation of nociception other than VGAT. One such mechanism for nociceptive modulation would be the effect of estrogen on glutamate levels. Estrogen has been shown to modulate nociception by decreasing glutamate levels in thalamus. Further studies are required to properly explain this phenomenon. In some studies estrogen has been shown to facilitate nociceptive transmission via membrane bound estrogen receptors (Zhang et al. 2012). 17β-estradiol, the most potent form of estrogen, has been shown to regulate gene expression of voltage gated sodium channels acting via estrogen receptors alpha and beta which may play a vital role in modulation of nociception (Hu et al. 2012). Thus, estrogen can modulate nociception acting through any of its receptors in a genomic or non-genomic way (Amandusson and Blomqvist 2013; Craft 2007; Deliu et al. 2012). We looked at the expression levels for androgen and estrogen receptors. There was no change in expression levels of these receptors after letrozole administration. Moreover, the receptor expression for aromatase also did not

show any difference due to virus or drug. These results are consistent with the idea that letrozole does not have any effect on the expression of these receptors and that these receptors might not function in the modulation of VZV associated.

In summary, after the administration of letrozole the animals in the virus group showed a greater response to nociception as compared to the ones receiving DMSO (vehicle) or control groups without virus. Inhibiting the activity of aromatase increased the nociceptive response in virus group suggesting testosterone might be attenuating the nociceptive response by its aromatization to estradiol.

5. CONCLUSION

Our behavioral results indicate that testosterone attenuated the VZV associated nociceptive response after conversion to estradiol suggesting that in humans, pain conditions attenuated by testosterone may actually be modulated by androgens being converted to estradiol rather than being effected by testosterone directly

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