ESTRADIOL CONTROLS VARICELLA ZOSTER VIRUS ASSOCIATED NOCICEPTION THROUGH GABAERGIC THALAMIC SIGNALING MECHANISM

A Dissertation

by

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ABSTRACT

Post-Herpetic Neuralgia (PHN), a chronic pain condition resulting from Herpes Zoster (HZ), is reported by females 3.75 times more often than men. Investigating the role of estradiol in thalamic gene expression, neuronal activity in the ventral posteromedial thalamus (VPM) and the Varicella Zoster Virus (VZV) associated pain response, can contribute to identifying mechanisms in which estradiol modulates the orofacial nociceptive processes of PHN and contributes to this disparity. VZV was injected into the whisker pad of adult Sprague Dawley rats. Changes in orofacial nociception and the affective nociceptive response, i.e. place escape avoidance paradigm (PEAP) were measured. Local field potentials (LFPs) of the VPM were recorded simultaneously while inhibiting neuronal activity to investigate its effects on the VZV associated nociception response.

Gene expression changes in cycling SD rats was analyzed by RT-PCR. Plasma estradiol levels were measured at different phases of the estrous cycle to monitor changes in thalamic gene expression during periods of low and high estradiol and identify key genes that modulate VZV associated nociception through estradiol induced changes in thalamic gene expression. Vesicular GABA Transporter (VGAT) expression was attenuated in the thalamus by injecting a VGAT silencing shRNA and the VZV associated nociception response was measured in both male and female rats. As seen in human PHN, infected rats showed neurite retraction in the whisker pad, translocation of VZV to neurons in the trigeminal ganglia (as evident by IE62 staining) and gabapentin amelioration of the nociceptive response. Increases in thermal hyperalgesia, mechanical

allodynia and PEAP were observed. Female rats showed a longer VZV associated affective response than males and when plasma estradiol concentrations were decreased, the affective response to VZV injection increased. In ovariectomized rats with hormone replacement, estradiol levels mirroring the proestrus phase of the natural cycle, reduced VZV associated nociception. The expression of VGAT was significantly increased in the lateral thalamus during the high estrogen phases (proestrus and estrus) of the estrus cycle. After VGAT silencing in the lateral thalamus, VZV associated nociception significantly increased in both male and female rats. Altogether exhibiting that estradiol controls VZV associated pain through VGAT GABAergic thalamic signaling.

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NOMENCLATURE

AAV	Adeno-Associated Virus
DREADD	Designer Receptor Exclusively Activated by Designer Drug
GABA	γ-Aminobutyric acid
GAD-1	Glutamate Decarboxylase 1
GAD-2	Glutamate Decarboxylase 2
GABARAPL1	GABA(A) receptor-associated protein-like 1
HZ	Herpes Zoster
LFP	Local Field Potential
OVX	Ovariectomized
RT-PCR/PCR	(Real Time)-Polymerase Chain Reaction
PEAP	Place Escape Avoidance Paradigm
PHN	Post-Herpetic Neuralgia
Rt	Reticular Thalamus
SD	Sprague Dawley
VGAT	Vesicular GABA Transporter
VPL	Ventral Posterolateral nucleus of thalamus
VPM	Ventral Posteromedial nucleus of the thalamus
VZV	Varicella Zoster Virus
ZI	Zona Incerta

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1. INTRODUCTION AND LITERATURE REVIEW

1.1.Varicella Zoster Virus and Post-Herpetic Neuralgia

Initial varicella zoster virus (VZV) infection typically occurs at an early age and results in viral latency in the thoracic and trigeminal ganglia neurons, during this latency period extrachromosomal VZV DNA and some viral protein are expressed [16; 77; 80; 81]. Typically, after age 50 this latent VZV becomes active and causes pain in up to 90% of the herpes zoster (HZ) patients [53]. This zoster-associated pain occurs during acute HZ (before or near the time skin lesions are observed) and then later is termed post-herpetic neuralgia (PHN); a pain defined to begin 3 months after the resolution of the lesions [26; 61; 89]. PHN remains several months after the rash from HZ clears up and in those that develop PHN about 30% have pain that lasts for more than one year after other disease symptoms dissipate [58]. Without taking the HZ vaccine one in three people will develop HZ or one million people annually in the US and more than 5 out of 100 will develop PHN [42; 58; 149]. The majority of HZ sufferers are age 60 or older [20; 56] and the number of people over 50 years of age will double in the next 50 years [58] indicating HZ and the associated pain will be an ever increasing problem in the future. Interestingly, females report PHN 3.75 times more often than men such that this pain has been reported to be 36% higher in females [2; 46], but little research has focused on this disparity. Although vaccines for both varicella (chicken pox) and HZ (shingles) are available the vaccines are effective in preventing about half of HZ cases and in reducing the clinical illness burden and the pain individuals experience by about

two-thirds [42]. Thus, with the vaccine 25 people out of 100 will develop HZ and more than 2 out of 100 will still develop PHN.

Mechanisms for VZV induction of pain are unclear but it appears that VZV does not need to replicate to induce PHN in humans. PHN patients given antiviral acyclovir showed no improvement [44; 134] suggesting replication of VZV would primarily occur only during the early acute phase of HZ and not play a role once PHN develops. In rats administration of antiviral agents also did not affect the allodynic response after VZV infection of the paw [21]. Replicating virus has not been demonstrated after VZV infection of the paw [29; 113; 114] suggesting lytic virus replication and necrosis of neurons is not a mechanism for induction of the nociceptive response in animal models. It is known that expression of replication genes is necessary to induce the PHN response in animals [40] and one clue about the mechanism inducing the nociceptive response after VZV infection is that inhibition of astrocytes in the spine dramatically reduces allodynic responses [150].

No animal models for zoster associated nociception of the face are currently available but in the paw VZV injection will result in mechanical allodynia and thermal hyperalgesia similar to humans with PHN [21; 29; 35; 40; 60; 110; 150]. Rat injection of VZV will increase ganglionic expression of calcium and sodium channels known to play a role in human neuropathic pain [35; 60; 150]. Moreover, treatment of VZV infected animals with antivirals, opioids or NSAIDS mirrors humans in that treatment with gabapentin or sodium channel blockers effectively ameliorated the pain [21; 35; 43; 110]. Together the results suggest a rodent model can be used to study the mechanism

of zoster associated nociception and be used as a tool for pre-clinical screening of analgesic drugs [21; 43]. Currently, neither the orofacial effects nor the sex differences have been determined in a zoster associated pain model.

1.2 Estrogenic Effects on Orofacial Pain

Epidemiological studies have demonstrated that certain pain disorders occur with higher prevalence, intensity, and/or duration in women as compared to men [17; 78; 10; 24; 86]. For example, trigeminal pain disorders, including migraine [84], trigeminal neuralgia [56] and Temporomandibular Joint Disorders (TMD) [143] are reported more often in females and the symptoms of the disorders are generally of greater magnitude [102; 10; 24; 86; 140; 19]. Steroid hormones, such as estrogen, have profound effects on gene expression, membrane-bond receptor/ion channels, and neuronal signaling both in the periphery and the central nervous system (CNS) as it can cross the blood-brain barriers [59]. It is important to understand how cycling estrogen can modulate orofacial pain and contribute to the sexual dimorphism of the orofacial pain response.

Estrogen receptors (ER), both alpha (α) and beta (β) subtypes, have been found in the both male and female rat Temporomandibular Joints (TMJs) and other orofacial structures [148]. A plasmatic- G protein coupled estrogen receptor 1 (GPER1) was also identified in addition to the two nuclear subtypes [33]. ERα immunoreactivity is highest in low estrogen states which may contribute to increase in the nociceptive response during certain phases of the estrus cycle. [103]. Notably, it has been shown that most

estrogenic effects actually occur via activation of estrogen receptors by intracellular aromatization of testosterone to estradiol [59].

The hormone changes during the rat estrous cycle are similar to those occurring during the human reproductive cycle [83]. The human cycle is characterized by a follicular phase in which estrogen and progesterone levels are low. Next during ovulation estrogen, follicular stimulating hormone and luteinizing hormone show marked increases that last for a few days. Following ovulation, in humans, is the luteal stage where progesterone rise and then drop during menstruation period. In the rat the stages or phases of the estrous cycle include first, diestrus (1-2 days), a time of low estrogen and progesterone (10 pg/ml). This is followed by proestrus (1 day) when estrogen, follicle stimulating hormone and luteinizing hormone increase abruptly leading to ovulation, which is similar to that which occurs during ovulation in humans (60-70 pg/ml) See Figure 1 [130]. All three hormones decrease during next phase, termed estrus (1 day), when the animal is sexually receptive [12]. It should be noted although direct correlations between rats and humans are often performed, such studies are complicated because there are subtle differences between the hormonal pattern of the rat estrous cycle and human ovarian cycle [31; 48]. There is evidence in the literature that the pain intensity varies over the menstrual cycle for women with orofacial pain conditions [70; 24], suggesting that changes in the estrogen levels modulate orofacial pain. Both human and rat studies have shown elevated pain thresholds to aversive stimuli during pregnancy, when estrogen level is high [37; 71; 47]. Women report increased orofacial pain just prior to menstruation when estrogen levels are low, during

or following ovulation and during rapid fluctuations of estrogen concentrations [70; 36; 18; 119]. When women were studied during periods of low and high estrogen, baseline μ-opioid receptor availability in-vivo and endogenous opioid neurotransmission was increased during pain states occurring when estrogen is increased [120]. Significant reductions in endogenous opioid tone were noted in various pain related CNS centers in periods of low estrogen that may account for the sex differences observed over the menstrual cycle [120]. Pain inhibition is more effective in the ovulatory phase of the menstrual cycle when estrogen levels are high than in the follicular phase when estrogen is low [106]. Pain thresholds in normally cycling healthy women were found to be lower in the follicular phase during low levels of estrogen when compared to other phases of the menstrual cycle [141]. However, the effects of estrogen on orofacial pain are complex as orofacial pain is attenuated during menopause and a reversal of these symptoms is observed with estrogen supplements [69]. Hormone replacement therapy (HRT) has also been associated with an increased risk of developing TMD, a common orofacial pain conditions, and those using estrogen replacement reported higher levels of pain than post-menopausal women not receiving HRT [119; 146]. This is not surprising as reproductive age women experience more orofacial pain than post-menopausal women. This observation may point to the cyclic nature and rapid fluctuations of estrogen as reasoning for increased pain sensitivity in low estrogen states for menstruating women and the attenuation on TMD and other types of orofacial pain seen in post-menopausal women [119]. HRT drugs, such as Premarin, are given in a constant dose which could lead to non-physiological levels of estrogen leading to increased

inflammation and pain experiences [69]. Myofascial pain patients who use oral contraceptives were significantly more stable over time than those reported by nonusers again suggesting decreasing rapid fluctuations in estrogen could play a role in the addressing the increased pain sensitivity evident in menstrual cycle phases with low and rapidly decreasing estrogen [24,118]. Experimentally induced pain studies in humans have failed to find the same differential pain experiences that is evident in epidemiologic studies of pain [18]. Thus, animal studies have been used to explore the complex nature of estrogens on orofacial nociception and have found that low levels and depleted levels of sex steroids causes increased orofacial hypersensitivity and the effects are site specific when comparing male and female rodents post gonadectomy [98].

1.3 Thalamus and GABAergic Signaling and Gene Expression

Thalamus

The thalamus processes, integrates and transmits information from multiple systems to the cerebral cortex. Peripheral sensory information such as visual, auditory, and tactile information is relayed to the thalamus for gaiting and projected to the cerebral cortex [133]. The lateral thalamus includes the ventral posterior medical nucleus (VPM) and the ventral posterior lateral nucleus (VPL). Sensory neurons in the craniofacial region, such as those areas innervated by the trigeminal nerve, project to the subnucleus caudalis (Sp5c). The VPM receives input from Sp5c and the principal nucleus (Pr5) [99]. In rats, the main projection of Sp5c is to the ipsilateral thalamus. The primary projection of Pr5 and some mandibular sensory neural projections terminate in the contralateral

thalamus. Efferent projections of the VPM are relayed to the primary somatic sensory cortex (S1), where pain is sensed by the brain [99].

The thalamus has a role in orofacial pain responses [11]. A facial HZ patient with PHN was found to have reduced thalamic activity on a PET scan [51]. Lesions in the lateral thalamic region, including the reticular thalamic nucleus, can heighten pain responses [112]. Estradiol has been shown to modulate pain responses by altering thalamic pain signaling [90].

γ-Aminobutyric acid

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain. GABA causes inhibition by binding one of two receptor subtypes: $GABA_A$ or $GABA_B$ See Figure 2 [97]. Activation of ionotropic $GABA_A$ receptors causes increased Cl^- ion conductance, whereas; activation of metabotropic $GABA_B$ receptors primarily activates a second messenger system and modulates G protein-gated calcium and potassium channels [138; 34; 101]. Previous investigation shows that activation of $GABA_A$ receptors outside of the CNS reduces pain [72; 92]. The reduction of GABA type A receptor-mediated inhibition has long been implicated in increased nociceptive response. For example, blockade of $GABA_A$ receptors within the TMJ will increase orofacial hypersensitivity [13; 14].

Interestingly, all neurons in the reticular thalamic region express GABA with only a miniscule population of cells in the adjacent ventroposterior nuclei [9]. GABA release in the thalamus regulates pain responses [95; 96; 100; 107; 108]. Neurons in the

ventroposterior send collaterals to the adjacent reticular thalamic nucleus and GABA positive neurons in the reticular thalamic nucleus send axons back to the ventroposterior thalamic nucleus regulating the "gating" of pain information passing through the thalamus to the cortex [141; 65]. Moreover, estradiol modulates GABA production in the brain [25] where the estrogen receptor is present [30; 45].

1.4 The Purpose of This Study

A gender disparity in orofacial pain conditions exists with epidemiological evidence that women report PHN more than men [2; 46]. Developing an orofacial animal model that can be utilized to study mechanisms of PHN and zoster-associated nociception will allow for investigating drug therapies to treat humans effectively. The animal model will also allow for the investigation of estradiol modulation of the VZV associated nociception responses. Estradiol modulates nociception through thalamic signaling and also modulates GABA production in the brain. These studies will also explore the estrogenic modulation of the zoster-associated nociception response. In addition it will investigate estradiol's control of GABAergic gene expression and thalamic signaling. This key sex hormone could be the physiologic mechanisms attributing to the sexual dimorphism in Zoster-associated pain and this study will strive to find the intricate link between estrogen, GABAergic thalamic signaling and VZV associated pain. Although the studies are specific to orofacial PHN, the indications could be non-disease specific and translate to other orofacial pain conditions displaying sex differences that predispose women to greater likelihood of increased pain experiences.

2. METHODS*

2.1 The PHN Rat Model

Cells and viruses

VZV Parent of Oka (pOka), a varicella isolate that was the parent strain used to derive the current VZV vaccine strain, was used in these experiments. pOka VZV induced chronic nociception indices in the rat model [40]. Virus was used at less than 12 passes beyond receipt from M. Takahashi (Osaka University, Japan) and propagated as detailed previously [61] in the VZV permissive MeWo human cell line (ATCC, Manassas, VA). Cells and virus were grown in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic and antimycotic mixture (Sigma, St. Louis, MO). VZV was prepared to high titer as previously detailed [40] on confluent monolayers of MeWo cells infected at ~0.1 plaque forming units (PFU)/cell. Virus infected cells incubated at 35 degrees Celsius for 48-72 hours post infection were harvested by trypsin digestion, and optimal VZV cell viability was maintained by slow freezing aliquots in MEM containing 20% FBS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO), followed by storage in liquid nitrogen.

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Virus titers of frozen stocks were assessed by an infectious center assay and VZV infectivity/cell ratios were estimated by immunostaining for VZV surface proteins and flow cytometry. VZV stocks used had >80% of cells infected. Uninfected cell equivalents were prepared using similar treatments for controls.

Animal studies

Male Sprague-Dawley rats (280-300 grams) from Harlan Industries, Houston, TX were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum. After a four day acclimation period experiments were completed in accordance with the NIH regulations on animal use with an approved TAMCOD IACUC protocol #16-327. One week after injection of a high (1900 pfu/µl with a 100µl total volume) dose of VZV (Poka strain), nociceptive assays were initiated.

Measuring thermal hyperalgesia and mechanical allodynia

The animals were trained to obtain a reward using the orofacial test chamber from Ugo Basile (Italy). The reward was sweetened condensed milk, 100 ml milk and 200ml water. Drinking time was measured by using an infrared beam that detects the animal has poked their nose into the spigot area. No thermal testing or filament testing was performed during the initial training period. Testing included 10 minutes of familiarization with the chamber environment and 10 minutes of active testing; so each animal was in the testing chamber 20 minutes each test day.

Once animals consistently drank milk for approximately 300 seconds out of a 600 second testing period further experiments were initiated, this training period took seven days. Thermal coils or filaments were added to the chamber and the animals were trained until they drank approximately 300 seconds out of a 600 second testing period, this training period took another 7 days. All testing was done in the beginning of the dark phase with red lights. Next, thermal hyperalgesia and mechanical allodynia were measured after injecting the whisker pad of the rats with control cells (human skin cell line) infected with a high dose of VZV or cells without virus (control), kindly provided by Dr. Kinchington. The contralateral whisker pad was not injected. The coils of the test chamber were set to 45 degrees Celsius for the thermal test. Twelve nickel/ titanium/chromium wires 0.16 mm in diameter were placed evenly around a circular opening of 3.5 cm in diameter for the filament test. Each wire projected 1.5 cm into the center of the opening. The test schedule included three days per week. Day one was a baseline with neither a thermal coil nor a filament present, a thermal test the following day and a filament test on the third day (Fig 6). Testing was done for six weeks, animals were then perfused and whisker pad tissue isolated for staining with PGP 9.5.

Place Escape/Avoidance Paradigm (PEAP) testing in male rats

A separate group of male rats were injected (whisker pad) with a high dose of VZV 1900 pfu/µl with a 100 µl total volume. The two other treatment groups were control cells or PBS and after seven days 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 Escape/Avoidance Paradigm (PEAP) test performed by the Fuchs' laboratory [63]. 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 [4; 7; 8; 63; 64; 137; 145].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 [63]. In this experiment the rat was placed into thechamber that has both a light and dark side and rodents being nocturnal prefer the dark side of the chamber. After placing the animal in the chamber the rat was immediately poked with 60 gram filament every 15 seconds such that when the rat is on the dark side of the chamber it was poked on the side that has been VZV injected. When on the light side of the chamber the rat was poked on the non-injected side (Fig 7). 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 V2. Because sensory neurons of the second branch (V2) innervate the whisker pad we expected sensitivity of the face below the eye [22]. The time spent on the dark side of the box was recorded in 5 minute intervals and testing was performed for a total of 30 minutes. Testing was performed once a week and continued for 8 weeks, the animals were then perfused and the trigeminal ganglia isolated for c-Fos or IE62 staining.

VZV injection in male rats to investigate analgesia efficacy

A separate group of male rats' whisker pads were injected with a low dose 360 pfu/µl of VZV or control cells and one week after injection the rats were given a 3.5 ml gavage of either 0.9% saline, or 50 mg/ml gapapentin (Amneal Pharmaceuticals, Branchburg, NJ). PEAP testing was performed 30 minutes after gavage for one week to test analgesic efficacy. No tissue was collected or analyzed from this group of male animals.

Immuno-fluorescent staining

After anesthetization with 100 mg/kg ketamine and 10 mg/kg xylazine the animals were perfused with 9% sucrose followed by 4% paraformaldehyde. Fixed tissues (spine, trigeminal ganglia, whisker pad) were stored in 25% sucrose, frozen, cryo-sectioned and the 20 µm sections placed on Histobond slides (VWR international, Radnor, PA). The tissue was blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for two hours at room temperature. The slides were incubated in a primary antibody solution overnight at 4°C. Primary antibodies consisted of a 1:200 dilution of the mouse monoclonal IE62 antibody (Acris Antibodies Inc., San Diego, CA) and a 1:250 dilution of the rabbit monoclonal NeuN antibody (Abcam, San Francisco, CA); or a 1:2000 dilution of the goat polyclonal c-Fos antibody and a 1:250 dilution of the mouse monoclonal NeuN antibody (Millipore, Billerica, MA); or a 1:500 dilution of the rabbit polyclonal PGP9.5 antibody (BioRad, Raleigh, NC) . Primary antibodies were diluted with PBS, 5% BSA and 0.3% Triton X-100.

After incubation in primary antibody the slides were rinsed three times in PBS and Triton-X 100 for a total of 45 minutes and placed for two hours in secondary antibody and PBS and 0.3% Triton X-100. After incubation in primary antibody the slides were rinsed three times in PBS and Triton-X 100 for a total of 45 minutes and placed for two hours in secondary antibody and PBS and 0.3% Triton X-100. Secondary antibodies included a mixture of goat anti-mouse 568 and goat anti-rabbit 488 or donkey anti-goat 488 and goat anti-mouse 568 (Invitrogen, Carlsbad, CA). After the PGP9.5 antibody incubation the slides were placed in a 1:500 dilution of the goat anti-rabbit 568 secondary (Invitrogen). After rinsing the slides three times in PBS for a total of 45 minutes 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).

Cell counting

Isolated whisker pads from virus and control rats were isolated after sacrifice. Counting of the PGP9.5 positive neurites in coronal whisker pad sections was performed by a blinded reviewer. PGP9.5 positive filaments that crossed the stratum basale/dermis border or were between the surface of the skin and this border were counted for every mm of skin. Neurite counts were completed on 10 sections taken from three different animals in each treatment group. The number of filaments counted per mm of skin for each section was reported.

Statistics

Data was analyzed with ANOVA and each time point was considered a replication. Independent variables included thermal hyperalgesia, mechanical allodynia, PEAP data, as well as, the estradiol concentration and the dependent variables were treatment, drug or estrous cycle. When a significant effect was observed Bonferroni post-hoc tests were completed (Prizm 5.04, GraphPad Software, La Jolla, CA, or Abstat, AndersonBell Corp, Arvada CO). When two groups were being compared a two-tailed Mann-Whitney t-test was performed.

2.2 Sex and Estrous Cycle Difference in the VZV Associated Nociception Response

Animal studies

Male and female Sprague-Dawley rats (280-300 grams) from Harlan Industries Houston, TX and Long Evans rats (280-300 grams) were purchased from Envigo (Indianapolis, IN) were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum. After a four day acclimation period experiments were carried out in accordance with the NIH regulations on animal use and with an approved TAMCOD IACUC animal protocol #16-327. 650 pfu/µl with a total volume of 100µl of VZV was inoculated into the whisker pad of the males as provided by Dr. Kinchington. PEAP testing was performed seven days after injection, continued once a week for six weeks. 360 pfu/µl with a total volume of 100µl of VZV was inoculated into the whisker pad of the female rats. PEAP testing was performed seven days after injection, continued once a week for five weeks.

Estrous cycle determination with vaginal cytology

Vaginal smears were performed daily on female rats to determine the stage of the estrous cycle, the estrous stage was recorded daily, and body weights were recorded weekly. Vaginal smears were taken immediately after PEAP testing each week. Plasma estradiol levels were measured via radioimmunoassay using blood collected after decapitation of the animal prior to dissection of needed tissues.

Ovariectomy

Animals were anesthetized with 2% isoflurane using a 2 liter per minute flow of air. The anesthetized female was placed in ventral recumbency with their tail toward the surgeon. The dorsal surgical area was shaved and swabbed with surgical scrub. Using sterile technique a short dorsal midline skin incision was made halfway between the caudal edge of the ribcage and the base of the tail. An abdominal muscle wall incisions was made bilaterally to the spine to provide access to the peritoneal cavity. The ovary and the oviduct were exteriorized through the muscle wall incision. A sterile silk ligature was placed around the oviduct. Each ovary and part of the oviduct was removed with a single cut through the oviduct near the ovary. The remaining tissue was replaced into the peritoneal cavity and the opening sutured. The ovary on the contralateral side was removed in a similar manner, the incision was closed and the animal was given a 2 mg/kg dose of nalbuphine. After a one week recovery period, 360 pfu/µl with a total volume of 100µl of VZV was inoculated into the right whisker pad and PEAP testing was performed seven days after injection, continued once a week for four weeks. Vaginal smears were done to ensure estrous cycle was disrupted by the ovariectomy.

Radio-Immunoassay

Plasma 17β-estradiol was quantitated in the trunk blood (blood collected from body of the animal after decapitation) by first, centrifugation and collection of the upper plasma layer, vigorously mixing 0.5 ml of plasma with 5 ml of ether. Second, the solution was frozen in an ethanol, dry ice bath and the ether phase decanted. The ether phase contains the lipid soluble hormones. Third, the ether was evaporated by nitrogen gas and the remaining residue suspended in radioimmunoassay buffer supplied by the manufacturer and assayed according to the manufactures directions (Beckman Coulter, Pasadena, CA).

2.3 GABAergic Gene Expression in the Thalamus

Animal studies

Female Sprague-Dawley rats (280-300 grams) from Harlan Industries, Houston, TX were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum and in accordance with the NIH regulations on animal use with an approved TAMCOD IACUC protocol #16-327. After a four day acclimation period the rats were smeared daily to determine the stage of the estrous cycle. Vaginal smears were performed for at least three consecutive days, the stage of the estrous cycle was determined and the rat sacrificed. In this study, the rats were sacrificed by exposure to CO_2 followed by decapitation. The brain was extracted using a rongeur and sliced on a slicer with 1 mm increments (Zivic, Pittsburgh, PA). Sections were 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, punches included the ventral posteromedial, ventral posterolateral and reticular thalamic nuclei. Tissue was stored in liquid nitrogen until RNA or protein could be isolated. The thalamic tissue was used for RNA isolation and transcript quantitation.

Radio-Immunoassay

17β-estradiol was quantitated in the trunk blood (blood from body of animal following decapitation) by first, centrifugation and collection of the upper plasma layer, vigorously mixing 0.5 ml of plasma with 5 ml of ether. Second, the solution was frozen in an ethanol, dry ice bath and the ether phase decanted. The ether phase contains the lipid soluble hormones. Third, the ether was evaporated by nitrogen gas and the remaining residue suspended in radioimmunoassay buffer supplied by the manufacturer and assayed according to the manufactures directions (Beckman Coulter, Pasadena, CA). Trunk blood was collected from both cycling animals and ovariectomized animals.

Statistics

The data was analyzed using ANOVA to determine significant estrous cycle effects. When significant ($p<0.05$), the test was followed by Tukey's post-hoc test. A two-tailed Spearman correlation statistic was used to compare the RIA and real time PCR data.

Real time PCR

RNA extraction was performed using the RNA Lipid Tissue Kit from Qiagen (Valencia, CA). The RNA concentration 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 from Qiagen. The thermal protocol was 30 minutes @ 50 °C for the reverse transcription reaction, 15 minutes @ 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 and reactions run in triplicate for quality assurance. Sample amount was adjusted according to total RNA concentration to obtain 20 ng of total RNA per well in the final reaction mix. For the RT-PCR data inflection point of each amplification curve and cycle number was determined as previously described in detail [41]. The Cɣ0 for GAPDH was subtracted from the Cɣ0 of each gene of interest to normalize the results (ΔCɣ0). A ΔCɣ0 value at a particular estrous cycle phase was subtracted from the mean ΔCɣ0 metestrus value (ΔCɣ0 metestrus average- ΔCɣ0 (cycle phase) = ΔΔCɣ0). To get the fold change the ΔΔCɣ0 was raised to the second power (2 ΔΔCɣ0).

2.4 Modulation of Lateral Thalamic Activity and VZV Associated Nociception

Animal studies

Male (280-300 grams) Sprague-Dawley rats from Envigo (Indianapolis, IN) were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum. After a 4 day acclimation period experiments were completed in accordance with the NIH

regulations on animal use and TAMCOD IACUC animal protocol #16-327. Thirty-two male rats were injected with an Adeno Associated Virus serotype 8 (AAV8) with inhibitory neuronal silencing virus and recording electrodes were implanted and then a week later the whisker pad injected with 100,000 pf/ ul of VZV. Local field potentials (LFP) recording in awake animals was completed for the rats (see below). The recording backpack restricts movement and significantly effects behavior (data not shown), thus behavioral nociception measurements were not reported. After three weeks the rats were sacrificed and tissues collected for molecular studies.

Thalamic AAV virus infusion an electrode placement

32 Adult male Sprague-Dawley rats (were anesthetized with 2% isoflurane and an air flow of 2 liter per minute. Stereotaxic (David Kopf Instruments, Tujunga, CA, Model 1460-61) injection of virus was performed with the needle tip (Hamilton #7002KH Neuros syringe, Reno, NV) at coordinates 3.6 mm posterior of Bregma, 3.0 mm from midline at a depth of 6.0 mm. A Stoelting stereotaxic syringe pump system was used to infuse 0.250 µl of 2-8 X1012 pfu/ml AAV8 at a rate of 20 nanoliters per minute. In the vehicle (no virus) group 0.250 µl of vehicle (350 nM NaCl, 5% D-Sorbitol in PBS) was infused. After infusion the needle was left in place for 5 minutes and then removed.

Male rats were infused in the right thalamus with AAV8 expressing a neuronal silencing construct Syn-hM4D(Gi)-mcherry (Gene Therapy Center Vector Core, University of North Carolina at Chapel Hill) or vehicle. The hM4D(Gi) gene was an engineered acetylcholine Gi-protein coupled receptor that inhibits neuronal burst firing when bound by clozapine-n-oxide (CNO). Upon CNO binding the receptor stimulates calcium release, Extracellular Signal Related Kinase (ERK1/2) activation, inhibits forskolin-induced cyclic-AMP(cAMP) formation and potentially G-protein coupled inwardly-rectifying potassium channels (GIRK) activation, thereby causing hyperpolarization and inhibition of basal action potential firing [109]. To affect change in neuronal activity, the modified acetylcholine Gi protein-coupled receptor was expressed primarily in the posterior, ventral posteromedial (VPM), ventral posterolateral (VPL) and reticular (Rt) thalamic nuclei, as well as the zona incerta (ZI) of Sprague-Dawley rats and activated by binding CNO.

Electrode placement

Immediately after infusion of AAV into the thalamus, two stainless steel bipolar twisted electrodes (MS303-1-B-SPC Elect SS 2C TW, Plastics One, Roanoake, VA) were implanted into the right hemisphere. Thalamic coordinates were 3.6 mm posterior of Bregma, 3.0 mm from midline at a depth of 6.0 mm. The electrodes were cemented with acrylic to jewelers screws attached to the skull. Histology is performed after sacrifice to verify electrode location was correct.

Recording electrical activity

LFP recordings were recorded using a customized wireless recording module [151] attached to the recording electrode implanted directly into the lateral thalamus. LFP was recorded using a low pass filter of 100Hz. Standard techniques of applying a Fast Fournier transform (FFT) were used [85]. The time resolution is 0.8192s and the frequency resolution is 1.221 Hz. Rats were briefly (5 minutes) anesthetized with 2% isoflurane and fitted with a backpack containing the LFP wireless recording module [151]. After waking from anesthesia a 10 minute LFP "Baseline" was recorded. Recording was completed for 30 minutes after intraperitoneally injecting a 1 mg/kg dose of CNO in 0.9% saline or 0.9% saline in a 0.5 ml volume [5]. PEAP testing was done for the last 30 minutes of the testing period (data not shown). Testing was completed for 3 weeks. Signals from the electrode were amplified and changed from volts to digital form by an Analog-to-Digital Converter (Multichannel Systems, Reutlingen, Germany) within the recording microcontroller module. Signals were transmitted by a receiver on a USB dongle to a laptop using customized software. The signals were saved as data in a raw waveform at a sampling rate of 4096 Hz (CED Spike2 V7 software, Cambridge, UK).

After anesthetization with 100 mg/kg ketamine and 10 mg/kg xylazine the animals were first perfused with 9% sucrose and then with 4% paraformaldehyde in 1X PBS, pH 7.4. Fixed tissues were stored in 25% sucrose, frozen, cryo-sectioned and the 20 μm sections placed on Histobond slides (VWR international, Radnor, PA). 7 rats were randomly chosen for the staining procedure. The tissue was then blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for 2 hours at

room temperature. The slides were incubated in primary antibody overnight at 4°C. Primary antibodies consisted of the mouse monoclonal NeuN antibody at a 1:250 dilution (Millipore, Billerica, MA), or the goat polyclonal VGLUT2 antibody (ab101760, Abcam, Cambridge UK) at a 1:150 dilution. Primary antibodies were diluted in PBS containing 5% BSA and 0.3% Triton X-100. After rinsing three times in PBS with 0.3% Triton X-100 for a total of 45 minutes, slides were placed for 2 hours in secondary antibody. Secondary antibodies (1:500 dilution) included goat anti-mouse 488 or donkey anti-goat 488 (Invitrogen, Carlsbad, CA). After rinsing the slides three times in PBS for a total of 45 minutes, 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).

Cell counting

Quantitation in the rats that received electrodes was completed by a blinded reviewer. Three rats were randomly chosen out of the control groups and four rats out of the VZV treated groups. Counts were completed for the number of AAV neurons transduced within a 0.5 mm^2 field adjacent to the injection site. On each section two randomly selected fields for each nuclei near the tip of the injection site was counted. Every other section lateral of the injection site was selected for staining. The injection site was the center point of the sections that were selected. Three sections were counted

for each animal. Cell counts from each section were averaged for each animal. Values were recorded as a mean and SEM.

Statistics

LFP and cell count data was analyzed by two-way ANOVA, the independent variables were virus (VZV, control) and drug (vehicle, CNO) and the dependent variables were the PEAP, power values and the cell counts. When a significant effect was observed Bonferroni post-hoc tests were completed (Prism 5.04, GraphPad Software, La Jolla, CA, or Abstat, Anderson Bell Corp, Arvada CO). All values are given as the mean and SEM.

2.5 GABAergic VGAT modulation in the VZV Associated Nociception Response

Animal studies

Male (300-350 grams) and female (280-300 grams) Sprague-Dawley rats from Envigo (Indianapolis, IN) were kept on a 14:10 light/dark cycle. The rats were given food and water ad libitum. After a four day acclimation period experiments were completed in accordance with the NIH regulations on animal use with an approved TAMCOD IACUC animal protocol # 16-327. AAV8 animals were injected with a high dose of VZV at >1000 pfu/µl. Male rats were injected with a lower concentration of VZV from a different preparation (650 pfu/µl).

Surgery and AAV8 injection

Adult male or female Sprague-Dawley rats were anesthetized with 2% isoflurane and an air flow of 2 liter per minute. Stereotaxic (David Kopf Instruments, Tujunga, CA, Model 1460-61) injection of virus was performed with the needle tip (Hamilton #7002KH Neuros syringe, Reno, NV) at coordinates 3.6 mm posterior of Bregma, 3.0 mm from midline at a depth of 6.0 mm from dura. A Stoelting stereotaxic syringe pump system was used to infuse 0.250 µl of 2-8 X1012 pfu/ml AAV8 containing a Designer Receptor Exclusively Activated by Designer Drugs (DREADD) or 1 X1013 pfu/ml AAV5 (see below) or AAV9 (see below) at a rate of 20 nanoliters per minute. In the vehicle (no virus) group 0.250 µl of vehicle (350 nM NaCl, 5% D-Sorbitol in PBS) was infused. After infusion the needle was left in place for 5 minutes and then removed. Both male and female rats were injected bilaterally with AAV8 expressing a neuronal silencing construct Syn-hM4D(Gi)-mcherry (Gene Therapy Center Vector Core, University of North Carolina at Chapel Hill) or vehicle. The gene contained an engineered acetylcholine Gi-protein coupled receptor that inhibits neuronal burst firing when bound by CNO. Expression of the receptor was driven by the neuronal synapsin-1 promoter, which is expressed in most neurons. Upon CNO binding the receptor stimulates calcium release, (ERK1/2) activation, inhibits forskolin-induced cAMP formation and potentially GIRK activation, thereby causing hyperpolarization and inhibition of basal action potential firing [109]. To affect change in neuronal activity, the modified acetylcholine Gi protein-coupled receptor was expressed primarily in the posterior, VPM and VPL thalamic nuclei of SD rats and activated by binding CNO [1].

The viral vector AAV9 contained either a verified VGAT shRNA construct (5'CACCGCATCATCGTGTTCAGCTACACTCGAGTGTAGCTGAACACGATGATGCTTTTT-3') driven by the U6 promoter with a mCherry tag or AAV5 containing a scrambled shRNA construct (5'-AGGATCC AGTACTGCTTACGATACGG-TTCAAGAGACCGTATCGTAAGCAGTACTTTTTTT-3') driven by the U6 promoter containing a GFP tag (Vector Biolabs, Philadelphia, PA). Animals were given nalbuphine after surgery and allowed to recover 7 days before injecting VZV or control cells.

Place Escape/Avoidance Paradigm testing in male rats

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 1000 pfu/µl VZV or control cells lacking virus [40]. The rats were ambulatory after five minutes following injection. Seven days following whisker pad injection rats were evaluated for nociception by individuals blinded to the groups using the PEAP test as detailed by the Fuchs' laboratory [63]. AAV8 rats received an intraperitoneal injection of 1 mg/kg clozapine-n-oxide dissolved in 0.9% saline or an injection of 0.9% saline in a 0.5 ml volume 30 minutes before testing to activate the DREADD complex. Testing was completed for four weeks in AAV8 males and for two weeks in experiments AAV8 females. AAV9 and AAV5 males were tested for only two weeks.

Tissue collection

Rats were sacrificed by exposure to CO_2 followed by decapitation. The brain was extracted using a rongeur and cut on a Zivic brain slicer (Zivic, Pittsburgh, PA). Sections were cut 2 mm thick 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 around the injection site, punches included the posterior thalamic nucleus, the ventral posteromedial and ventral posterolateral thalamic nuclei and a portion of the reticular thalamic nucleus. Tissue was stored in liquid nitrogen until RNA or protein extraction.

Real time PCR

RNA extraction was performed using the RNA Lipid Tissue Kit from Qiagen (Valencia, CA). RNA concentration 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 (VGAT primer catalog # QT00378413, GAPDH primer catalog # QT00199633). The thermal protocol was 30 minutes @ 50 °C for the reverse transcription reaction, 15 minutes @ 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. 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. For each sample, the threshold

Ct (threshold cycle) value for GAPDH was subtracted from the Ct of value for VGAT to give a ΔCt. The mean ΔCt from the right thalamus was subtracted from the left thalamus to give a ΔΔCt. To get the fold change the ΔΔCt was raised to the second power (2-ΔΔCt). Values for decreased expression were calculated by using the formula (-1/fold change).

Immunofluorescent staining

After anesthetization with 100 mg/kg ketamine and 10 mg/kg xylazine the animals were first perfused with 9% sucrose and then with 4% paraformaldehyde in 1X PBS, pH 7.4. 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 blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for 2 hours at room temperature. The slides were incubated in primary antibody overnight at 4°C. Primary antibodies consisted of the mouse monoclonal NeuN antibody at a 1:250 dilution (Millipore, Billerica, MA), 1:200 dilution of the rabbit polyclonal c-Fos antibody (Cell Signaling, 96F) or the rabbit polyclonal VGAT antibody (AB5062P, Millipore) at a 1:200 dilution. Primary antibodies were diluted in PBS containing 5% BSA and 0.3% Triton X-100. After rinsing three times in PBS with 0.3% Triton X-100 for a total of 45 minues, slides were placed for two hours in secondary antibody. Secondary antibodies (1:500 dilution) included goat anti-mouse 488, goat anti-mouse 568, goat anti-rabbit 488 or goat anti-rabbit 568 (Invitrogen, Carlsbad, CA). After rinsing the slides three times in PBS for a total of 45 minutes, 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).

Cell counting

Quantitation was completed by a blinded reviewer. Counts were completed for the number of AAV and NeuN positive cells within a circular field 0.5 mm in diameter. On each section a randomly selected field near the tip of the injection site was counted. Three sections were counted per animal. Every other section was selected for staining. The injection site was the center point from which sections were selected. Cell counts from each section were then averaged for each animal. Values were given as a mean and SEM for the three animals per treatment group.

Statistics

PEAP data was analyzed by two-way ANOVA with repeated measures the independent variables were the PEAP data and the dependent variables were virus (VZV, control and drug (no CNO, CNO) or drug (no CNO, CNO) and sex (male, female). NeuN cell count data was analyzed by two-way ANOVA and the independent variable was cell counts and the dependent variable was treatment (VGAT shRNA, control shRNA). 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). c-fos cell count data and PCR data were analyzed using a unpaired t-test. All values are given as the mean and SEM.

3. RESULTS*

3.1 The PHN Rat Model

Animal model histological studies show human PHN disease hallmarks

Interestingly, skin biopsies of zoster patients suffering PHN have reduced peripheral innervation of the skin compared to zoster patients not suffering from PHN [93; 111]. To measure peripheral innervation, neurites were identified with PGP9.5 staining (Fig. 3A, B red). Rats showing hypersensitivity due to VZV injection had significantly reduced peripheral innervation of the whisker pad skin (Fig. 3C). Trigeminal ganglia neurons were IE62 positive, a marker for VZV expression (Fig. 4). IE62 is an essential intermediate early gene in the production of VZV and localizes to both the nucleus and cytoplasm [15]. c-Fos was present in neurons within the caudalis of VZV injected rats that were still responsive to the PEAP assay eight weeks after VZV injection of the whisker pad (Fig. 5).

VZV infection decreased nociception thresholds in the trigeminal V2 region

After whisker pad injection of VZV facial thermal hyperalgesia of male rats was significantly increased $F(1,170)=7.1$, $p<0.01$ ($n=10$) in comparison to rats injected with uninfected control cells (Fig. 6A). In Figure 6, fewer seconds drinking is an indicator of greater hyperalgesia or allodynia.

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There was no significant interaction of treatment and time $F(1,170)=0.63$, $p=7.2$. Testing for VZV induced mechanical allodynia revealed the rats showed a significant response over the 51 day testing period $F(1,170)=16.3$, $p<0.0001$ with no significant interaction between treatment and time $F(1,170)=1.7$, $p=0.1$ (Fig. 6B). Post-hoc testing revealed a significant difference between the VZV and Control treatment groups at days 15 and 23 (Fig. 6B).

VZV infection increased PEAP affective nociception response

In addition to measuring thermal hyperalgesia and mechanical allodynia in male rats a PEAP test was completed after whisker pad injection. Fewer seconds on the dark side indicates a greater affective nociception response [63]. Compared to the controls the thermal hyperalgesia response following VZV injection was significant for the first seven weeks of the eight week experimental period. (Fig. 7): significance was observed in week one $F(2,75)=19.9$, $p<0.001$ (Fig. 7A); two $F(2,75)=196.1$, $p<0.001$ (Fig. 7B); three $F(2,75)=31.3$, $p<0.001$ (Fig 7C); four $F(2,75)=14.7$, $p<0.001$ (Fig. 7D); five $F(2,75)=20.1$, $p<0.001$ (Fig. 7E); six $F(2,75)=5.2$, $p<0.05$ (Fig. 7F); and seven $F(2,75)=6.4$, $p<0.01$ (Fig. 7G), $n=6$ rats per group. The effect of virus in week eight was no longer significant, $F(2,75)=2.4$, $p=0.12$ (Fig, 7H). A significant interaction between the PBS, control and VZV groups was observed in weeks two $F(10,75)=5.8$, $p<0.001$ (Fig. 7B) and three $F(10,75)=2.5$, $p<0.05$ (Fig. 7B). No significant interaction was observed in the other weeks (data not shown). Post hoc testing demonstrated a difference between the VZV and control groups for seven out of the eight weeks (Fig. 7A-G);

but starting with week eight no significant differences were determined (Fig. 7H).

In a control experiment, when no filament was applied, rats in the virus group spent a majority of their time on the dark side such that there was no significant difference between the virus and control groups (data not shown). These PEAP assay results demonstrate that the PEAP method was very sensitive to nociceptive changes resulting from virus injection and thus, the PEAP assay was continued throughout the rest of the following experiments.

Gabapentin decreased the PEAP nociception response

Seven days after injecting the whisker pad with VZV, gabapentin was administered orally. Injection of VZV significantly increased the nociceptive response in the PEAP assay $F(2, 171), 13.41, p<0.01$ (Fig. 8), n=5 per group. Gabapentin significantly reduced the nociceptive response in the PEAP assay $F(2, 171)=6.75$, $p<0.01$. Post-hoc testing demonstrated that gabapentin significantly reduced the response at the 15, 20, 25 and 30 minute testing periods (Fig. 8). A significant virus/drug interaction was observed $F(2, 171)=7.77$, $p<0.01$. No significant differences were observed between the control treatment groups (data not shown).

3.2 Sex and Estrous Cycle Differences in the VZV Associated Nociception Response

Female rats experience longer PEAP nociception from VZV infection

Virus was injected into female and male rats and the nociceptive response was measured over 6 weeks.

One week after virus injection the PEAP response significantly increased $F(1, 395)=19.1$, $p<0.001$ (Fig. 9A) but there was no significant difference between the sexes $F(1, 395)=0.03$, $p=0.85$, see figure legend for number of animals per group. However, starting at week two after injection there was a significant nociceptiveeffect due to virus in the females but not in the males [week two $F(1,443)=16.6$, $p<0.001$ (Fig. 9B), three $F(1, 473)=15.4$, $p<0.001$ (Fig. 9C), four $F(1, 473)=10.6$, $p<0.01$ (Fig. 9D) and five $F(1, 473)=8.4$, $p<0.01$ (Fig. 9E). This sex difference was no longer significant in week six (Fig. 9F)]. No significant interaction between sex and virus was observed in week one and five but in week two, three and four a significant interaction was measured $F(1,443)=4.9$, $p<0.05$, $F(1, 473)=16.8$, $p<0.001$, $F(1, 473)=11.0$, $p<0.01$, respectively

Low Estradiol increases PEAP nociception in female rats

Comparison of 17β-estradiol over the estrous cycle indicates that the plasma level of 17β-estradiol was significantly greater at proestrus and estrus versus diestrus I and diestrus II (Fig. 10, n=15). The behavioral response in the PEAP assay was determined for the control and virus injected animals over the estrous cycle. In Figure 11 the estrus and proestrus data were combined and the diestrus I and diestrus II (i.e., metestrus) data were combined because of their similar estradiol levels. A significantly greater response was observed in rats with low estradiol (Diestrus VZV) versus the rats with higher plasma estradiol (Proestrus/estrus VZV) in week one $F(1, 281)= 5.8$, $p<0.05$ (Fig. 11A, $n\geq8$), two $F(1, 281)= 4.4$, $p<0.05$ (Fig. 11B),

three $F(1, 281)= 61.6$, $p<0.001$ (Fig. 11C) and four $(1, 281)= 12.4$, $p<0.01$ (Fig. 11D). No significant cycle response was observed in weeks five and six (data not shown). During the first $F(1, 281)= 19.2$, $p<0.001$, second $F(1, 281)= 11.2$, $p<0.01$, third $F(1, 281)= 21.2$, $p<0.001$, fourth $F(1, 281)= 4.3$, $p<0.05$ week there was a significant virus effect. A significant interaction between cycle and virus treatment was observed in week two $F(1, 281)= 9.4$, $p<0.01$, three $F(1, 281)= 21.3$, $p<0.001$ and four $(1, 281)= 14.6$, $p<0.001$. Post-hoc testing indicated a significant decrease in the Diestrus VZV group versus the Diestrus control group at the 15, 20, 25 and 30 minute time points in weeks one, two, three, and four. No significant difference was observed in week five and six.

Ovariectomized (constant low estradiol) rats have more PEAP nociception from VZV infection

An additional experiment was performed to determine the VZV response of OVX females in comparison to males because a majority of human females suffering from PHN are post-menopausal and have low levels of plasma estradiol. The PEAP assay was performed for two weeks and then the animals were sacrificed to determine viral infection during the active behavioral response. Sprague Dawley rats injected with VZV showed a significant response $F(1, 144)=117.0$, $p<0.001$ in the first week, but there was no significant nociceptive sex difference (Fig. 12A). Two weeks after VZV inoculation the intact female and OVX female rats had a significantly greater response than males (Fig. 12B)

3.3 GABAergic Gene Expression in the Thalamus

Increased estradiol levels increased GABAergic Gene Expression

When comparing thalamic transcript at proestrus and diestrus the genes that increased at least two fold on a large gene array were mostly related to GABA function(Table 1).Measurement of plasma estradiol indicated rats in the proestrus or estrus phase of the estrous cycle had a significantly greater amount of estradiol in comparison to rats in either diestrus or metestrus (Fig. 13).

Transcript for the GABA related genes GAD1, GAD2, GABARAPL1 and VGAT was significantly elevated at the estrus phase of the estrous cycle (Fig. 14 A, B, C and D). There was a direct relationship between the amount of transcript and the plasma estradiol. A significant correlation between GAD1 ($r=0.35$, $p<0.05$, Fig. 14A), GAD2 ($r=0.36$, $p<0.05$, Fig. 14B), VGAT ($r=0.42$, $p<0.01$, Fig. 14D) and estradiol was measured but no significant relationship was observed for GABARAPL1 and estradiol ($r=0.25$, $p>0.1$, Fig. 14C).

3.4 Modulation of Lateral Thalamic Activity and VZV Associated Nociception

Excitatory neurons in the VPM were transduced with Gi construct

Thalamic AAV infection of the Syn-hM4D(Gi)-mcherry construct resulted in cells expressing the fluorescent virus tag. These fluorescent cells were located in the posterior, ventral posteromedial and ventral posterolateral thalamic nuclei (Fig. 15). Many of the cells infected with virus were neurons as indicated by the staining of axons (Fig. 15). Cell quantification showed Thalamic neurons were transduced with the Gi construct after injecting the VPM with AAV (Figure 15E). Following AAV injection the whisker pad of these rats were injected with either VZV or control (i.e., MeWo carrier cells). One week following whisker pad injection the groups were divided, half received the drug CNO and half vehicle. The average number of NeuN positive cells in a 0.5 mm^2 field was not significantly different between the treatment groups $F(3, 31)=0.94$, $p=0.42$ (n=3 control groups, n=4 VZV groups) or between the different nuclei (VPM, Rt or ZI) $F(2, 31)=0.32$, $p=0.72$. No significant interaction between treatment and location was observed $F(6, 31)=0.25$, $p=0.95$. The Gi construct with a fluorescent tag (mcherry) was observed in cells positive for the neuronal marker NeuN (Fig. 17A-D). Cell counts of these fluorescent cells indicated that the VPM had a significantly greater number of AAV transduced neurons $F(2, 31)=36.66$, $p<0.0001$ than the Rt or ZI (Fig. 17E).

There was no effect of treatment $F(3, 31)=0.33$, $p=0.80$ and no interaction between treatment and location of the cells $F(6, 31)=0.35$, $p=.90$.

Decreasing lateral thalamic activity decreased the VZV associated nociception response

In the nociception assessment studies, rats injected with cell-associated VZV showed significantly greater nociception over three weeks when compared to rats injected with an equivalent number of uninfected control cells (Fig. 16A-D). The VZV effect was significant in weeks one $F(1, 14)=17.4$, $P=0.0009$, two $F(1, 14)=37.5$, $P<0.0001$ and three $F(1, 14)=137$, $P<0.0001$ but not in week four $F(1, 14)=3.9$, $P=0.07$.

However, activation of Gi by CNO administration significantly reduced nociception in the VZV inoculated male rats as compared to vehicle inoculated rats (Fig.16A-D) in weeks one $F(1, 14)=24.2$, $P=0.0002$, two $F(1, 14)=12.5$, $P=0.003$ and three $F(1, 14)=107$, $P<0.0001$ but not in week four $F(1, 14)=0.009$, $P=0.97$. A significant interaction between VZV and drug (CNO) was observed in weeks one $F(1, 14)=23.3$, $P=0.0003$, two $F(1, 14)=16.6$, $P=0.001$ and three $F(1, 14)=99$, $P<0.0001$, but not in week four $F(1, 14)=0.13$, $P=0.72$. Female rats also developed nociception indicators after VZV injection and also showed reduced nociception after CNO activation of hMD4i(Gi) in week one $F(1, 10)=29.6$, $P=0.0003$ and two $F(1, 10)=36.4$, $P=0.0003$ (Fig. 17 A, B). For the first week after injecting VZV no sex difference in nociception was indicated $F(1, 17)=0.24$, $P=0.62$, but there was a significant sex difference in week two $F(1, 17)=13.7$, $P=0.002$ (compare Fig. 16A, B to Fig. 17A, B)

VZV infection increased local field potentials in VPM

LFP is the summation of extracellular currents recorded at subthreshold levels and offers a uniquely integrated view of the activity in a specific brain area [87]. VZV injection significantly increased activity at the theta frequencies in the VPM the first week after injection $F(1,27) = 3.89$, $p = 0.05$, n=8. VZV also increased VPM activity at the gamma frequencies in week one $F(1, 27) = 4.18$, $p=0.05$. A significant interaction between VZV and DREADD (CNO) treatment was observed $F(1, 27) = 5.10$, $p=0.03$. Post-hoc analysis demonstrated VZV significantly increased activity at the gamma frequency and CNO treatment decreased this response (Fig. 18). In weeks two no significant effect on LFP activity in the VPM was measured for the virus or the DREADD.

3.5 GABAergic VGAT modulation in the VZV Associated Nociception Response

The VGAT shRNA adequately targeted VGAT cells and decreased VGAT expression

The VGAT shRNA construct co-localized to VGAT expressing cells in the reticular thalamic nucleus (Fig. 21B-D, arrows). A significant >3 fold reduction ($p<0.05$, n=7) in VGAT expression was observed in the lateral thalamic region after infection of VGAT shRNA (Fig. 20).

Decreasing VGAT expression increased the VZV associated nociception response

PEAP test was performed once a week for two weeks starting 7 days after VZV or control injection. A reduction in thalamic VGAT expression coincided with an increased nociception resulting from VZV infection (Fig. 19).

4. DISCUSSION*

4.1 The PHN Rat Model

Persistent mechanical allodynia and thermal hyperalgesia have been associated with VZV injection in the paw and this study demonstrates a significant sensory response in the facial region as well [35; 39; 43]. The PEAP assay is considered a measure of affective nociception [63] and the affective response, as measured by the PEAP assay, lasted several weeks longer than the sensory response (Ugo Basile chambers). The longer PEAP response suggests that either the PEAP assay was more sensitive than the Ugo Basile chambers in measuring responses or that the affective component is present longer than the sensory component after VZV injection. Previous studies injecting compete Freund's adjuvant into the paw showed that sensory responses (von Frey) and affective responses (PEAP test) lasted for a similar amount of time [63]. In contrast, the affective aspect of nociception remained longer or was more intense than the sensory aspect of nociception in this VZV model. Behavioral tests such as the PEAP require supraspinal nociception processing and are expected to be useful in development of analgesics for patients [7; 137]. Administration of gabapentin has been effective in treating PHN in HZ patients and consistent with human treatment gabapentin reduced the PEAP response in this rat model [44]. It is interesting to note that anxiety like behaviors were not reversed by gabapentin treatment after VZV injection of the paw but did reverse the response in the PEAP assay [43],

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consistent with the result that anxiety does not appear to bias the PEAP measurement [7; 144]

Most cases of PHN has been shown to cause life altering consequences such as depression, suicide and withdrawal due to debilitating pain making research into this disease process and development of a treatment modality crucial [53; 55; 57; 150]. In a study of 658 HZ patients and 1974 controls, the risk of stroke was 4.52-fold higher in the HZ patients than in controls. HZ, commonly known as shingles, affects about a quarter of the population in their lifetimes, with incidence rising with age and immune decline [42; 149]. Most adults are at risk for developing HZ and PHN because once infected with the virus, wild-type VZV resides within their ganglia in a latent state [149]. Even if all individuals in the US over the age of 60 received the zoster vaccine, more than 500,000 cases annually would be at increased risk of stroke within the following year indicating the need to study HZ and the development of PHN [91].

4.2 Sex and Estrous Cycle Differences in the VZV Associated Nociception Response

VZV injected female rats experienced the same level of nociception as the VZV injected males post-injection week 1 but during post-injection week 2 the females experienced a higher level of sensitivity. Sex differences in pain responses have been previously shown to affect nociception in the TMJ region [62] and several past studies have determined that female subjects experience more pain-related sensations but very few studies determined the mechanism causing this difference between males and females [10; 117; 136].

One reason for the sex difference observed in this study could be related to estradiol in the females. Previous studies have suggested that estrogen enhances nociception, whereas progesterone attenuates nociception [32; 94; 105; 131].This data supports a role for estrogen in that during the estrous cycle when estradiol levels were reduced (diestrus) there was a significantly greater PEAP response in comparison to when estradiol was higher (proestrus/estrus). This data is consistent with the nociceptive response in an inflammatory TMJ model where rats given a diestrus level of estradiol had a greater nociceptive response in comparison to rats given a proestrus dose of estradiol [62]. Although the mechanism is unclear as to why the nociceptive response is higher in females, it is important to notice that the PEAP assay post VZV injection can be used to further explore the mechanistic differences in pain processing between males and females. Determining a mechanism for the sex difference in PHN post-HZ has not been investigated but is warranted as PHN is reported 3.75 times more often in women than in men after HZ infection [46]. This study presents a promising new model reflecting the male/female differences in zoster associated pain. For the first time a sex difference is shown after VZV injection consistent with epidemiological evidence that women report PHN more than men [2; 46]. This model can be utilized to study mechanisms of zoster-associated pain and test drug therapies to treat humans effectively.

4.3 GABAergic Gene Expression in the Thalamus

Screening of thalamic genes in proestrus and diestrus rats revealed changes in expression for GAD1, GAD2, GABARAPL1 and VGAT.

Expression of these four genes was studied further at each stage of the estrous cycle using real time PCR. The results indicate that all four genes significantly increased at the proestrus or estrus stage,a period when estradiol is elevated. GAD1, GAD2, GABARAPL1 and VGAT show a similar pattern of change across the estrous cycle and the pattern of transcript expression correlated to the change in estradiol during the cycle. Importantly, elevated estradiol was associated with elevation of these GABA genes in the thalamus; consistent with the idea that estradiol can modulate nociception responses by increasing activity in the GABA pathway.Transcript levels for GAD1, GAD2 GABARAPL1 and VGAT were not significantly elevated in proestrus rats but the protein content was elevated at proestrus (data not shown). One explanation for this result would be that the transcript was measured in early proestrus when estradiol was less. Whereas the protein was isolated from rats in late proestrus and plasma estradiol was greater. The greater amount of estradiol could explain the higher amount of GAD1, GAD2 GABARAPL1 and VGAT protein versus transcript.

Changes in gene expression typically takes several hours after binding ERα [66; 68] and this delay, compounded with a lower level of estradiol, could have contributed to the lower amount of transcript observed at proestrus. It is known that sex hormones (notably, estrogen) affect nociception and pain perception across mammalian species [3; 27; 50; 118]. For example, orofacial nociceptive responses are reduced in proestrus rats when estradiol levels are elevated [28; 62].

Elevated nociception sensitivity at diestrus and metestrus has been linked to sex steroid modulation of GABA activity [134]. Viral introduction of GAD1 into the sciatic nerve has been shown to reduce neuropathic nociception [54]. A recent study has shown that altering the activity of GAD1 positive cells in the cingulate cortex can effect nociceptive responses [38]. Moreover, a link between GAD2 expression and nociception was observed in the spinal cord [74; 88]. In mice where VGAT levels are reduced thermal nociception and inflammatory nociception were enhanced [148]. Together these results suggest altered expression of GAD1, GAD2 and VGAT can affect nociception responses.

Increased production of GABARAPL1, a GABAA receptor subunit recycling protein [82], during proestrus/estrus would increase turnover of the GABA receptor on the postsynaptic membranes. However, a higher turnover would decrease receptor density and reduce neuronal sensitivity to GABA resulting in attenuation of GABA's inhibitory effect. during estrus and proestrus, periods of high estradiol, GAD1, GAD2, VGAT and GABARAPL1 were elevated. GAD1, GAD2, VGAT and GABARAPL1 all have estrogen response elements within their promoters [45; 49; 142] and co-localization of GAD2 and ERα in the thalamus, as well as, ERα antagonist studies suggests regulation could involve the estrogen receptor. Elevated GAD1, GAD2, and VGAT expression has been shown to attenuate nociception responses [38; 54; 74; 88; 148] and previous evidence indicates that elevated estradiol will reduce orofacial nociception [28; 62] consistent with the idea that elevated GAD1, GAD2, and VGAT, at proestrus/estrus contributes, in part, to the reduced nociception response.

4.4 Modulation of Lateral Thalamic Activity and VZV Associated Nociception

CNO administration and activation of the engineered Gi coupled receptor (DREADD) reduced the behavioral response to VZV infection in the whisker pad. Because this engineered receptor has been shown to reduce neuronal activity [5] these results suggests inhibition of neuronal activity in the lateral thalamus reduced VZV induced nociception. Male and females both developed affective nociception responses to VZV inoculation, and both showed a decrease in nociception after activation of Gi in the thalamic region, suggesting the thalamus functions in processing VZV nociception in the face for both sexes. This work extends the previous rat model of PHN from the footpad to the whisker pad [29; 21]. It is likely there are some differences between males and females in the thalamic processing of the nociception response because previous work from our lab indicated that GABA gene expression in the thalamus differs during the estrous cycle [139]. Affective and motivational aspects of nociception resulting from VZV infection of the face can be reduced by modulating neuronal activity in the thalamus, which suggests that affective and motivational aspects nociception signals from the face are transmitted by the lateral thalamus. This is consistent with studies demonstrating noxious orofacial input from the Vc are conveyed to the VPM [116].

The low-frequency activity, local field potential (LFP), contains information about how neurons integrate synaptic inputs [73]. Local field potential recordings are useful to provide measures of general electrical activity in localized brain areas. While LFP changes are less clearly understood and involve different bands in which meanings are not completely understood, it is worthwhile to investigate the changes within the

different frequency bands to further the understanding of how the electrical changes in specific brain areas may correlate with the behavior or experience of pain. In this study the thalamus, which is a major gaiting area in the brain, was recorded after inducing pain with VZV infection.

Rats that exhibited more escape-avoidance behavior demonstrated higher levels of LFP activity but only in the VPM gamma frequencies. This is consistent with literature that suggests that the gamma peak frequency has been linked to sensory processing and cognitive function. This increase in only VPM gamma waves could be suggestive of an increase in the processing of sensory information in the VPM caused by VZV associated nociception. Gamma frequency variations are highly structured, shared between areas and shaped by the theta rhythm [76]. A significant increase in the theta rhythm was found when measuring LFP in the Anterior Cingulate Cortex (ACC) in week one (data not shown). Gamma oscillations are controlled by both excitatory and inhibitory mechanisms almost exclusively with no cholinergic influence [79]. The activation of the Gi-neuronal silencing DREADD that decreased excitatory activity in the VPM also decreased gamma activity suggesting that the increase in the gamma activity with VZV infection could likely be due to increase in nociception sensory information. The decrease in nociception and gamma activity in VPM with the inhibition of excitatory activity using the DREADD complex further implicates VZV and increased excitatory input as the cause for the increase in gamma activity.

4.5 GABAergic VGAT Modulation in the VZV Associated Nociception Response

Most VGAT in the lateral thalamic region is expressed in the reticular thalamic nucleus [67]. Because GABA positive neurons in the reticular thalamic region send collaterals to the ventroposterior thalamic nucleus [9; 65] it is possible that inhibition of VGAT in GABA positive neurons of the reticular thalamic region reduced inhibitory signaling to the ventroposterior thalamic nucleus. This reduced inhibition of the excitatory neurons within the ventroposterior thalamic nucleus [132] would result in an increase in the behavioral response, as was observed in this study. The results suggest that the lateral thalamus functions in controlling VZV nociception in the face and that GABA in the thalamus inhibits the VZV nociception response. Studies from our lab suggested thalamic expression of VGAT modulates nociception in the orofacial region [62] but it was unclear if VGAT would alter orofacial nociception or if VGAT could affect VZV induced nociception. To address this question neurons were transduced with various constructs after injection of a viral vector into the VPM. No viral fluorescent signal was observed in the somatosensory cortex, a region projecting to the thalamus, supporting the idea that virus did not transport from the injection site but caution must be taken as the AAV5 and AAV6 serotype can result in axonal transport [6; 75; 115].

The results indicate VZV and VGAT shRNA are necessary for a greater nociceptive response in week two because treatment with the control (data not shown) and VGAT shRNA did not increased the behavioral response. Indicating VGAT shRNA treatment alone would increase the behavioral response. In the first week there was no increase in nociception resulting from VGAT inhibition, this phenomenon is potentially due to the behavioral response being at a minimum or "bottomed out" with this assay. Note the reduction in VGAT was over 3 fold after infection with VGAT shRNA, the response could have been much greater in specific sub-nuclei the tissue plug used for analysis was large and included posterior, ventroposterior and reticular thalamic nuclei. We have not tested the response of females to the VGAT shRNA virus but future work will determine if any sex differences exist.

The reduction of thalamic VGAT increased nociception suggesting that GABA signaling in the lateral thalamic region gates or inhibits VZV facial pain; potentially through GABA positive interneurons in the reticular thalamic region [65].

5. CONCLUSION

Epidemiological studies have demonstrated that Zoster-associated pain occurs with a higher prevalence, intensity, and/or duration in women as compared to men [2, 46]. Studies of other orofacial pain conditions have reported that pain intensity varies over menstrual cycle [70; 62; 10] pointing to estradiol status as a possible mechanism involved in the sex disparity in this orofacial pain condition. Steroid hormones, such as estrogen, have profound effects on gene expression, membrane-bond receptor/ion channels, and neuronal signaling both in the periphery and the CNS as it can cross the blood-brain barriers.

The thalamus is important in pain control but became the primary area of interest when GABAergic gene expression in the lateral thalamus (ventral posteromedial, ventral posterolateral, reticular thalamic nuclei) significantly increased due to increases in estrogen. The genes GAD1, GAD2, GABARAPL1 and VGAT were all increased in the lateral thalamus due to increased estradiol levels. Other major participants in the orofacial pain pathway such as the zona incerta, spinal nucleus caudalis and periaqueductal gray did not display any significant changes due to estradiol.

LFP changes in the thalamic area after injection of the AAV8 Gi-inhibitory DREADD indicated that thalamic inhibition decreased the VZV associated nociception response. It also indicated that VZV infection caused increased VPM activity more than likely due to the increase in nociception sensory information due to the infection.

VGAT was silenced using a shRNA construct and resulted in increased VZV associated nociception. The significant decrease in VGAT expression decreased the GABAergic

The result of these studies indicates estradiol is modulating the VZV associated pain response in females. This study also concluded that estradiol is modulating GABAergic gene expression in the thalamus and thalamic signaling. Further studies are indicated to investigate if estradiol is modulating the VZV associated pain response by exerting changes in GABAergic gene expression in the thalamus to cause the increased VZV pain reported by females.

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APPENDIX A

FIGURES

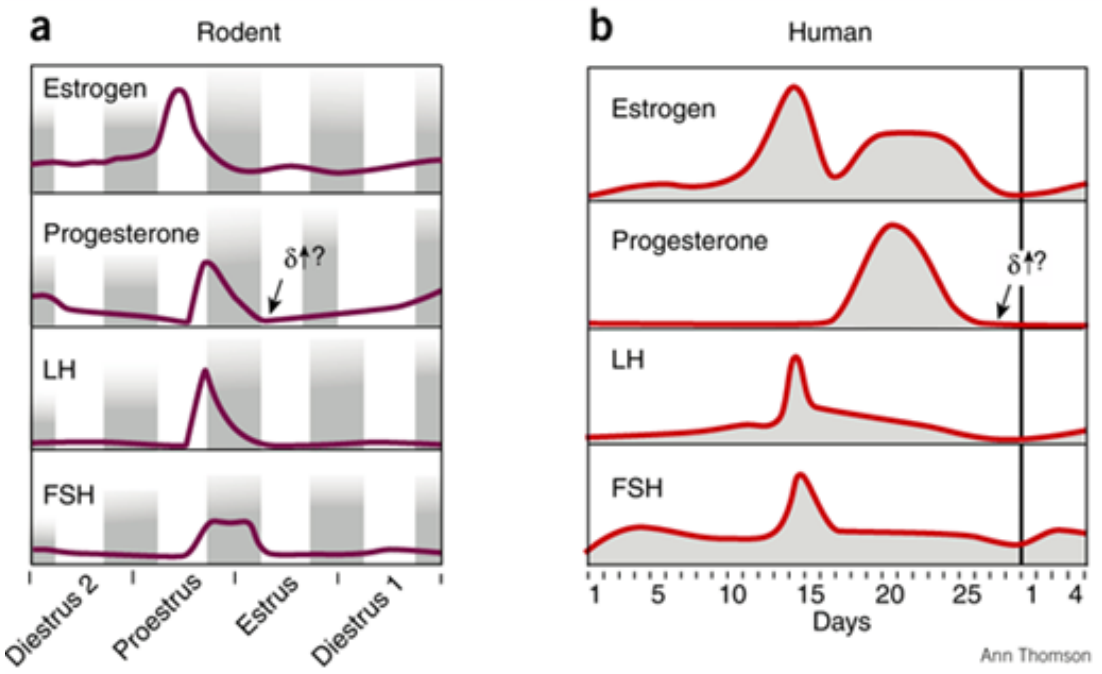

Figure 1. Rat and human hormone cycles: [130] (**a**) The 4-day estrous cycle of the rat (gray bars indicate night, 6 p.m. to 6 a.m.), showing fluctuations in estrogen, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The arrow indicates the approximate time of progesterone withdrawal and potential change in δsubunit expression suggested by the findings of Maguire *et al*. In the mouse, the estrous cycle is similar, but varies in length (4–7 days) and is less well defined. (**b**) The human 28-day menstrual cycle. The time of progesterone withdrawal is thought to be at the end of the cycle, approximately at the onset of menses.

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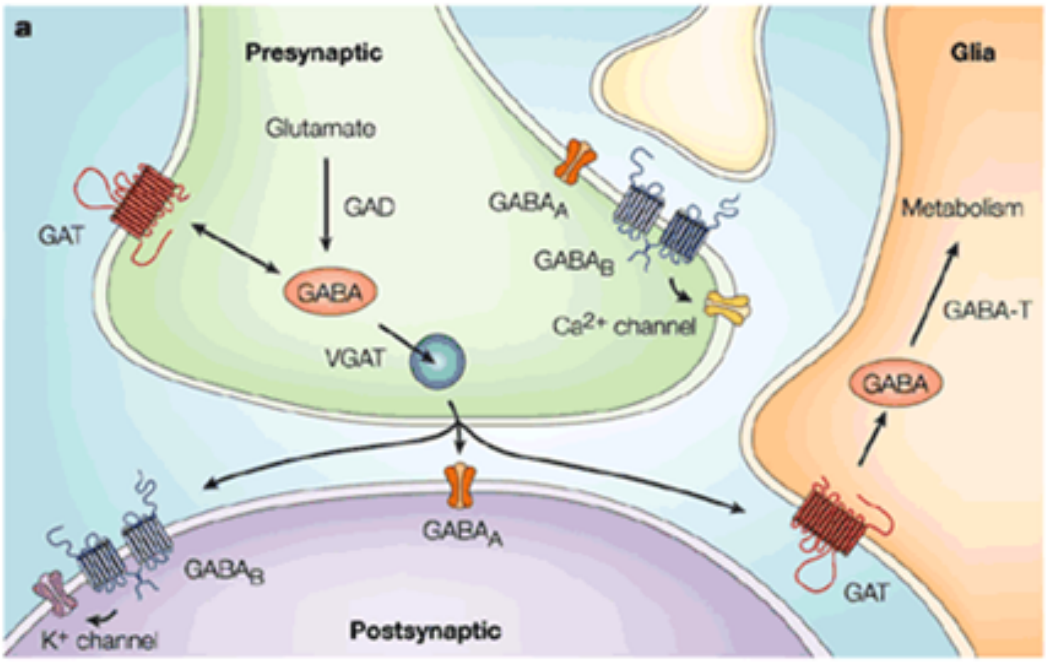

Figure 2. Schematic diagram of the synthesis and transport of GABA at synapses: ɣ-aminobutyric acid (GABA) is synthesized in inhibitory neurons from glutamate by the enzyme glutamic acid decarboxylase (GAD), and is transported into vesicles by a vesicular neurotransmitter transporter (VGAT). GABA can be released either vesicularly or non-vesicularly (by reverse transport). GABA receptors are located at pre- and postsynaptic sites. $GABA_B$ receptors are metabotropic receptors that cause presynaptic inhibition by suppressing calcium influx and reducing transmitter release, and achieve postsynaptic inhibition by activating potassium currents that hyperpolarize the cell. Reuptake of GABA by surrounding neurons and glia occurs through the activity of GABA transporters (GAT). Subsequently, GABA is metabolized by a transamination reaction that is catalysed by GABA transaminase (GABA-T). The metabolism of GABA occurs in both neurons (not shown) and glia.

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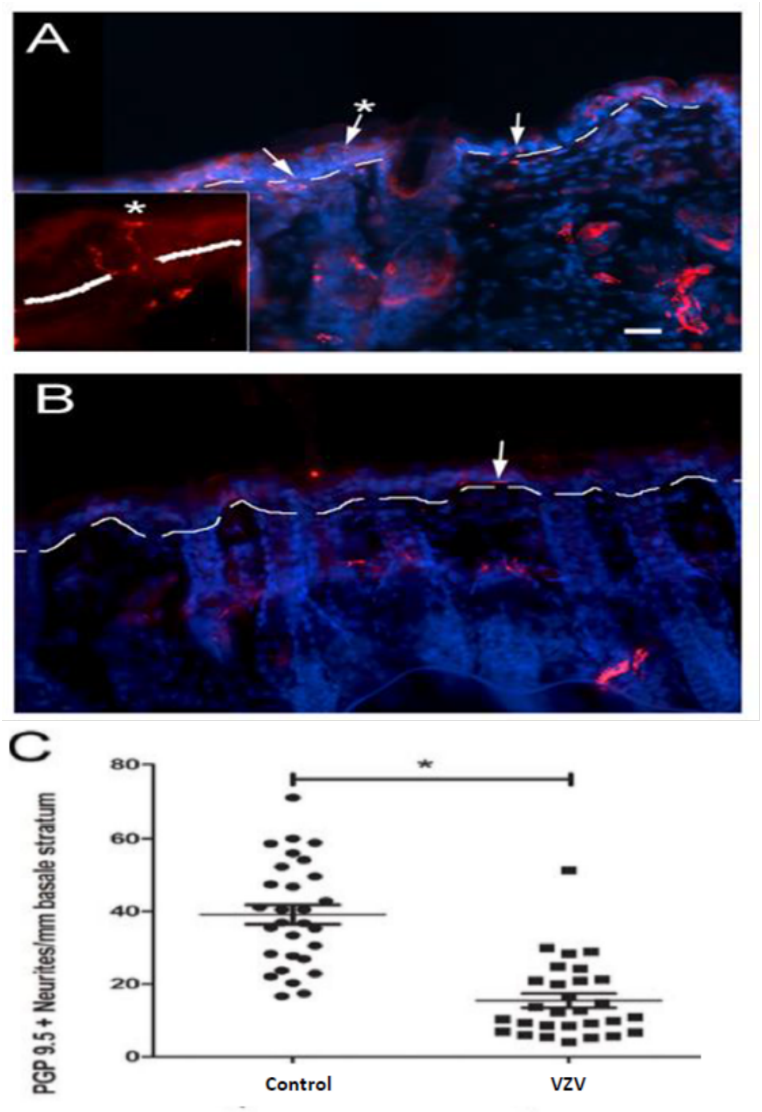

Figure 3. Peripheral innervation in whisker pad decreased due to VZV infection: PGP9.5 positive neurites in the whisker pad six weeks after injection. Animals were injected with either control cells (A) or VZV (B). 20 µm coronal sections were cut from each injected whisker pad. Representative images for control injected (A) and VZV injected (B) rats are shown after staining with the PGP9.5 antibody (red), cell nuclei are stained blue. The border between the epidermis and dermis (epidermal/dermal junction) is shown as a white dotted line. Arrows point to PGP9.5 positive filaments. Asterisk above arrow indicates enlarged region within box (asterisk). Bar = 50 µm. In panel C, each data point on the histogram shows the average number of PGP9.5 positive neurites per mm that are above or cross the epidermal/dermal junction for each section. There were ten sections per rat and three rats per treatment group. Statistical significance was determined with a Mann-Whitney t-test. Mean and SEM are shown

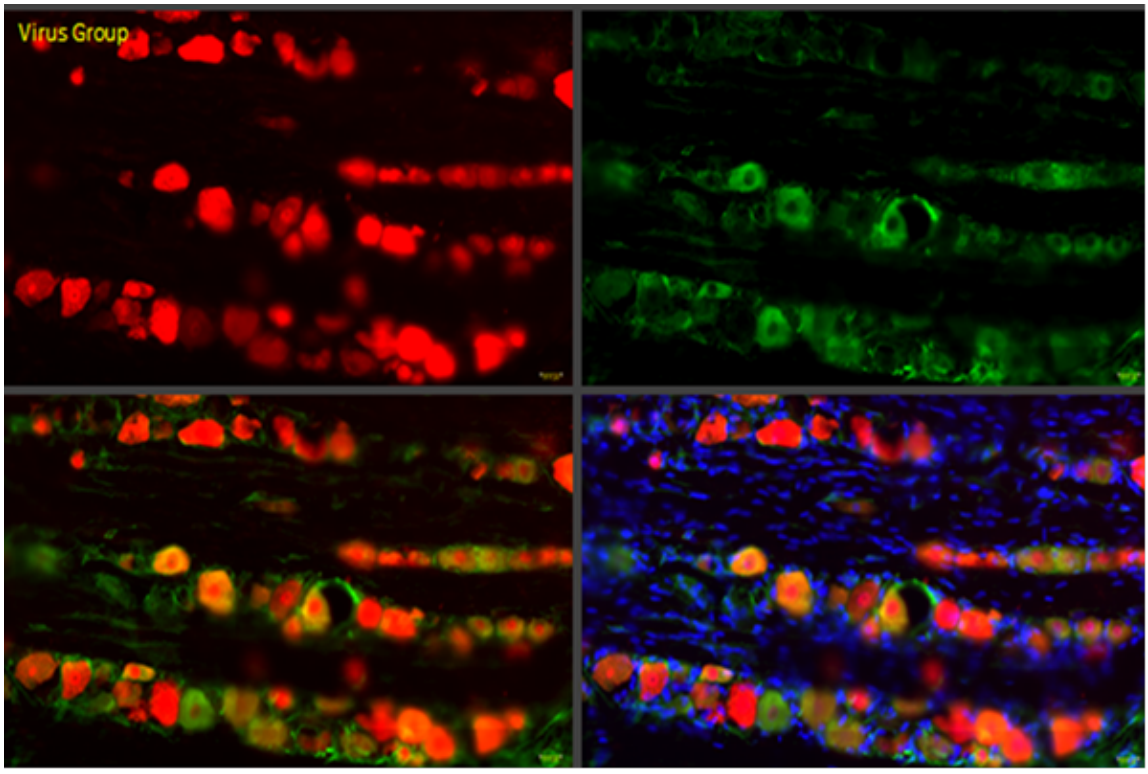

Figure 4. VZV infection of neurons in the trigeminal ganglia: shown by immunofluorescent stain of fixed frozen trigeminal ganglia sections from rats injected with 360 pfu/ µl VZV. Image is from the trigeminal ganglia of a female rat 5 weeks after injecting VZV into the whisker pad. The trigeminal ganglia was taken ipsilateral to the injection. The saggital section was stained with the neuronal marker NeuN (green) and the VZV marker IE62 (red). Co-localization of NeuN and IE62 is shown in yellow. Blue is nuclear Hoeschst stain.

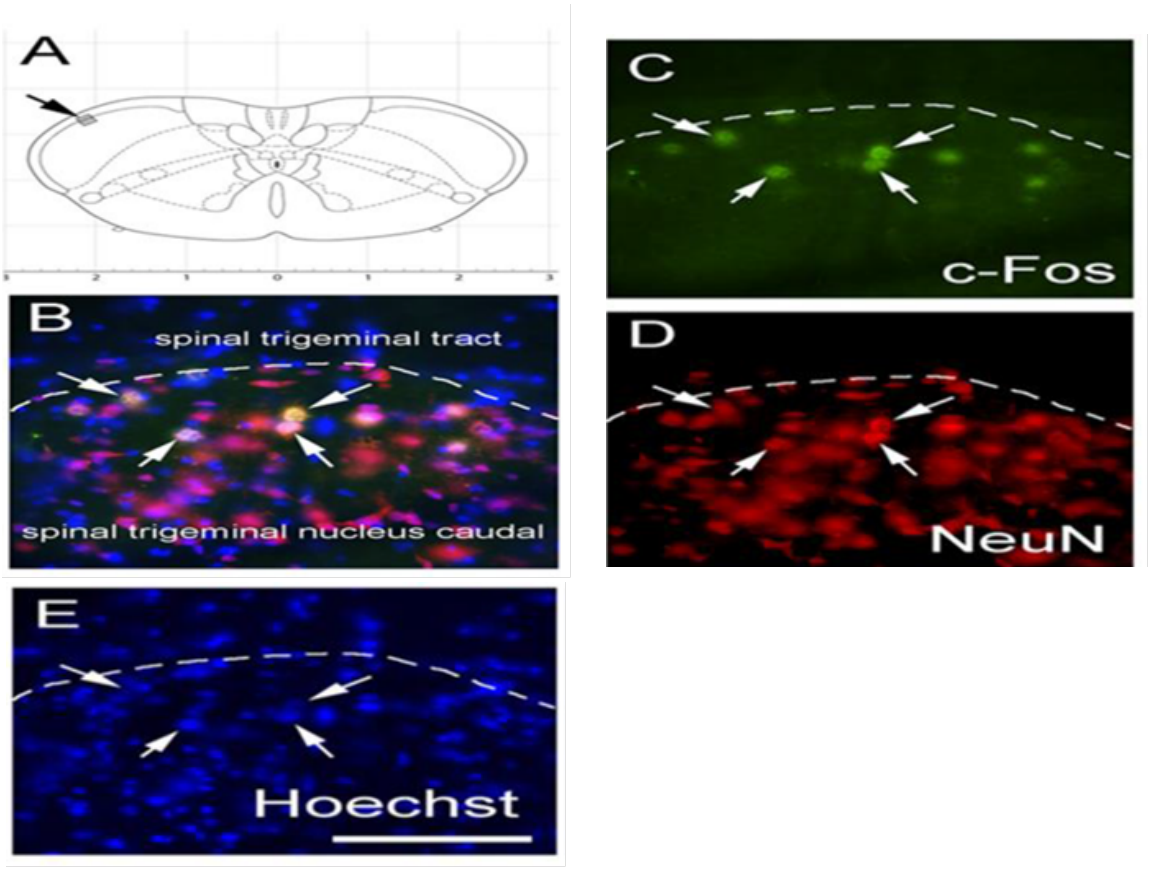

Figure 5. c-Fos expression in neurons in the trigeminal nucleus caudalis: located in the ipsilateral caudal portion of the trigeminal nucleus: 8 weeks after VZV injection, 1900 pfu/ µl. While a majority of rats no longer responded in the PEAP assay this particular rat continued to respond to the PEAP assay eight weeks after VZV injection and was sacrificed and the caudalis stained with c-Fos and the neuronal marker NeuN. The region imaged is delineated by a box in panel A (black arrow). Neurons (red) double stained with c-Fos (green) are shown in yellow in panel B (yellow cells in focus are indicated by white arrows). Staining for c-Fos (green) is shown in panel C, neuronal marker NeuN staining (red) is shown in panel D and Hoechst nuclear stain (blue) in panel E. Bar equals 100 µm.

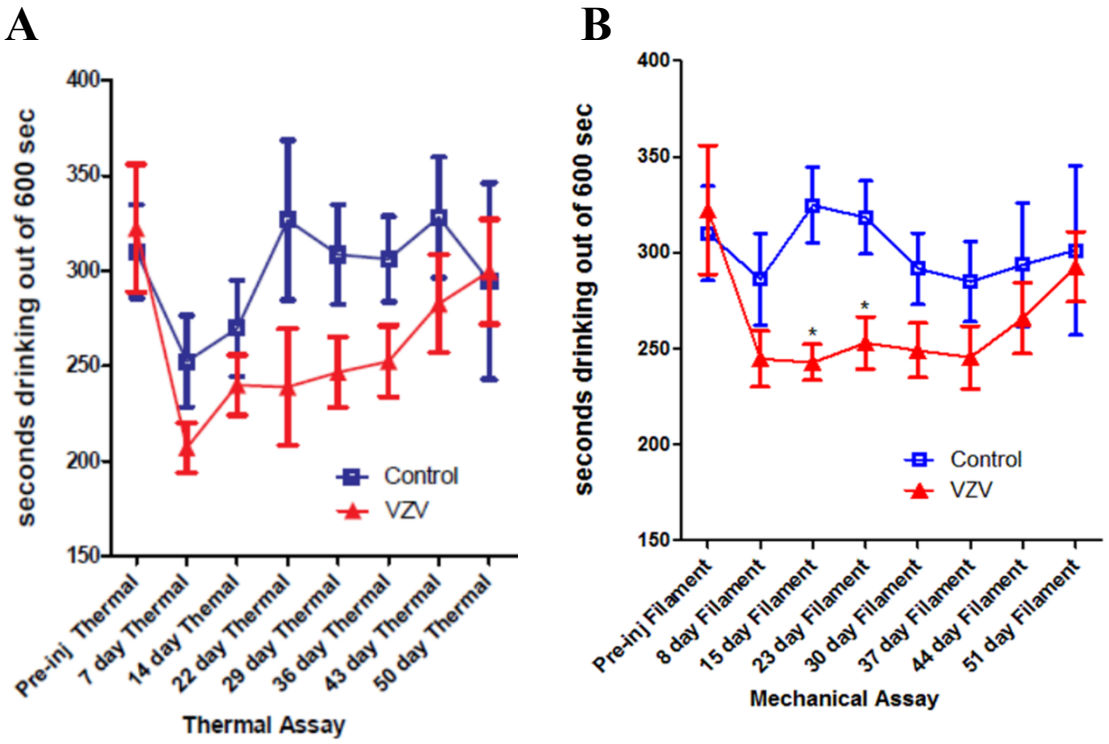

Figure 6. VZV infection decreased nociception sensitivity thresholds: thermal hyperalgesia and mechanical allodynia was tested in rats before and after whisker pad injection of either control cells or VZV, 1900 pfu/ µl. Testing was performed for 50-51 days post injection. Testing was completed using the orofacial test module from Ugo Basile. Thermal testing was completed at 45 °C and filament testing was performed with a custom circular opening containing 12 filaments. Asterisk indicates a significant difference ($p<0.05$) between the control and VZV treatment groups. There were 10 animal per treatment group.

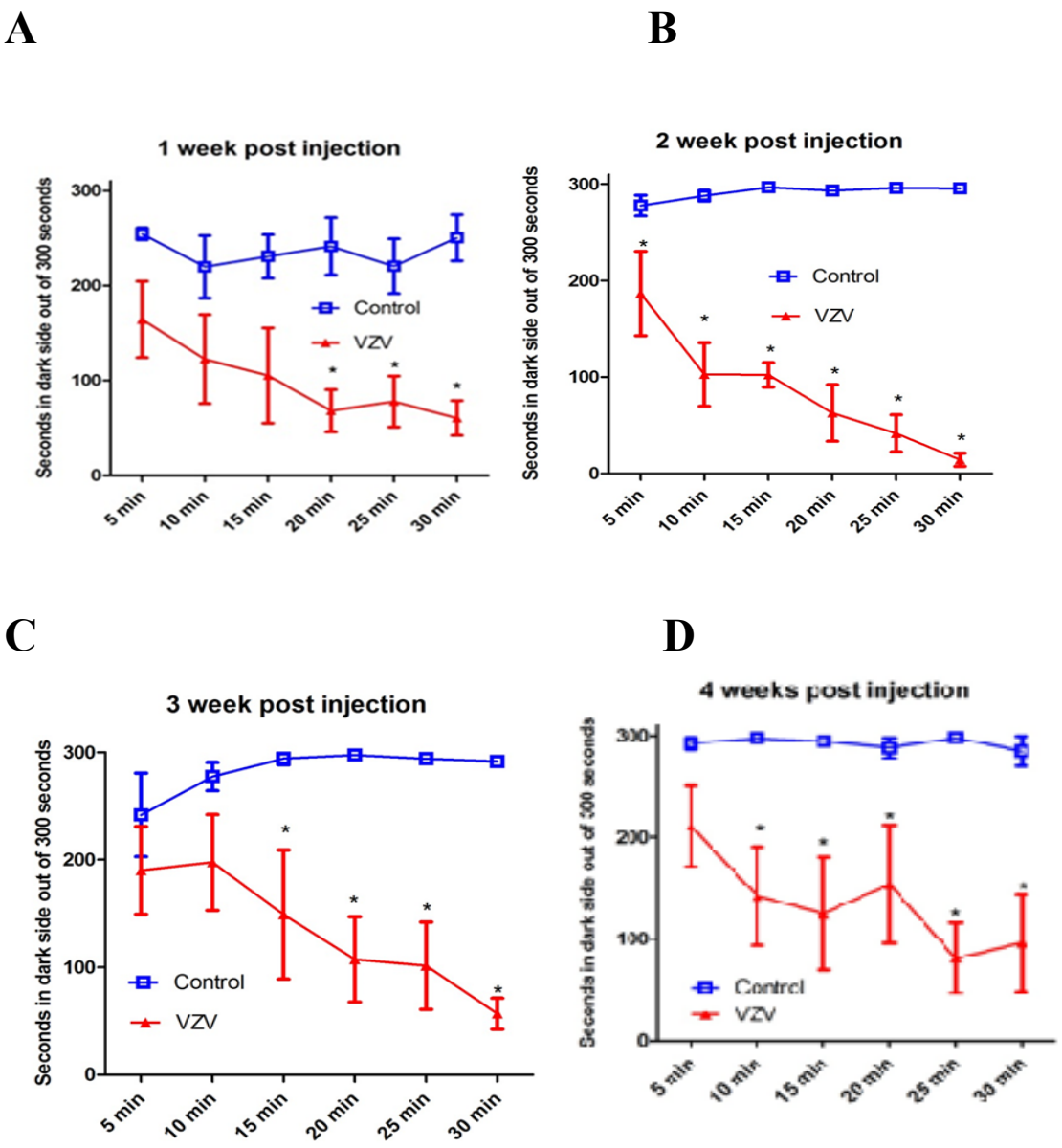

Figure 7A-7D. VZV infection increased affective PEAP Pain: Place Escape/Avoidance Paradigm (PEAP test) was completed on males whose whisker pad was injected with either PBS (vehicle), control cells or with 1900 pfu/ µl VZV. The PEAP test was performed for eight weeks, each panel shows data for a different week. The upper row of asterisks indicate a significant difference ($p<0.05$) between the PBS and VZV treatment groups. Asterisks on the lower row indicate a significant difference ($p<0.05$) between control and VZV treatment groups. There were 6 animals per treatment group

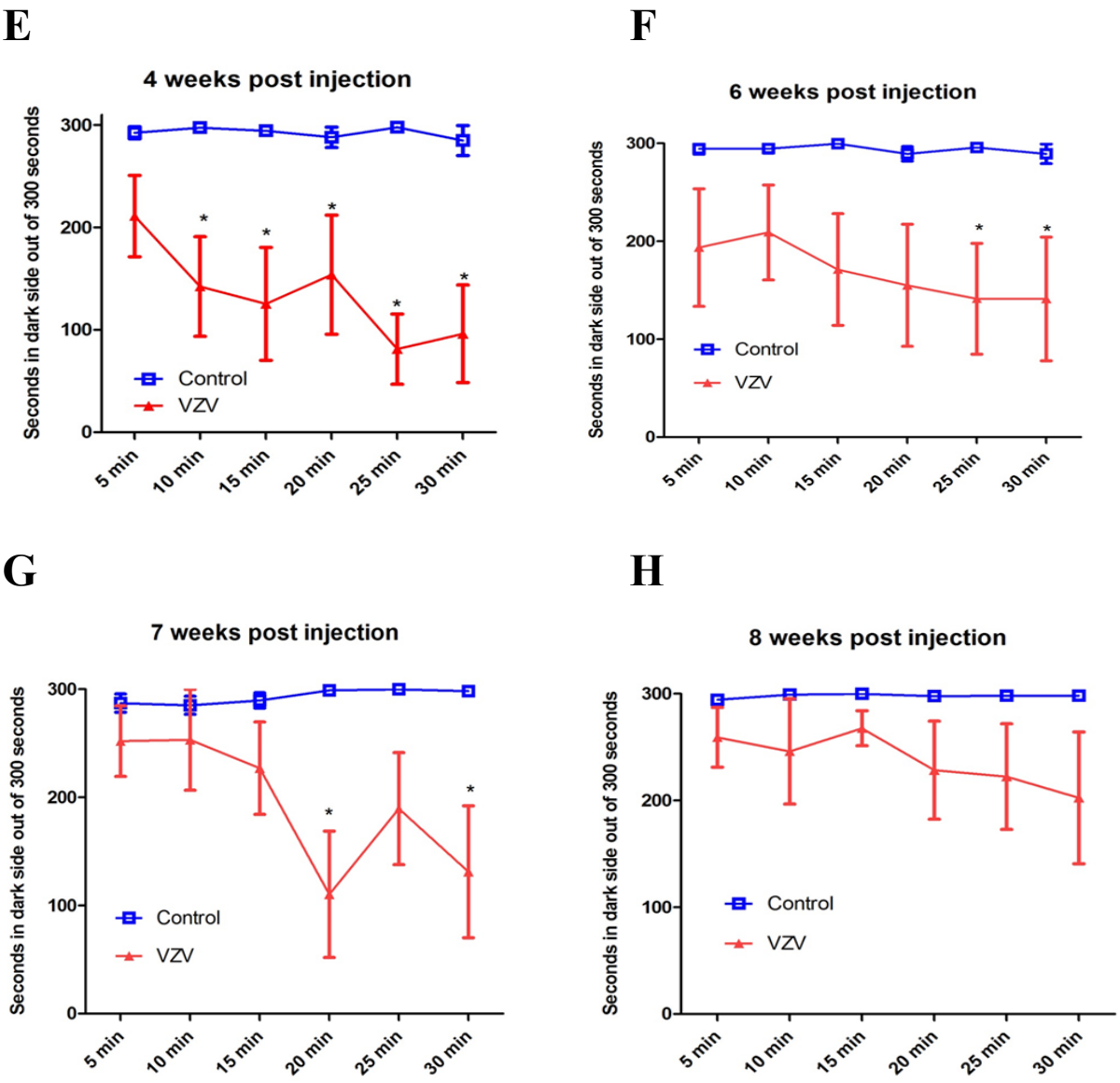

Figure 7E-7H. VZV infection increased affective PEAP Pain: Place Escape/Avoidance Paradigm (PEAP test) was completed on males whose whisker pad was injected with either PBS (vehicle), control cells or with 1900 pfu/ µl VZV. The PEAP test was performed for eight weeks, each panel shows data for a different week. The upper row of asterisks indicate a significant difference ($p<0.05$) between the PBS and VZV treatment groups. Asterisks on the lower row indicate a significant difference ($p<0.05$) between control and VZV treatment groups. There were 6 animals per treatment group.

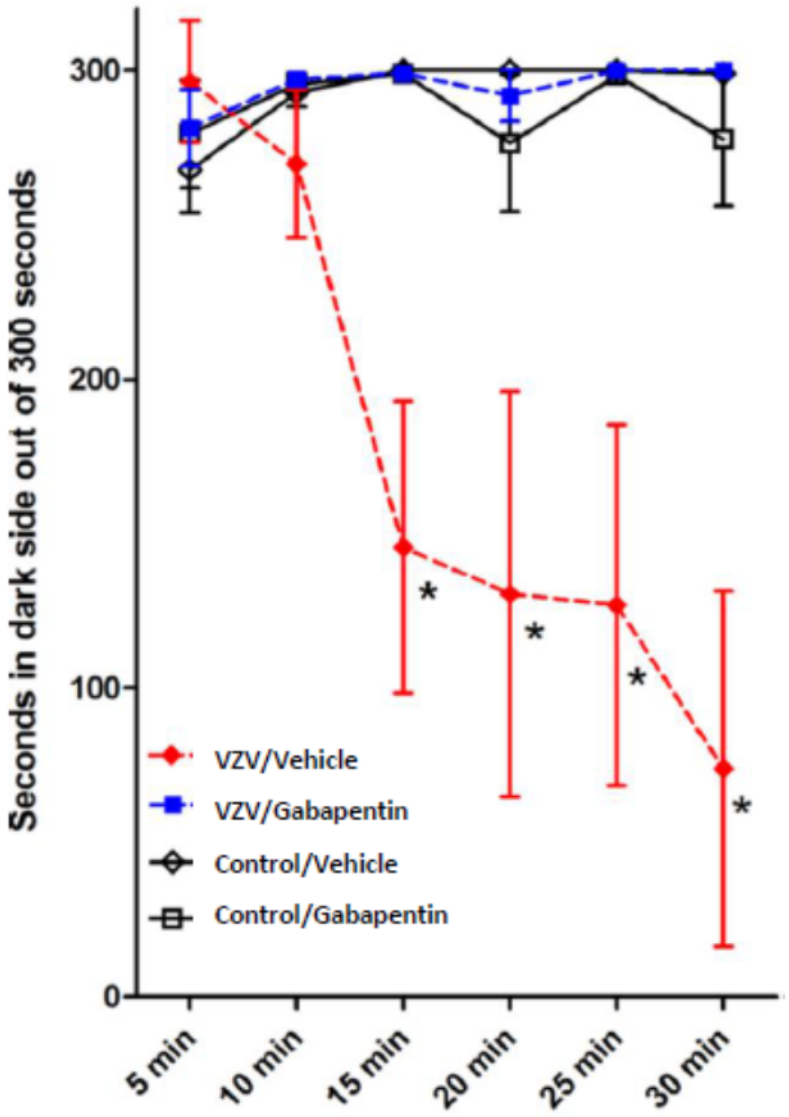

Figure 8. Gabapentin reduced VZV associated PEAP nociception: Treatment with gabapentin reduced the PEAP response in rats whose whisker pad was injected with control cells or 360 pfu/ µl VZV. Testing was completed 7 days after injection. An asterisk indicates a significant difference between the VZV/vehicle group and the VZV/gabapentin group, as well as, a significant difference between the control/vehicle group and the VZV/vehicle group. There were 5 animals per treatment group.

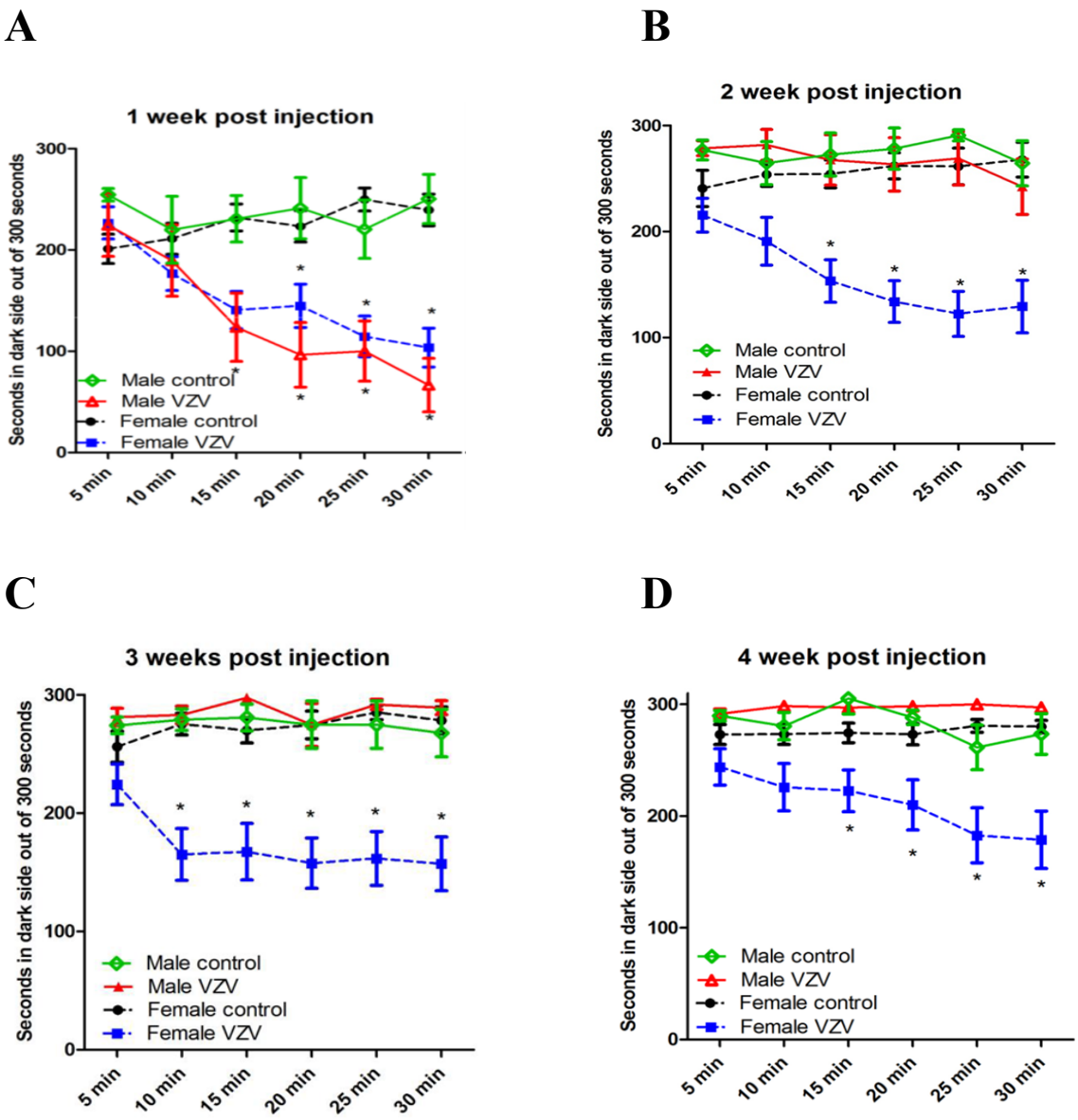

Figure 9. Sex differences in the VZV associated nociception response: Male and female whisker pads were injected with either control cells or 360 pfu/µl VZV. PEAP testing was completed weekly over six weeks, each panel shows data for a different week. In panel A the upper row of asterisks indicate a significant difference between the Female control and Female VZV treatment groups and the lower row of asterisks indicate a significant difference between the Male control and Male VZV treatment groups. In panels B-D an asterisk indicates a significant difference ($p<0.05$) between the Male control and Male VZV treatment groups. There were 24 control and 25 virus infected females and the number of males in both the control and VZV treatment groups was 7 for week one, 10 for week two and 15 for weeks three, four, five and six.

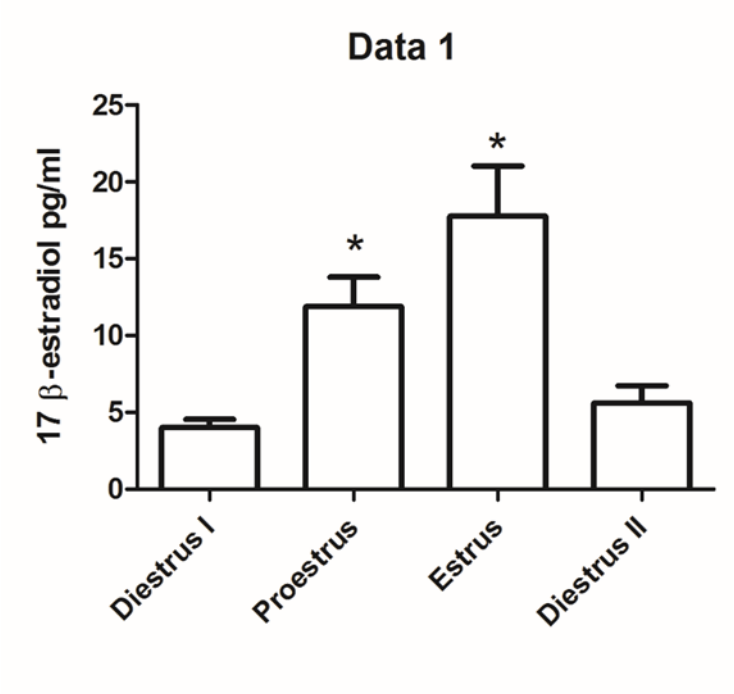

Figure 10. Measured estradiol levels in VZV infected animals after PEAP testing: Cycling females had vaginal smears completed and then plasma was collected after sacrifice. 17 β-estradiol concentrations in the blood plasma were determined by radio-immuno assays. An asterisk indicates a significant difference ($p<0.05$) between the proestrus and diestrus I and diestrus II animals and between the estrus and diestrus I and diestrus II animals. There were 15 rats per treatment group.

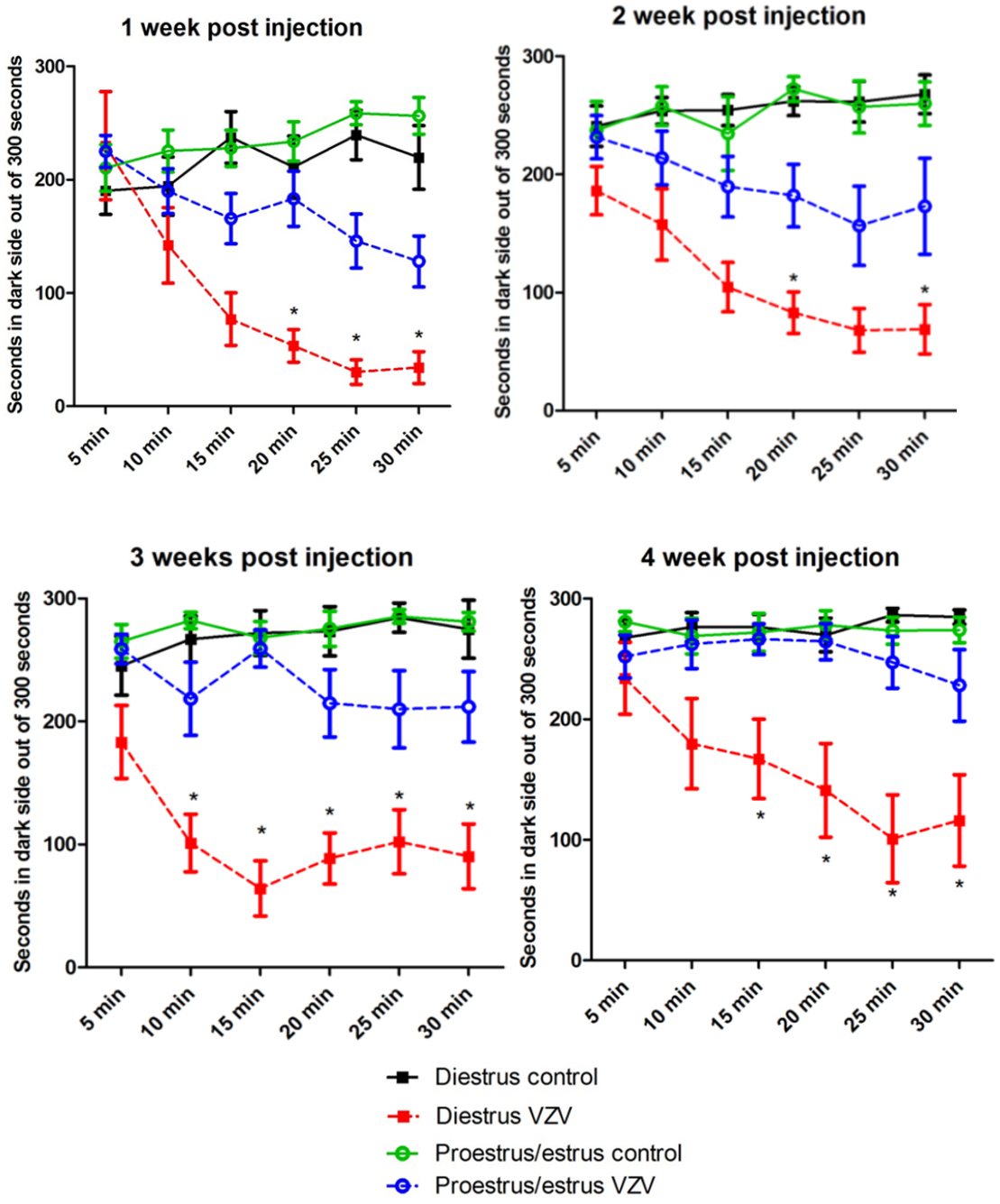

Figure 11. VZV associated nociception is modulated by estradiol levels: Weekly PEAP testing was completed on cycling female rats after VZV injection. A total of 24 rats whose whisker pad was injected with control cells and a total of 25 rats whose whisker pad was injected with 360 pfu/µl VZV were included in this experiment. Smears were completed on the day of testing and the number of animals at each estrous cycle phase (i.e., diestrus or proestrus/estrus) was a minimum of 8 but would vary from week to week depending on the estrous

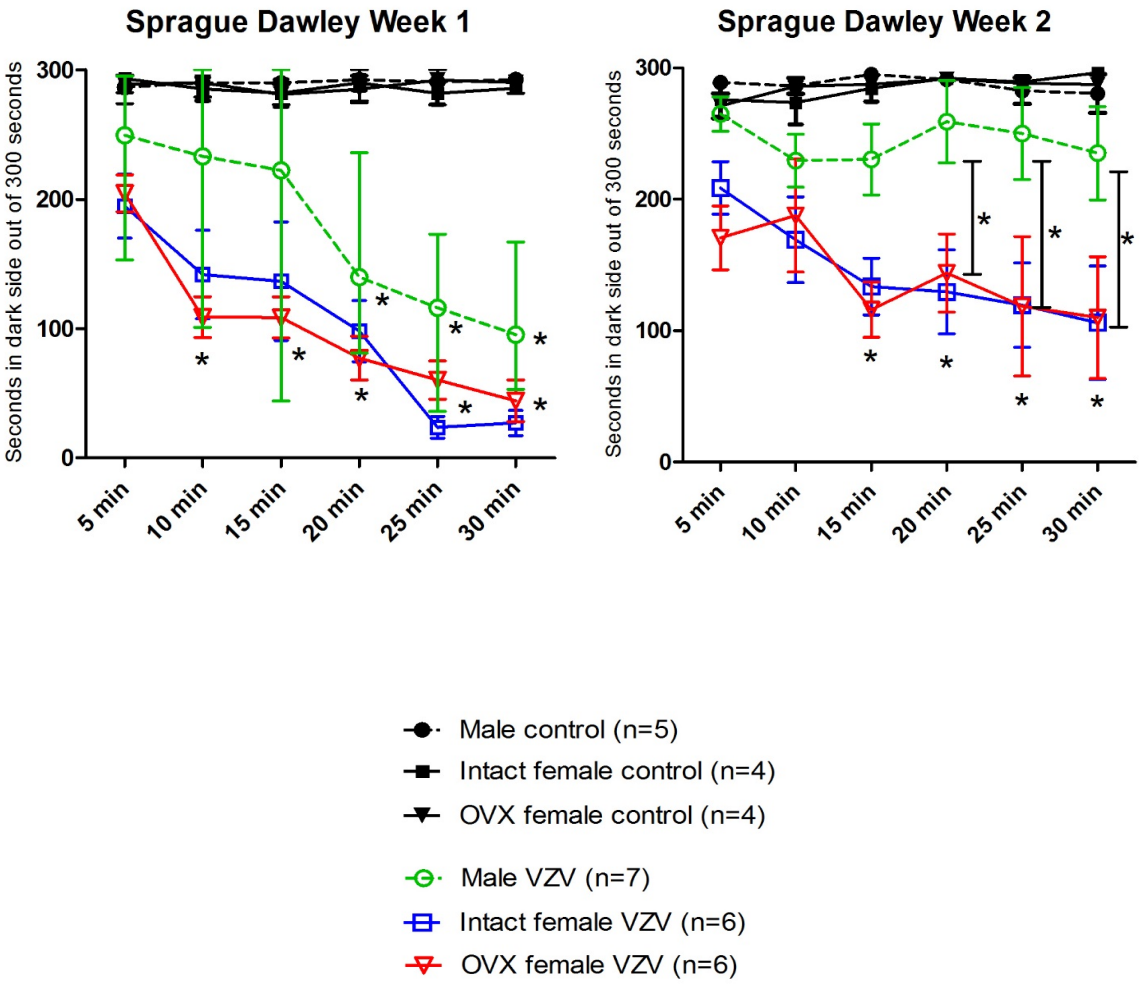

Figure 12. OVX females experienced more VZV nociception than males: The whisker pads of intact or ovariectomized (OVX) female rats were injected with either control cells or 650 pfu/µl VZV. PEAP testing was completed weekly over two weeks. The lower row of asterisks indicate a significant difference between the Female control and Female VZV treatment groups (both intact and OVX) and the upper row of asterisks indicate a significant difference between the Male control and Male VZV treatment groups. In panels B and D the bar with an asterisk indicates a significant difference ($p<0.05$) between the Male VZV and both intact and OVX Female VZV treatment groups. In panel C the bar with an asterisk indicates a significant difference ($p<0.05$) between the Male VZV and the intact Female VZV treatment groups. The number of animals in each treatment group is shown on the figure in parentheses.

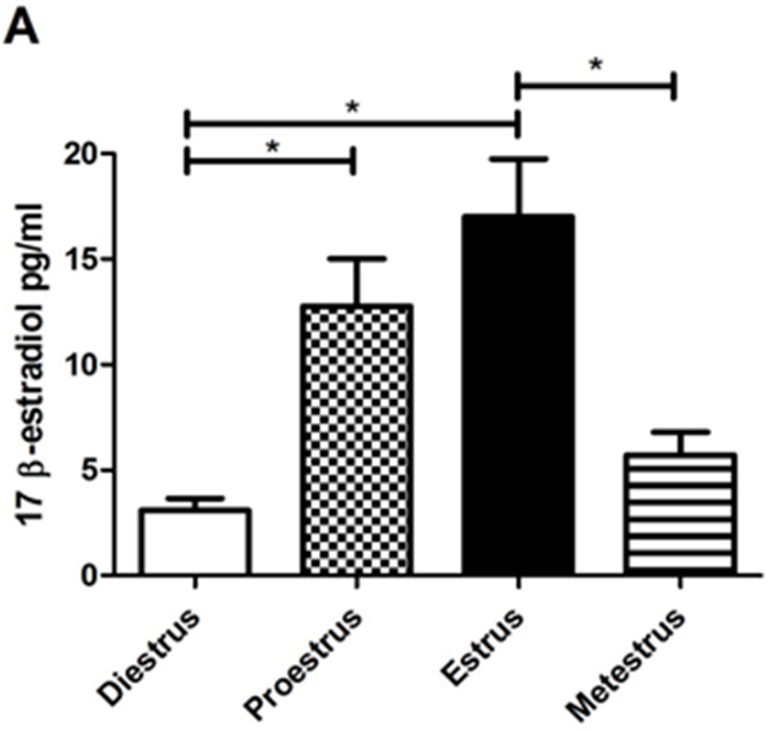

Figure 13. Estradiol measured in cycling animals for GABAergic gene expression analysis: Vaginal smears were performed and plasma was collected within one hour. Plasma estradiol was measured by RIA. Significant differences of $p<0.05$ are indicated by an asterisk. Seven animals were in each of the four estrous cycle phases.

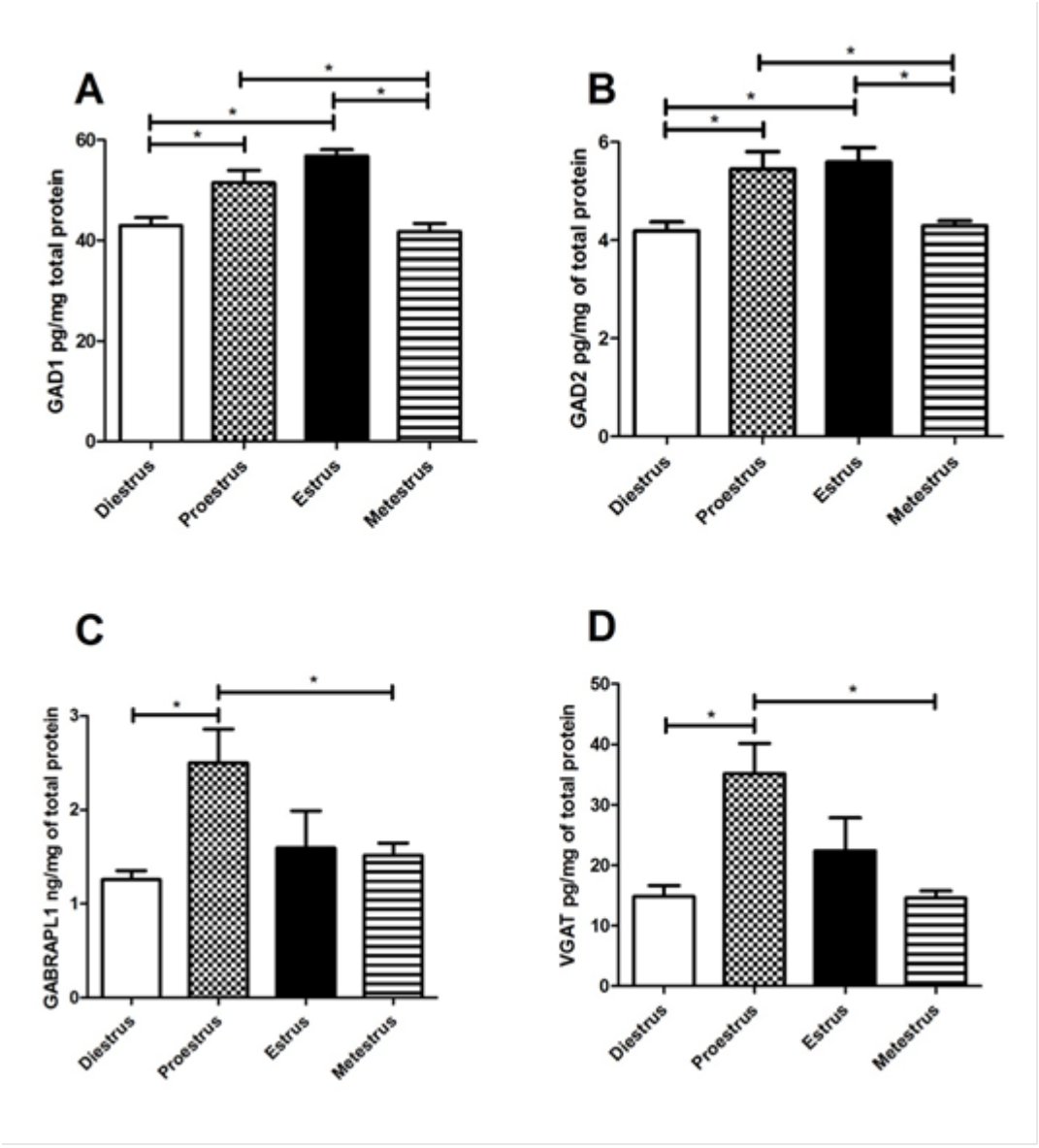

Figure 14. Transcript levels of GAD1, GAD2, GABARAPL1 and VGAT in the thalamus of cycling female rats: Estrous cycle phases were determined by vaginal smears and the thalamic tissue was isolated within one hour. RT-PCR analysis of the thalamic tissue was then performed. Panel A shows GAD1 transcript levels, Panel B shows GAD2 transcript expression, Panel C shows GABARAPL1 transcript expression and Panel D shows VGAT transcript expression. Significant differences of $p<0.05$ are indicated by an asterisk. There were 7 animals in each of the three estrous phases except estrus where there were 8 animals.

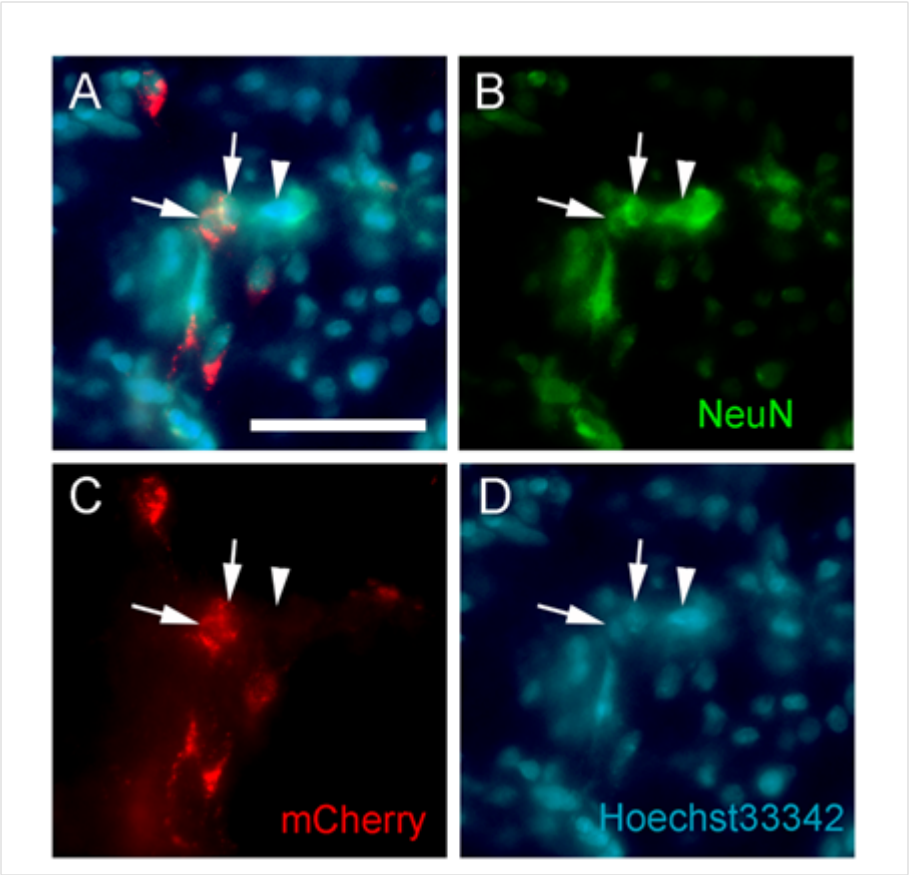

Figure 15. Neurons infected with AAV8 neuronal silencing complex: A-D) Immunofluorescent staining of the ventral posteromedial (VPM) thalamic nucleus of a rat four weeks after infusion of 0.25 microliters of AAV8-hSyn-hM4D(Gi)-mCherry. The representative brain slice was collected from a rat that whose whisker pad was injected with control cells and given an IP injection of vehicle (0.5% saline). Arrows point to NeuN positive cells expressing mCherry, arrowhead points to NeuN positive cell that did not express mCherry. Nuclei were stained blue with Hochst33342. Bar equals 50 micrometers.

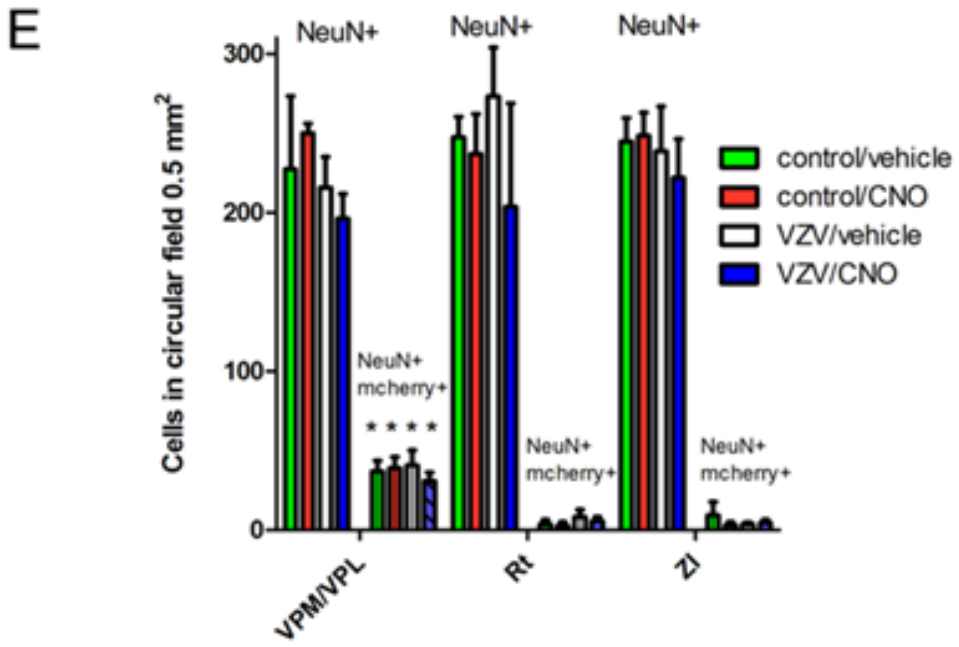

Figure 15.E Histogram of cell counts in the VPM: circular field 0.5 mm2 in area, counts were completed in randomly chosen fields adjacent to the injection site in several thalamic regions; the VPM, ventral posterolateral (VPL) and reticular (Rt) thalamic nuclei, as well as the zona incerta (ZI). Asterisk indicates $p<0.05$ when comparing the NeuN+/mCherry positive cells in the VPM/VPL to the NeuN+/mCherry positive cells in the Rt and ZI. n=3 for the control/vehicle and control/CNO groups and n=4 for the VZV/vehicle and VZV/CNO groups. Values are the mean and SEM.

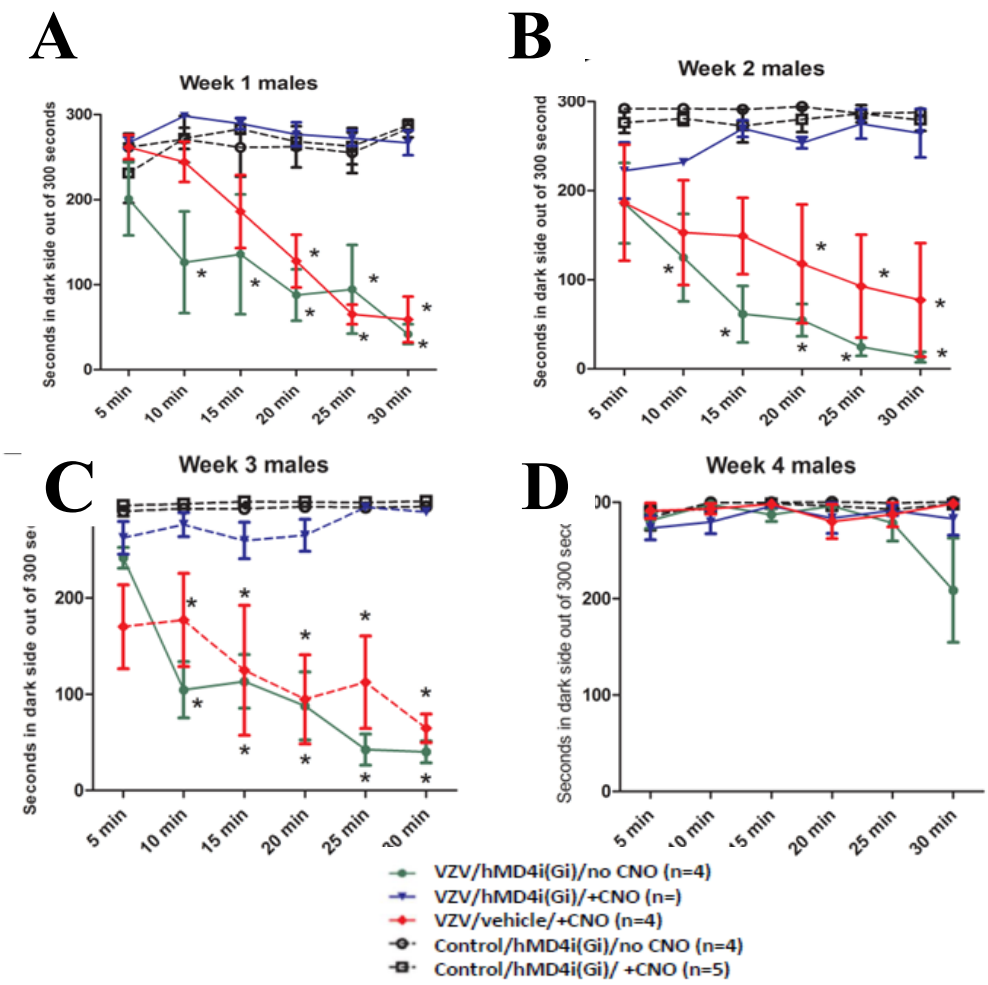

Figure 16. Activation of DREADD decreased VZV associated nociception in males: In panels A-D, nociception was measured in each rat using the Place Escape/Avoidance Paradigm (PEAP). Two weeks before nociception was measured the thalamus was injected with AAV8-Syn-hM4D(Gi) or vehicle bilaterally. PEAP was completed one (A) two (B) three (C) or four weeks (D) after injecting the left whisker pad with VZV (pOka strain) (>1000 pfu/µl) or equivalent uninfected control cells. Thirty minutes before PEAP testing the rats were intraperitoneally injected with a 1 mg/kg dose of clozapine-n-oxide (CNO) or 0.5 ml of saline. ANOVA and Bonferroni post-hoc testing was performed and an asterisk indicates a significant difference ($p<0.05$) between the VZV/hMd4i(Gi)/no CNO and the VZV/vehicle/ +CNO group versus the control injected groups, as well as, the VZV/hMd4i(Gi)/ +CNO group. The predetermined number of animals in each group is shown in parenthesis in legend.

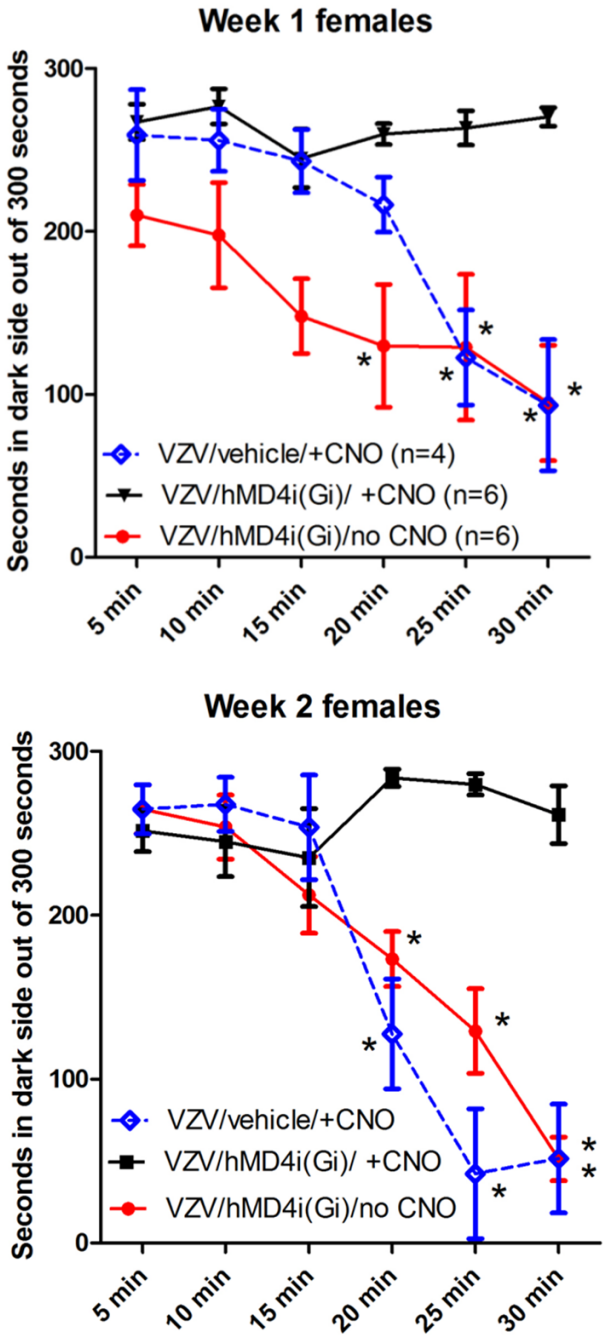

Figure 17. Activation of DREADD decreased VZV associated nociception in females: Sprague Dawley female rats were injected with AAV8 containing an Syn-hM4D(Gi) construct and the whisker pad was injected with VZV as described in figure 16. At one and two weeks after injection, the animals were given an injection of CNO 30 minutes prior and PEAP testing was performed. An asterisk indicates a significant difference ($p<0.05$) between the VZV/hMd4i(Gi)/no CNO and the VZV/vehicle/ +CNO group versus the VZV/hMd4i(Gi)/ +CNO group. The number of a

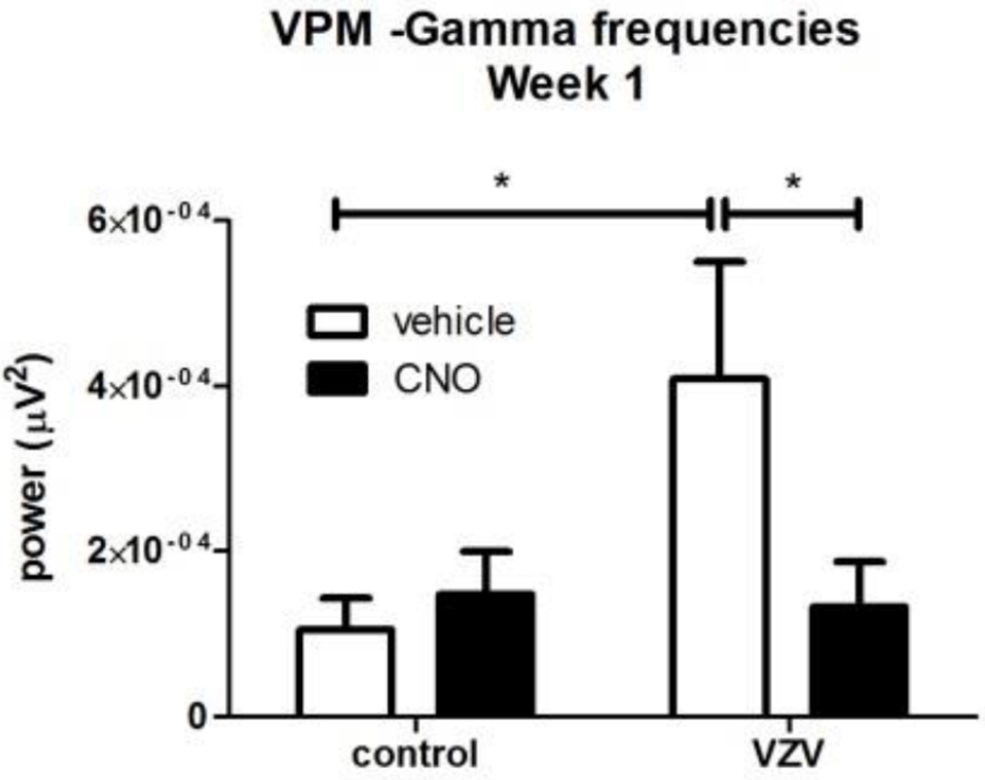

Figure 18. VZV infection increased local field potentials in VPM: Rats had electrodes placed in the VPM and were treated as described in the legend to Figure 13. LFP recordings were completed in the VPM. Thirty minutes before LFP recording the rats were given an injection of either CNO or vehicle (0.9% saline). Testing was completed once a week for thirty minutes. Testing was performed on three consecutive weeks. Asterisk indicates a significant difference of $p<0.05$. There were eight animals in each treatment group. The values are the mean ± SEM.

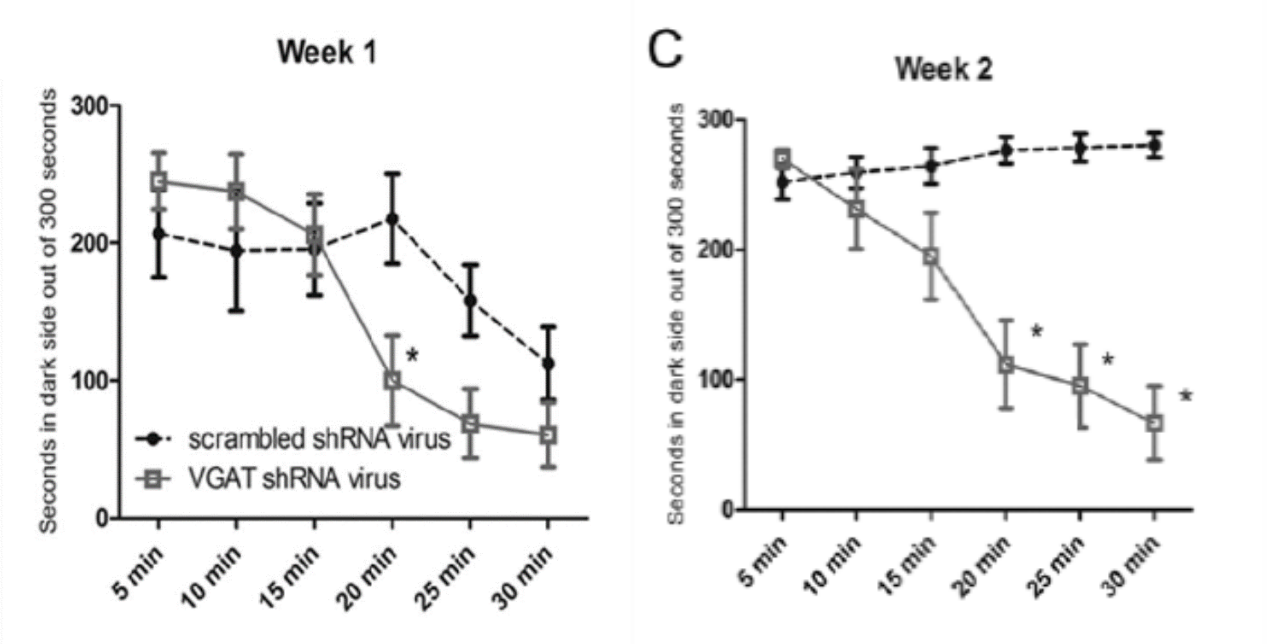

Figure 19. VGAT silencing shRNA increased VZV associated nociception: Male rats were injected with AAV containing the scrambled shRNA or VGAT shRNA construct and tested using PEAP after whisker pad injection of VZV (650 pfu/µl). The PEAP test was performed once a week for two weeks starting 7 days after VZV injection. Panel A shows the data for week one and panel B shows the data for week two. The asterisks indicate a significant difference ($p<0.05$) between the scrambled shRNA and the VGAT shRNA group. There were 10 animals per treatment group. See figure 3 for details.

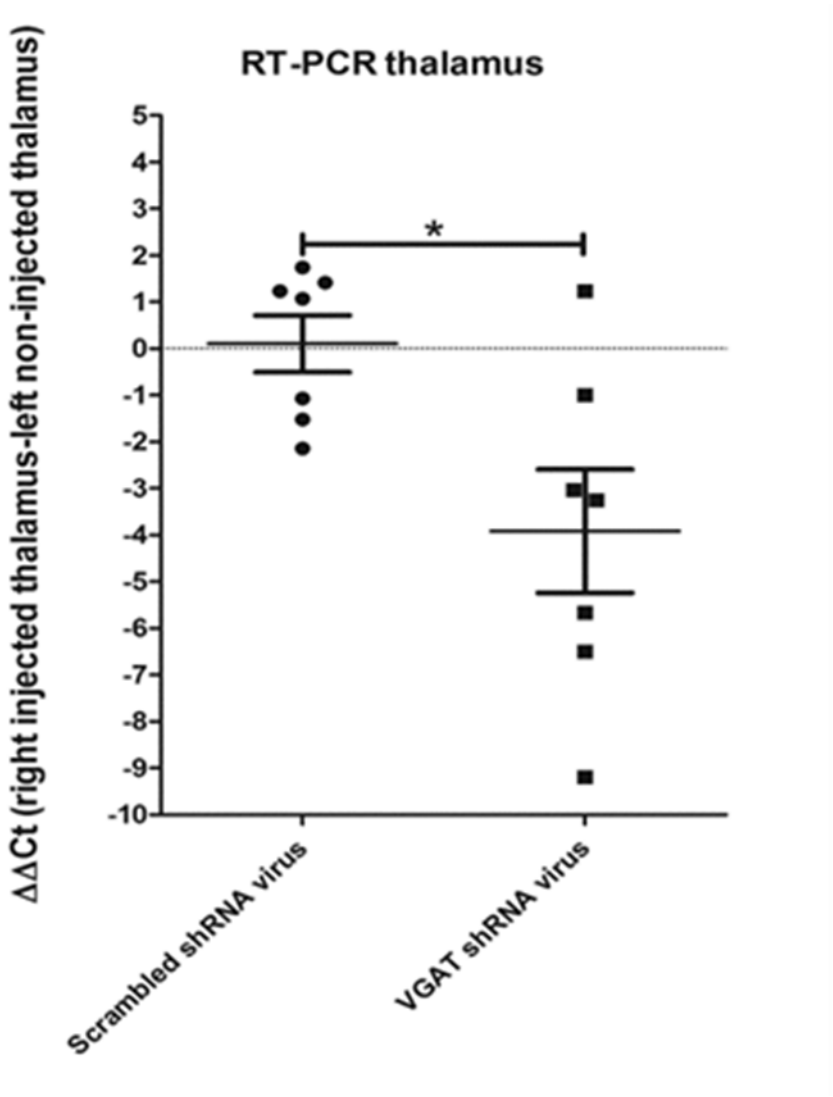

Figure 20. VGAT silencing shRNA decreased VGAT expression significantly: Two weeks after whisker pad injection the animals were sacrificed and the lateral thalamic region was isolated by punch biopsy. RT-PCR for VGAT expression from this tissue indicated a significant reduction after VGAT shRNA treatment. There were 7 animals per VZV treatment groups and 4 animals in the control treatment groups.

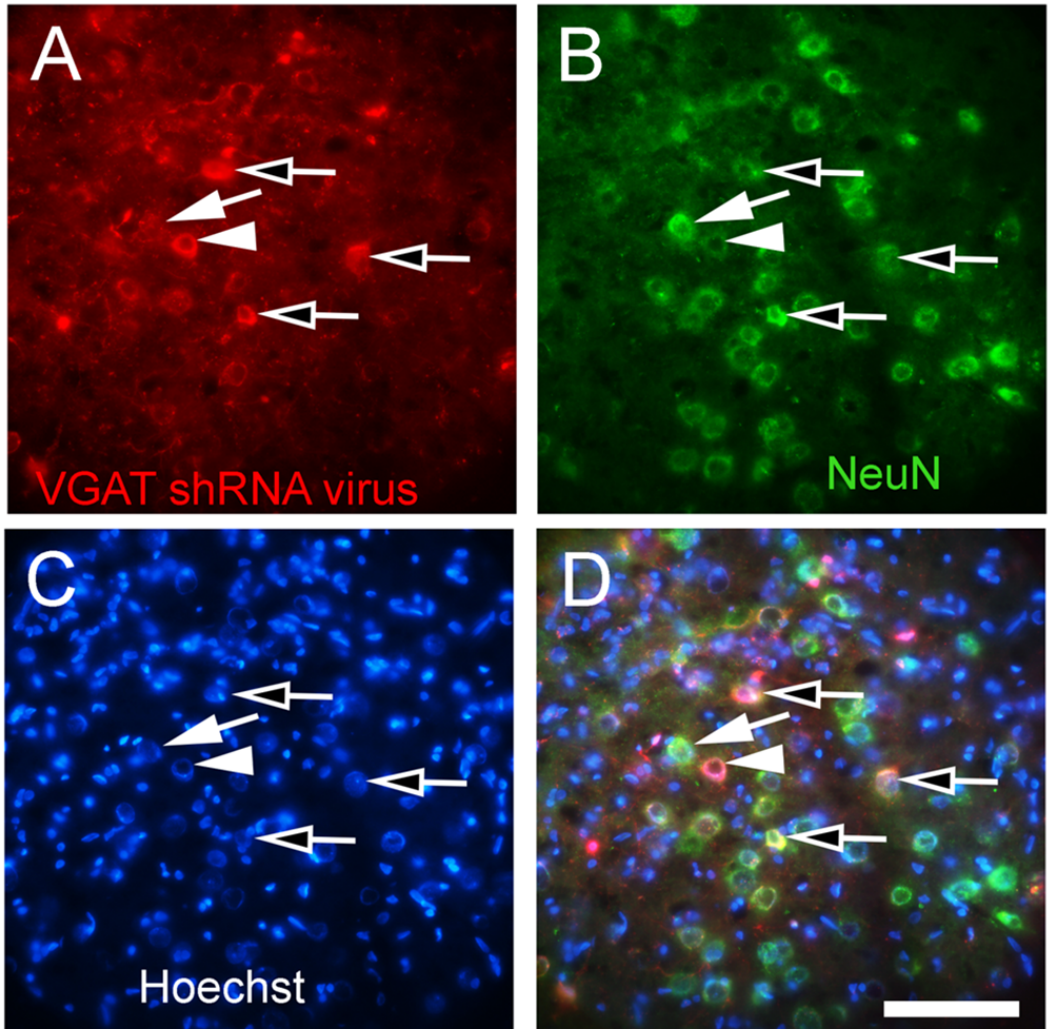

Figure 21. AAV transduced neurons in the lateral thalamic region: Male rat's right thalamus was injected with AAV9 and 7 days after surgery the left whisker pad was injected with control cells infected with 650 pfu/ µl VZV (pOka strain). Twenty-one days after AAV9 injection three randomly chosen animals were selected from each of the two treatment groups for sectioning and staining. In panel A the image shows a representative section for a rat injected with AAV9 containing the VGAT shRNA construct driven by the U6 promoter; this construct had a mcherry tag (red, arrow head, open arrows) and panel B shows NeuN positive neurons (green, arrow, open arrows). Hoechst stain is shown in blue, panel C (arrow, arrow head, open arrows). NeuN positive neurons co-expressing the mCherry tag 21 days after injecting the right thalamus with AAV9 virus are yellow (panel D, open arrows). The image is taken near the border of the ventroposterior nucleus and the reticular thalamic nucleus. Bar equals 50 µm.

APPENDIX B

TABLES

Table 1. Thalamic gene expression changes between proestrus and diestrus

Table 1. Thalamic gene expression changes over 2 fold between proestrus and diestrus

Fold change	Proestrus signal	Diestrus signal	Genbank#	Gene Description	Gene symbol
3.46	311.32	180.74		Similar to 40S ribosomal protein S3	RGD1560831
3.18	1519.41	678.48	M72422	Glutamate decarboxylase 2	Gad2
2.8	881.36	366.14	M34445	Glutamate decarboxylase 1	Gad1
2.05	502.57	275.51	AF030253	Solute carrier family 32 (GABA vesicular transporter) member1	Slc32a1 (vGAT)
2.05	392.20	145.97		Meishomeobox2	Meis2
2.03	103.05	197.57	FQ221570	Thyrotropin releasing hormone	Trh
2.01	222.72	185.77	FQ221553	Ubiquitin associated and SH3 domain containing, B	Ubash3b