BEHAVIORAL AND LOCAL FIELD POTENTIAL CHANGES IN THE THALAMUS AND ANTERIOR CINGULATE CORTEX OF BEHAVING RATS EXPERIENCING POST HERPETIC NEURALGIA

By

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THESIS

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ABSTRACT

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OF BEHAVING RATS EXPERIENCING POST HERPETIC NEURALGIA

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The University of Texas at Arlington, 2016

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Post herpetic neuralgia (PHN) is experienced as burning pain in the facial region, caused by nerve damage from the herpes zoster virus. The pain often becomes chronic and debilitating. Investigations into the pain pathway become vital. Pain itself is multidimensional, consisting of both the sensory and affective experiences. One of the primary brain substrates for transmitting sensory signals in the facial muscles is through ventral posterior medial thalamus (VPM). Anterior cingulate cortex (ACC) has been shown to be vital in the affective experience of pain, so investigating both of these areas in freely behaving animals can provide important information. Local field potential (LFP) recordings measure changes to the summation of subthreshold neuronal activity. A new investigational method of using a designer receptor activated by a designer drug (DREADD) (Armbruster et al, 2007) has been shown by our lab to inhibit both neuronal activity in the VPM and spontaneous pain behavior in a formalin pain model in rats. By application of this DREADD, a modified G Protein Coupled Receptor (mGPCR), the LFPs of both VPM and ACC were recorded simultaneously to see how the inhibition of neuronal activity in the VPM affects neuronal activity in the ACC in freely behaving rats in a more clinically relevant pain model, PHN. Additionally, a place escape-avoidance

paradigm was used to measure affective experience of pain (Fuchs et al, 2014). mGPCR was infused into the VPM of Sprague Dawley rats and LFP recording electrodes were implanted into the VPM and ACC. A week later, the whisker pads were injected with either varicella zoster virus (VZV) infected cells or MeWo (control) uninfected cells. Within 7 days, the LFP for both VPM and ACC were recorded, mGPCR was activated via IP injection (or saline for control) of clozapine N oxide. A new baseline was recorded and the animals were tested using a preferred escape-avoidance paradigm. We found that, similar to the spontaneous pain behavior, escape-avoidance behavior is significantly reduced in the drug group compared to the no drug group. Changes in LFP activity of delta, theta, and alpha wavelengths were noted with increased activity observed in PHN rats compared to control and also between the drug and no drug group in PHN. Overall thalamocortical coherence between VPM and ACC also increased over time. Understanding how manipulation of thalamic activity can affect changes in both neuronal activity and pain behavior can bring us closer to identifying novel ways to relieve post herpetic neuralgia in humans.

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Chapter 1

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Merskey, 1986). The primary purpose of pain is adaptive, limiting mobility and changing autonomic systems to allow an organism time and resources to heal. While the adaptive component of pain cannot be overlooked, it can quickly become maladaptive, lingering when the tissue damage has healed or the threat of damage has passed. For patients with a primary diagnosis of pain, this maladaptive level is currently costing Americans $560-635 billion annually, accounting for loss of productivity and cost of medical care (Gaskin & Richard, 2012).

Research has historically focused on the sensory component of pain, but the focus began to change in 1968 when Melzack and Casey proposed a model that included three major determinants of pain; sensory, affective and cognitive. Since then, the field of pain research has delved deeper into understanding the reciprocal relationship between pain sensations and the pain experience.

Orofacial pain is represented by pain above the neck, below the orbitomeatal line, in front of the ears and includes the oral cavity; areas innervated by the trigeminal system, and includes some of the most prevalent pain conditions experienced, affecting both physical and psychological health (Madland & Feinmann, 2001; Romero-Reyes & Uyanik, 2014). It is estimated that 25% of the population reports non-dental orofacial pain, with 11% reporting chronic pain (Bender, 2014; Benoliel, Birman, Eliav, & Sharav, 2008; Zakrzewska & Linskey, 2015). Orofacial pain can be caused by a variety of pathologic conditions, so investigating the underlying neuronal circuitry of this type of pain is vital (Giannakopoulos, Keller, Rammelsberg, Kronmüller, & Schmitter, 2010; Conti, Pinto-Fiamengui, Cunha, & Conti, 2012).

Post herpetic neuralgia (PHN) is a specific form of chronic orofacial pain that can be especially debilitating. PHN is a common complication of herpes zoster, and is the continued sensation of pain after the rash has healed. Up to 50% of patients who contract shingles will experience postherpetic neuralgia, with the pain lasting months and sometimes years (Mounsey, Matthew, & Slawson, 2005).

Herpes zoster (HZ), commonly referred to as shingles, is a painful rash that is caused by the reactivation of the latent varicella-zoster virus (VZV). This varicella-zoster virus lies latent in the dorsal root and cranial root ganglia in over 90% of adults in America (Oxman, 2009). While varicella (chickenpox) is commonly a childhood disease, herpes zoster is more commonly found in people over the age of 50. Key risk factors for shingles include age, heightened stress level, immunosuppression and altered cell-mediated immunity (Drolet et al., 2010; Gnann & Whitley, 2002).

There is a paucity of research into animal models of post herpetic neuralgia. VZV appears to be a generally human species specific virus. Some replication has been found in guinea pigs and rats (Guedon, 2015), While VZV has been shown to produce mechanical allodynia in the hind paw of rats (Fleetwood-Walker et al, 1999; Garry et al, 2005) with similar results in the whisker pad (Stinson et al, 2016), recent data also suggests that the virus does not replicate well in rats, with limited viral gene expression, and that the pain behavior may be due more to peripheral neurite retraction than neuronal modification (Guedon, 2015). With this in mind continued investigation into a rat model of PHN, primarily in the orofacial region, is vital, both to validate the model and to understand the mechanisms.

1.1 DREADD and modified G protein coupled receptors

Nociceptive signaling is dependent on different second messenger pathways, proteins and organelles within the cells. G protein coupled receptors (GPCRs) are part of a superfamily of seven transmembrane receptor proteins. These receptors are actively involved in the

nociceptive process, acting as a second messenger system to facilitate the release of prostaglandins, histamine, and bradykinin; among others. Activation of a transmembrane GPCR begins a process that releases a G protein into the cell to activate a secondary process. These secondary processes are central to the regulation of cellular function involving hormones, neurotransmitters, and many large and small molecules involved in nociception, vision, olfaction, and taste. (Deupi & Kobilka, 2007; Gainetdinov et al, 2004; Katritch, Cherezov, & Stevens, 2013; Pitcher, Freedman, & Lefkowitz, 1998; Alexander et al, 2013; Strader et al, 1994) By bridging molecular events to nociceptive signaling and behavior we can identify specific pathways in which to focus pharmacological intervention (Hucho & Levine, 2007).

A recently developed investigational approach involves using a Designer Receptor Exclusively Activated by Designer Drugs (DREADD). In this approach, a protein receptor is modified to activate by binding to a specific ligand that is not naturally occurring, allowing greater experimental control over the receptor activation (Armbruster et al, 2007). In this case, the modified receptor will be Gi, which is an inhibitory protein in the GPCR family (Pei, 2008). The Gi protein-coupled receptor, driven by neuronal specific synapsin-1 promoter, was modified to bind to clozapine-N-oxide (CNO) to induce activation rather than its naturally occurring ligand, acetylcholine.

Activation of the mGPCR triggers the inhibitory Gi protein and is expected to facilitate neuronal silencing in the targeted area as it did in the hippocampus. (Alexander et al., 2009; Armbruster et al., 2007).

1.2 Thalamus

The ventral posterior medial nucleus (VPM) of the thalamus lies in the most medial portion of the ventrolateral nuclei. The VPM receives input from the principal (Pr5) and spinal (Sp5) trigeminal nuclei (Paxinos, 1995). Trigeminal thalamic cells project to the VPM beginning as sensory neurons in the craniofacial region and terminating in the subnucleus caudalis (Sp5c),

a subgroup of the spinal trigeminal nuclei, often referred to as the medullary dorsal horn (Craig & Dostrovsky, 1999). Pr5 and Sp5 have major projections to the VPM. In rats, the main projection of Sp5 is to the ipsilateral thalamus, although some mandibular input is projected to the contralateral VPM, and the primary projection of Pr5 is to the contralateral thalamus with minor projections to the ipsilateral thalamus (Paxinos, 1995), as shown in Figure 1.

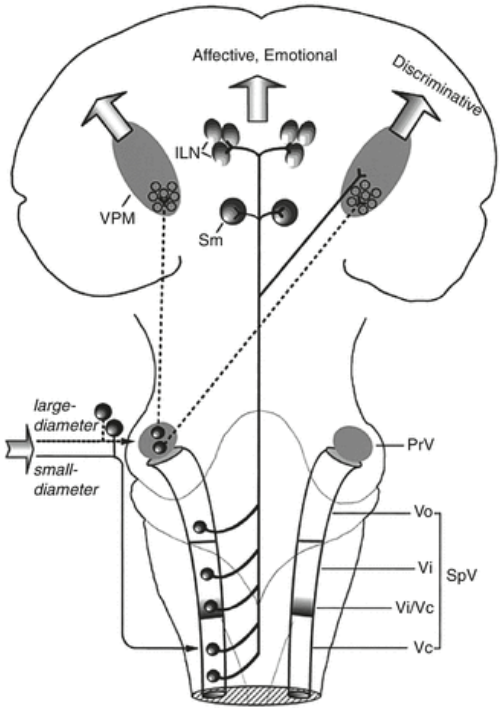

Figure 1. Ren, 2007

Efferent projections of the VPM are relayed to the primary somatic sensory cortex (S1), which in turn provides reciprocal afferent projections (Paxinos, 1995).

The thalamus processes, integrates and transmits information from multiple systems to the cerebral cortex. This includes peripheral sensory information such as visual, auditory, and tactile information and also internal information from the limbic and motivational systems, and vital intra-cortical information (Strominger, Demarest, & Laemle, 2012). Any sort of information that is received by the cortex benefits from thalamic processing (Sherman, 2005).

Thalamic nuclei are considered relay, association, or projection nuclei. Relay nuclei are the first order drivers, activating specific areas in the cerebral cortex, association nuclei are modulators and projection nuclei are the higher order processes that influence corticocortical signaling. The VPM is considered a the primary relay nuclei for somatic sensation to the face and head muscles (Sherman, 2005, 2007; Strominger et al., 2012; Paxinos, 1995).

The ventral posterior medial thalamic nuclei (VPM) processes somatosensory information from the head and facial muscles, and is therefore an ideal place to investigate orofacial pain. One of the key features of the thalamus is it's interconnectedness with the cerebral cortex (Yen & Lu, 2013), so reducing nociceptive signaling in the VPM should reduce the amount of nociceptive information relayed to the cerebral cortex.

1.3 Anterior Cingulate Cortex

The cingulate cortex in the rat represents the medial part of the prefrontal cortex (Paxinos, 1995). The anterior cingulate cortex (ACC) is the most anterior segment of this cingulate cortex. The ACC receives afferents from the visual cortex, hippocampus, subiculum, amygdala, basal ganglia, globus pallidus, pars reticulum, insula, ventral tegmental area, superior colliculus, parabrachial nucleus, and nociceptive input from the thalamic midline and mediodorsal nuclei (Neafsey et al, 1993).

The anterior cingulate cortex does not have a layer IV, a layer that receives significant input from thalamic sensory neurons (Allman et al., 2001), but it does contain high density of neurons in layer V (Vogt, 1993), allowing for diffuse projections into subcortical and brainstem areas such as amygdala, hypothalamus, insular cortex, mammillary nuclei, periaqueductal gray, nucleus ambiguous, and the dorsal motor nucleus of the vagus nerve (Neafsey et al, 1993). Efferent output is also closely tied to the motor cortex in controlling the head and eye movements.

The structure and cytoarchitecture of the ACC in rodents is homologous to those in humans and therefore allows rodent modeling of human conditions in the ACC, with human area d32 and rat area 24b expressing high degrees of similarity (Vogt & Paxinos, 2014). See Figure 2.

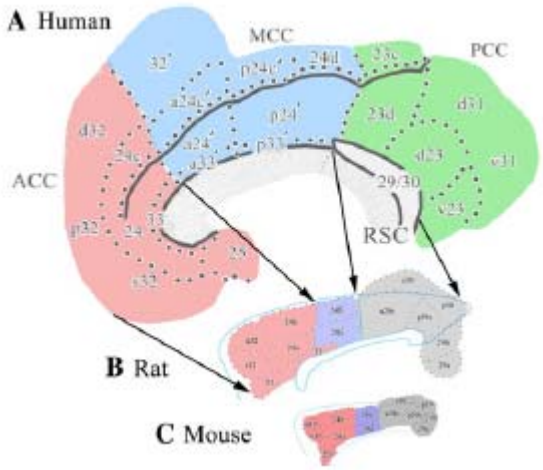

Figure 2. Vogt & Paxinos, 2014

Understanding the ACC is key in providing insight into the emotional aspects of pain and pain processing. The ACC is central to emotional responses, attention, reward, self-control (Allman, Hakeem, & Hofetal, 2001), and has been linked to autonomic activity (Neafsey et al, 1993). These components play a role in pain perception.

ACC is activated by nociceptive stimulation. Wall and Melzack (1999), note robust activity in the ACC under evoked acute pain. Neuroimaging studies have consistently found that the ACC is activated both during a painful stimulus and during persistent chronic pain conditions (Fuchs et al, 2014).

Differentiation can be made, however, between pain sensation and pain affect. Pain patients treated with small bilateral lesions in the ACC reported, more than a year later, that while they still felt the pain they were no longer bothered by it (Allman, Hakeem & Hofetal, 2001). Studies of humans having undergone cingulectomy or cingulotomy have found that they

can still localize the sensation of pain, however the pain affect is blunted or gone completely (Fuchs et al, 2014). A human PET study used hypnotic suggestion to alter the unpleasantness of pain, while leaving the pain intensity unaltered. They observed significant changes in ACC activity but none in the somatosensory cortex (Rainville et al, 1997). In an similar study, PET results of a hypnotic suggestion to alter the intensity of pain elicited significant change in the activity of the somatosensory cortex (Hofbauer et al, 2001), further illuminating the distinctive function of the ACC in processing pain affect. This pain unpleasantness can be modeled as a moderator of the final pain perception when experiencing nociceptive stimuli (Price, 2002).

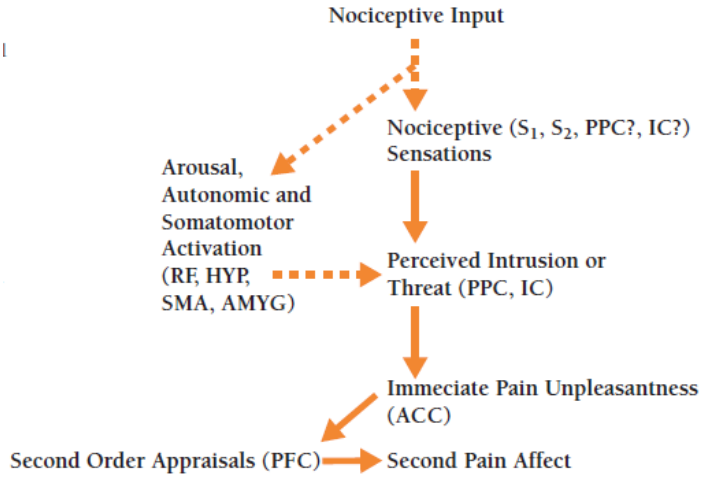

Figure 3. Model depicting direct and indirect projections from the thalamic relay of nociceptive stimulation, into the ACC, culminating in the experience of pain (Price, 2002).

1.4 Local Field Potential Response

One measure of brain activity is local field potential (LFP). LFP is electrophysiological recording that potentially offers information about the neuronal activity of larger networks in specific brain areas and can provide a picture of how all the cellular activity works together to process important sensory and motor functions. LFP records the summation of electrical currents in active cellular processes (Zheng et al., 2012),

The primary contribution of the currents is found in the EPSPs and IPSPs(Buzsáki, Anastassiou, & Koch, 2012), but also includes other low frequency activity such as non-synaptic calcium spikes, glial cell fluctuations and other subthreshold membrane oscillations, somatodendritic afterpotentials, and $GABA_A$ receptor inhibitory input (Berens et al, 2008) Understanding how the extracellular field activity reacts and changes in concert with behavioral changes can provide with a greater depth of understanding concerning the relationship between the network activity and studied process such as pain.

1.5 Behavioral Response

Previous research from this lab looked at the sensory component of pain behavior by allowing the rat to move freely around its cage during the pain condition and monitoring the number of pain behaviors that the animals exhibited such as twitching and rubbing or licking the affected area (Whittaker & Howarth, 2014). However, spontaneous pain behavior may not tell the entire story of such a multidimensional concept as pain and research is showing differentiation between pain sensations and pain unpleasantness.

The Placement Escape Avoidance Paradigm (PEAP) is a behavioral test designed to investigate the dimension of pain unpleasantness in rats (LaBuda & Fuchs, 2000). This paradigm consists of a chamber that has a dark side and a light side in equal dimensions. The rat is allowed to move freely around in the chamber. When the rat is on its preferred dark side of the chamber, it encounters noxious stimuli. The only way to avoid this pain is to escape to the non-preferred light side in which the noxious stimulus is absent. The amount of time the animal spends in the light side of the chamber reflects the degree of aversion the animal has to the noxious stimuli, or the level of pain unpleasantness (Fuch & McNabb, 2012).

The PEAP test has been helpful in showing the differentiation between pain sensation and pain unpleasantness. With increasing evidence showing that the ACC is instrumental in detecting pain unpleasantness (Rainville et al, 1997), further evidence shows that manipulation

of the ACC changes the affective pain behavior such as escape and avoidance activity without changing sensory responses such as hypersensitivity to mechanical stimulation (Uhelski et al, 2012).

1.6 The Purpose of this Study

The ability to look at the summation of activity simultaneously in two specific brain areas while manipulating targeted GPCR is a novel viewpoint that is expected to provide important new information. The rationale is that the inhibition of LFP in the VPM could indicate changes in the nociceptive function of the thalamus and subsequently change the function in higher processing areas. Once it is effectively understood how mGPCR acts to inhibit neuronal activity in key nociceptive processing areas, such as the thalamus, targeted therapeutic strategies can be developed for pain management in clinical populations of post herpetic neuralgia.

Previous research conducted in collaboration between molecular biology labs at Baylor College of Dentistry and electrophysiology neuroscience labs at University of Texas at Arlington investigated the use of a designer receptor activated by a designer drug (DREADD) in a pre-clinical model of orofacial pain by injecting formalin into the masseter tendon of a freely behaving Sprague Dawley rat. Using a DREADD designed to inhibit neuronal activity in a specific brain area; we found that activation of the inhibitory drug in the thalamus reduced both pain behavior and thalamic activity, as measured by local field potential. We specifically found a significant pain behavior response to formalin injected into the masseter tendon of rats in a model of orofacial pain and significant inhibition of pain behavior to formalin injected into the masseter tendon in the presence of DREADD activated Gi protein.

In the present study, our goal was to investigate both the sensory and affective components of pain using a more clinical model of orofacial pain, post herpetic neuralgia. The first main goal was to investigate individually the activity of the VPM and the ACC in response to nociceptive stimulation in freely moving animals and determine how the LFP in the individual VPM and ACC

activity changes interact with behavioral measures of affective pain. The second goal was to determine if the manipulation of the sensory component of pain (VPM) had an effect on the affective component of pain (ACC) when Gi protein is activated in the thalamus by investigating the LFP of both the VPM and ACC and behavioral changes, as measured by PEAP testing.

The near-term goal in this study was to increase the understanding of how activated mGPCR affects the neuronal activity in the thalamus and anterior cingulate cortex. The final goal will be targeted inhibition of synaptic activity in the thalamus, resulting in clinical pain management treatments.

1.7 Specific Aims and Hypotheses

The first specific aim was to investigate the electrophysiological activity of the thalamus and anterior cingulate cortex by looking at local field potential changes when Gi protein is activated in the thalamus in a model of neuralgic orofacial pain. To this end we looked specifically at two hypotheses. First, we expected that there would be greater LFP activity in the rats with VZV infected cells than with rats with MeWo uninfected cells in both the thalamus and the ACC. Second, we expected that the VZV/Saline condition would have more LFP activity in the thalamus and ACC than the VZV/CNO condition.

The second specific aim of this study was to investigate the relationship between pain behavior and LFP activity in the thalamus and ACC in freely moving rats. Three hypotheses fell under this aim. One, the VZV infected cell rats would exhibit more escape-avoidance behavior and spend more time in the light side of the PEAP box than the MeWo uninfected cell rats. Two, the VZV infected cell rats in the CNO condition would exhibit less escape avoidance behavior and spend less time in the light side of the PEAP box than the VZV infected cell rats from the no CNO condition. Three, the rats that exhibit more escape-avoidance behavior would demonstrate more LFP activity in the thalamus and ACC.

Chapter 2

METHODS

2.1 Subjects & Groups

Two separate experiments were conducted, using a total of 62 male Sprague-Dawley rats (280-325 grams). These rats were ordered from Harlan Industries in Houston, Texas and were freely given food and water. They were allowed a four day acclimation period before any surgeries were performed. The experimental protocol was approved by The Texas A&M University Baylor College of Dentistry Institutional Animal Care and Use Committee. Guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983) were closely followed. The first experiment designed to provide preliminary behavioral data used 22 of these rats in 4 conditions; with 5-6 animals per group. The final 40 rats were used for the primary experiment, testing both behavior and electrophysiological responses. These 40 male Sprague-Dawley rats were placed into 4 separate experimental and control conditions. Four rats died prior to testing and one rat removed his head electrodes and bone cement immediately following the first week of testing. This left a remaining 35 rats; allowing for 8-9 rats per condition. See Table 1.

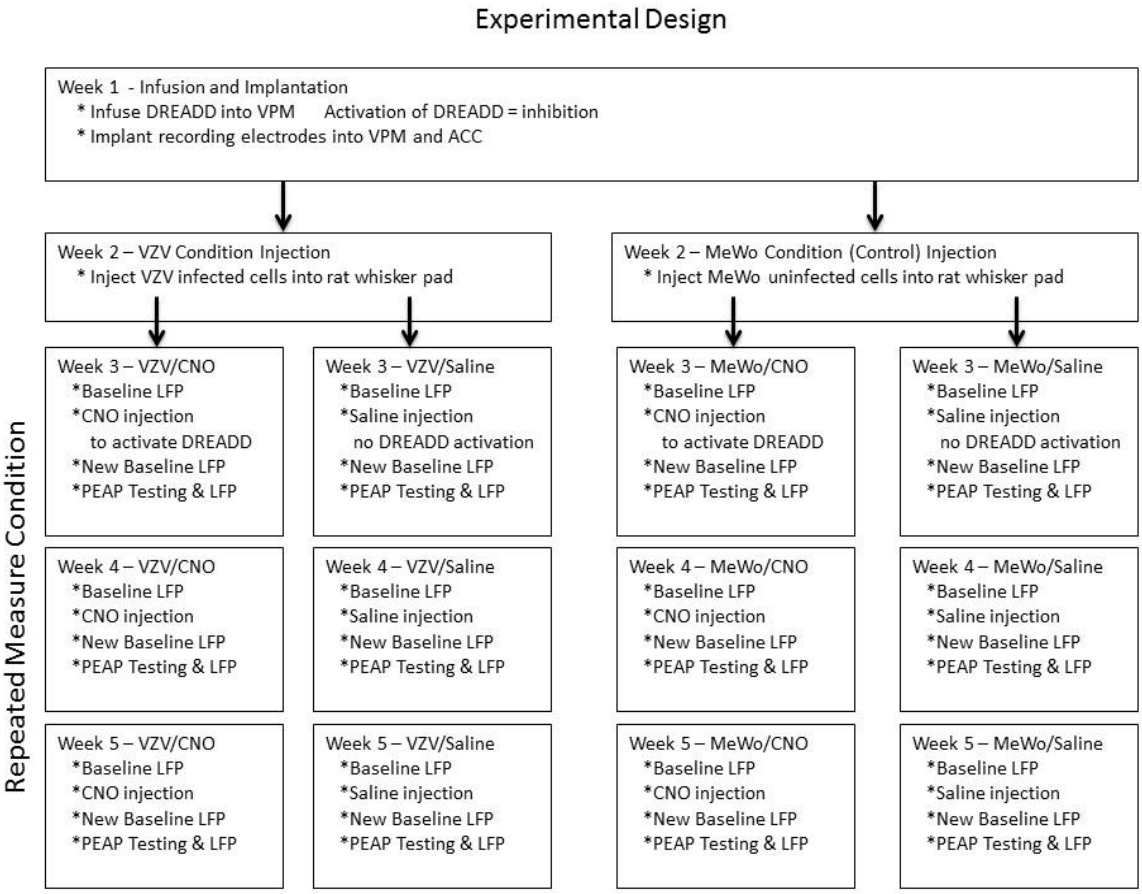

Table 1. Outline of experimental design.

2.2 Animal Preparation

Adult male Sprague-Dawley rats (280-325 grams) were anesthetized with 2% isoflurane with an air flow of 2 liter per minute. A 22 gauge cannula/electrode (C313G-MS302-2-SPC, Plastics One, Roanoke, VA) was inserted stereotaxically, with the tip placed in the lateral thalamic region (from Bregma posterior 3.6 mm, lateral 2.7 mm, depth 6.0 mm). Once inserted, a Stoelting stereotaxic syringe pump system was used to infuse 0.250 µl of 2-8 $X10^{12}$ pfu/ml AAV8 at a rate of 20 nanoliters per minute. After the infusion was complete, the syringe pump needle was left in place for 5 minutes and then removed. AAV8 is the adeno associated virus containing a neuronal silencing construct Svn-hM4D(Gi)-mcherry which contains an engineered

acetylcholine Gi-protein coupled receptor (mGPCR) designed to inhibit neuronal burst firing when bound by CNO (Gene Therapy Center Vector Core, University of North Carolina at Chapel Hill). The Svn promoter in the construct induces expression in most neurons.

Immediately after the mGPCR was infused 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. One electrode was implanted into the thalamus using the same coordinates as the needle that was removed after infusing AAV8 (from Bregma posterior 3.6 mm, lateral 2.7 mm, depth 6.0 mm). The second electrode was implanted into the ACC (from Bregma posterior 1.0 mm, lateral 0.7 mm, depth 3.1 mm) at a 26 degree angle. Three anchor screws were placed into the skull. Electrodes and anchor screws were affixed with dental cement. See Figure 4.

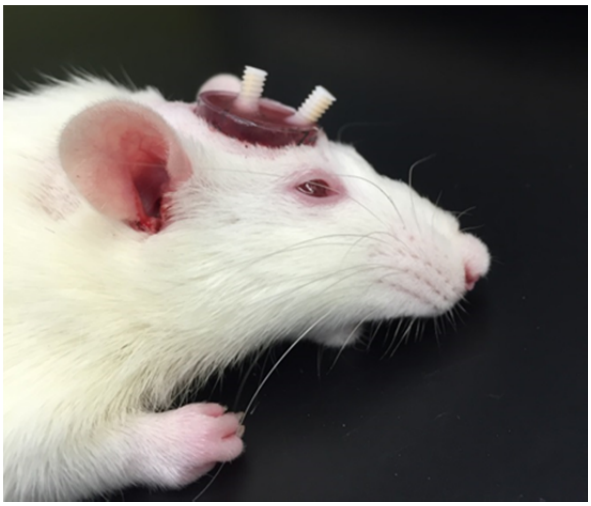

Figure 4. Sprague Dawley with implanted electrodes

After a 7 day recovery period, the animals were injected with VZV infected or MeWo uninfected cells in the left whisker pad. The rats were allowed 7-14 days for the cells to develop in the desired area. Food and water were freely given during this time and the rats were closely monitored for signs of infection or disease.

2.3 Local Field Potential

The rats were placed into 4 separate experimental or control conditions while both electrophysiological and behavior responses were recorded simultaneously.

In each condition the rat was very lightly anesthetized with 2% isoflurane to fit the rat into the backpack equipped with the LFP recording headstage, recording, ground, and reference wires (Multichannel Systems, Germany) and to connect the module to the surgically implanted electrodes. See Figure 5.

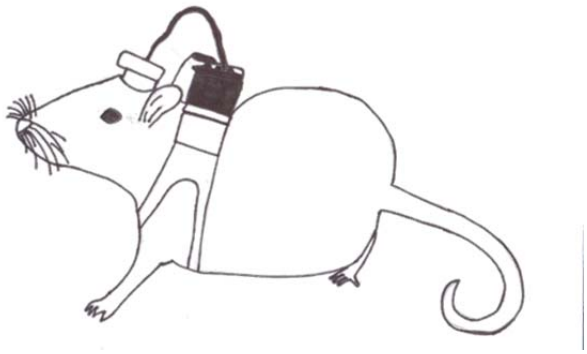

Figure 5. Illustration of backpack and LFP recording apparatus.

Once awake, the rat was first placed in a separate box near the PEAP testing chamber to record a 10 minute baseline for the local field potential activity of the VPM and ACC in a freely moving rat. Then 0.03 mL of either saline or CNO was given intraperitoneally to the awake rat. The rat was returned to the same box for a new 10 minute LFP baseline recording. Finally, the rat was placed in the PEAP testing chamber for 30 minutes. In this chamber the field potential of both the ACC and VPM was recorded while the rat received mechanical stimulation to the whisker pad via VonFrey Filament level 5.88 (60 grams force) every 30 seconds. These measurements were taken 3 times at weekly intervals.

Signals from the electrode were transmitted wirelessly to a receiver connected to a laptop by using MultiChannel systems equipment and MC_Rack software. The signals were

recorded as raw electrode waveforms at a sampling frequency of 10kHz and filtered using a Butterworth second order low pass filter with a cut off frequency of 200Hz. See Figure 6.

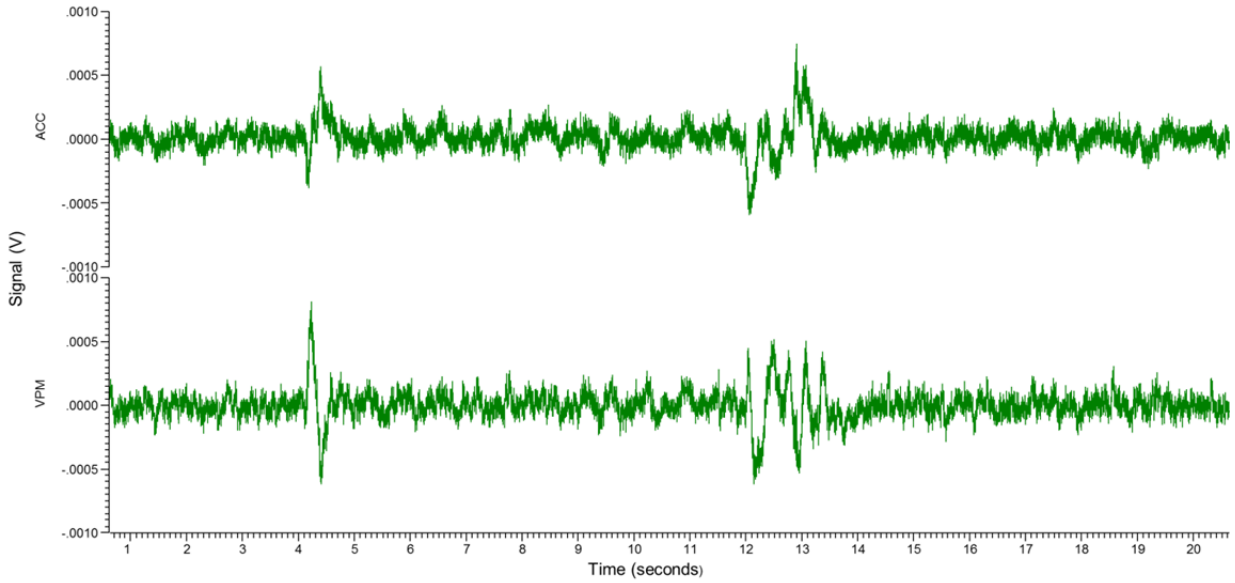

Figure 6. Representative raw traces

This raw electrode waveform was then imported using CED Spike2 V7 software and analyzed using power spectrum analysis with a Hanning window and FFT size of 8192, with the results shown in bins reflecting 1.22Hz. The power spectrum was then imported into Excel as a text file and grouped according to frequency bands of Delta (0-4Hz), Theta (4-8Hz), Alpha (8-13Hz), Beta (13-30Hz), and Gamma (30-100Hz). See Figure 7.

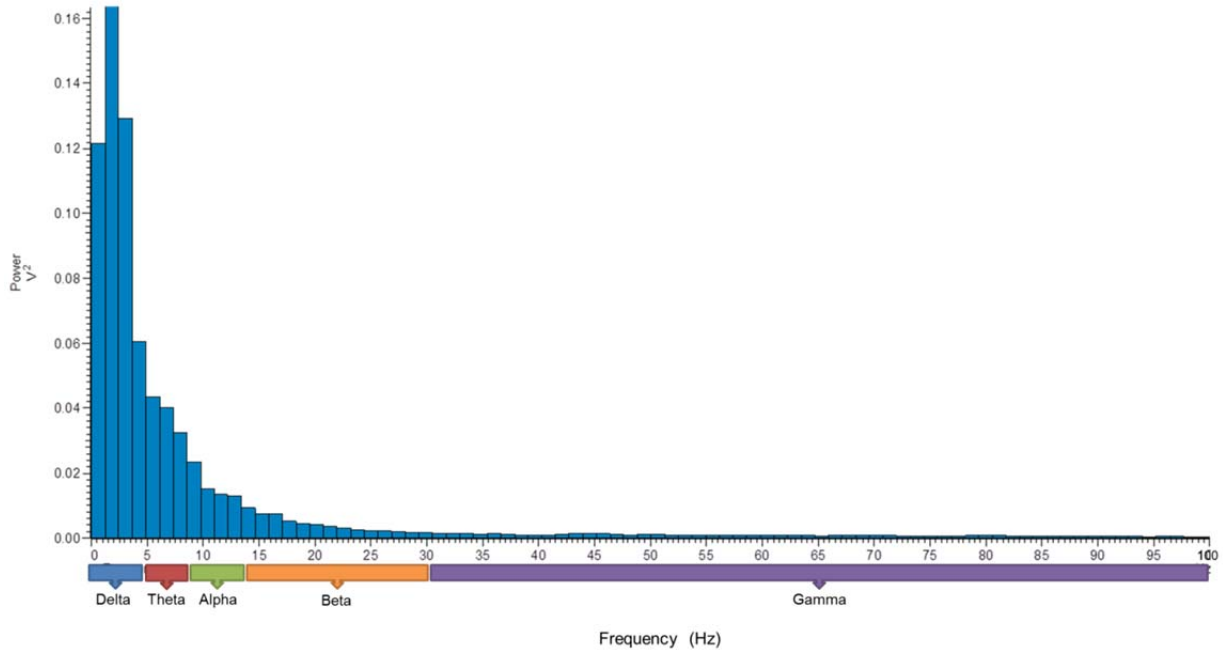

Figure 7. Representative power spectrum.

2.4 Place Escape Avoidance Paradigm

Behavioral responses were measured using the Place Escape Avoidance Paradigm (PEAP). After the second baseline was recorded, the rats were placed into the PEAP chamber. This chamber is a box divided equally into dark and light sides to which the rat has free access. The rat received mechanical stimulation to the whisker pad via VonFrey Filament level 5.88 (60 grams force) every 30 seconds for 30 minutes. When the rat remained in the dark side of the chamber, the VonFrey filament was administered to the left whisker pad, the side previously injected with VZV/MeWo. When the rat remained in the light side of the chamber, the VonFrey filament was administered to their right whisker pad. These measurements were taken 3 times at weekly intervals.

Pain behavior was analyzed in 5 minute time bins and expressed as a percentage of how much time the rat spent in the dark side of the box during each of those time bins. The interpretation was that animals spending more time in the dark side experienced less pain unpleasantness and animals that spent less time in the dark side were experiencing more pain

(LaBuda & Fuchs, 2000). Animals in the light side exhibit the escape-avoidance behavior, leaving their preferred side to reduce the pain experience.

The PEAP chamber was also utilized in a preliminary experiment without field potential recordings. These 22 rats were grouped into the same experimental/control conditions with the only differences being that they received the PEAP chamber testing without implantation of the recording electrodes or use of the backpack and recording module.

2.5 Histology

After the final LFP recording the rats were perfused with 9% sucrose followed by 4% paraformaldehyde. The brain was extracted using a rongeur and stored in 25% sucrose at 4°C. Fixed whole brains stored in 25% sucrose were frozen, cryo-sectioned and the 20 μm sections placed on Histobond slides (VWR international, Radnor, PA). . The tissue was then blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for 2 hours at room temperature. Following three rinses in PBS with 0.3% Triton-X 100 the slides were incubated in a primary antibody solution overnight at 4°C. Primary antibodies consisted of a 1:300 dilution of the mouse monoclonal VGLUT2 antibody (ab79157, abcam, Cambridge, MA) and a 1:300 dilution of the rabbit polyclonal VGAT antibody (AB5062P, Millipore). 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 60 min and placed for 2 hours in a 1:500 dilution of secondary antibody in PBS and 0.3% Triton X-100. Secondary antibodies were either goat anti-mouse 488 or goat anti-rabbit 488 (Invitrogen, Carlsbad, CA). After rinsing the slides three times in PBS for a total of 60 min, the slides were then 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). See Figure 8 for placement.

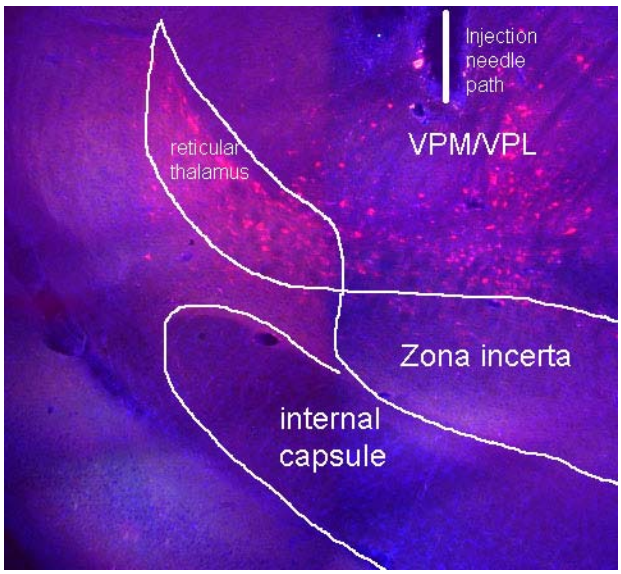

Figure 8. Placement of thalamic infusion cannula and LFP recording electrode.

Chapter 3

RESULTS

3.1 Specific Aim 1

This first specific aim was intended to understand changes to the field potential activity of the VPM and the ACC in the presence of post herpetic pain and also when the mGPCR is activated in the thalamus. In hypothesis 1, it was expected that there would be greater field potential activity in rats with the VZV infected cells than with rats in the MeWo uninfected cell group in both the VPM and ACC. In hypothesis 2, it was expected that the VZV/saline group would have more activity in the VPM and ACC than would the VZV/CNO condition. Both hypotheses expected that the activity of the VPM and ACC would show similar changes in LFP activity. However, since both the VPM and ACC produced their own independent results, the data was analyzed independently of each other. A mixed repeated measure analysis of variance was run with VZV/MeWo and CNO/saline as the between subjects groups and condition (baseline, CNO/saline baseline, and PEAP testing) as the within subjects measure. These ANOVA's were analyzed individually with each frequency band (delta, theta, alpha, beta, and gamma) as the dependent variable.

3.1.1 Anterior Cingulate Cortex

When comparisons were investigated across the entire three week experiment there were no significant local field potential differences for the ACC found in our between subject conditions (VZV/MeWo or CNO/saline). There were some significant differences in the within subject condition of week.

In the delta waveband there was a main effect for week $F(2,22) = 3.82$, $p = 0.038$, partial $\eta^2 = .26$, which indicates a large effect size (Cohen, 1988). Post hoc comparisons found that

field potential activity was higher overall (p = 0.01) during week one (M = 0.180 ± 0.002) compared to week three (M = 0.172 ± 0.002).

The gamma waveband also showed a main effect for week $F(2,21) = 5.436$, p = 0.013, partial η^2 = .341, indicating a large effect size. Further investigation found significantly lower (p = 0.035) activity in week one (M = 0.013 ± 0.001) compared to week three (M = 0.022 ± 0.002). See Figure 9.

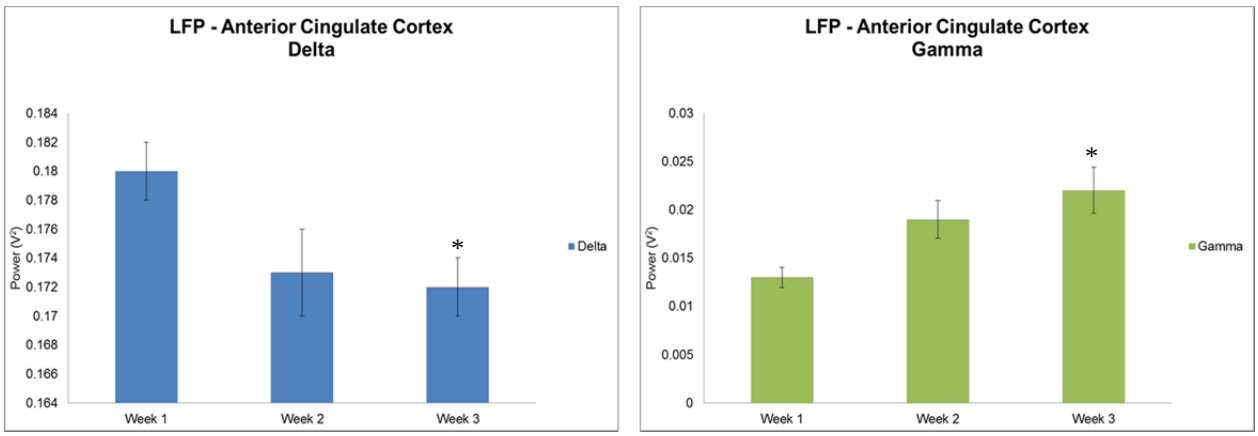

Figure 9. Power spectrum analysis results by week across condition.

However, when comparisons were investigated through a repeated measure ANOVA using only the first week of testing, there were significant between subject effects seen. In the delta frequencies a main effect was seen in the CNO/saline condition $F(1,30) = 5.205$, p =0.03, partial η^2 = .148, indicating a medium effect size. Pairwise comparisons indicate that the field potential for CNO (M = 0.176 ± 0.003) is smaller than for saline (M = 0.184 ± 0.002) across all conditions.

In theta frequencies a main effect was seen in the CNO/saline condition $F(1,30) = 5.331$, p =0.028, partial η^2 = .151, indicating a medium effect size. Pairwise comparisons indicate that the field potential for CNO (M = 1.68 ± 0.052) is smaller than for saline (M = 1.84 ± 0.049).

The alpha waveband was nearing a significant main effect in the CNO/saline condition $F(1,30) = 3.391$, $p = 0.075$, partial $\eta^2 = .102$, yet still indicating a medium effect size. Pairwise comparisons indicate that the field potential for CNO (M = 2.31 ± 0.062) is smaller than for saline (M = 2.469 ± 0.058). See Figure 10.

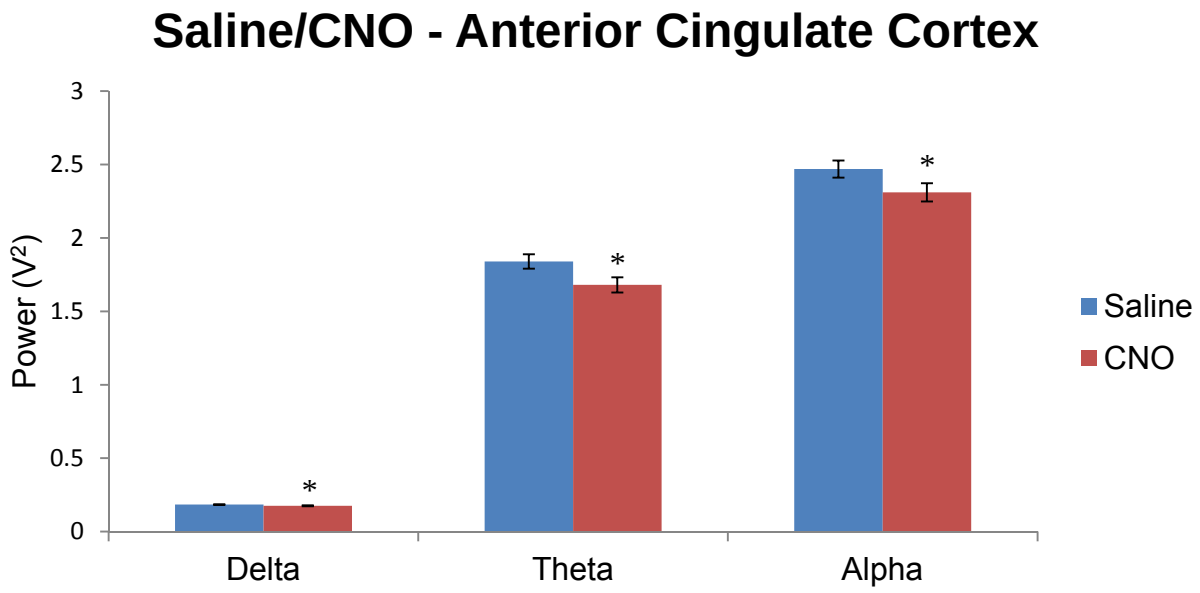

Figure 10. Power spectrum analysis comparing CNO to saline during the first week of testing.

For the Anterior Cingulate Cortex, when looking at VZV infection as a chronic PHN pain state there were no between group differences and neither hypothesis 1 nor hypothesis 2 were supported. There were some overall differences in activity by week, indicating a change in ACC field potential over the course of the experiment. In looking at the field potential activity during the first week alone, a main effect of CNO/saline was seen in the delta, theta, and alpha bands. In each of these cases, the field potential activity was smaller in the CNO groups than in the saline groups. In this case, the expectation that the VZV/saline condition will have more LFP activity than the VZV/CNO condition (H2) is supported in the ACC.

3.1.2 Thalamus

When comparisons were investigated across the entire three week experiment there were no significant local field potential differences for the thalamus found in our between subject conditions (VZV/MeWo or CNO/saline). There was one significant difference in the within subject condition of week. In the gamma frequencies waveband there was a main effect for week $F(2,19) = 3.743$, $p = 0.043$, partial $\eta^2 = .28$, which indicates a large effect size (Cohen, 1988). Post hoc comparisons found that field potential activity was higher overall ($p = 0.025$) during week three (M = 0.000198 ± 0.00001418) compared to week one (M = 0.000123 ± 0.000016). See Figure 11.

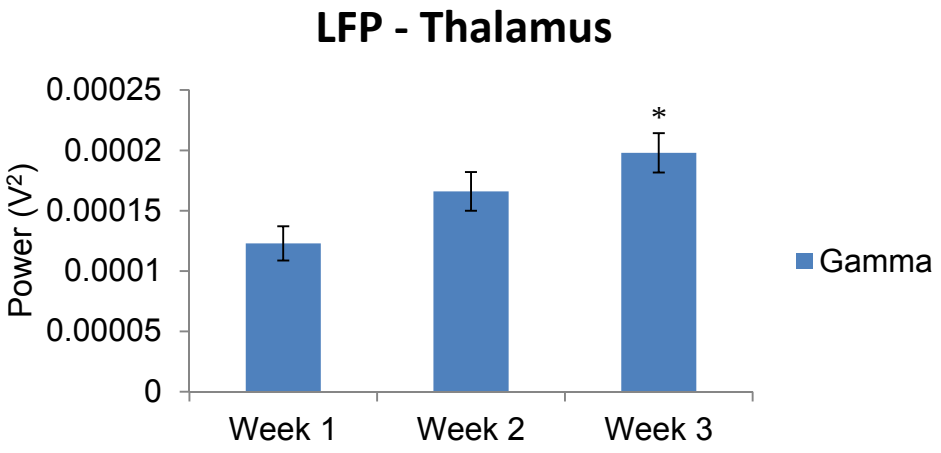

Figure 11. Power spectrum analysis results by week across condition.

Again, when using a repeated measure ANOVA to investigate data from only the first week of testing, there were significant between subject effects seen in thalamus. In the delta frequencies a main effect was seen in the VZV/MeWo condition $F(1,29) = 4.723$, $p = 0.038$, partial $\eta^2 = .140$, indicating a medium effect size. Pairwise comparisons indicate that the field potential for VZV (M = 0.173 ± 0.003) is smaller than for MeWo (M = 0.184 ± 0.003) across all conditions.

In theta frequencies a main effect was seen in the VZV/MeWo condition $F(1,29) = 4.085$, $p = 0.05$, partial $\eta^2 = .123$, indicating a medium effect size. Pairwise comparisons indicate that the field potential for VZV (M = 0.021 ± 0.002) is greater than for MeWo (M = 0.017 ± 0.001).

The alpha waveband was contained a significant main effect in the VZV/MeWo condition $F(1,29) = 4.701$, $p = 0.038$, partial $\eta^2 = .139$, indicating a medium effect size. Pairwise comparisons indicate that the field potential for VZV (M =0.006 ± 0.001) is greater than for MeWo (M = 0.004 ± 0.001). See Figure 12.

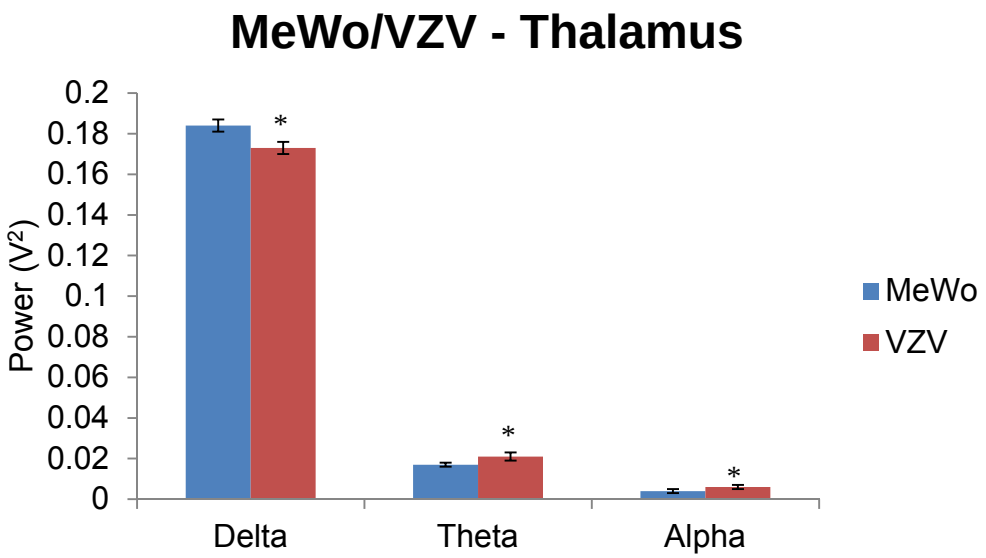

Figure 12. Power spectrum analysis comparing VZV to MeWo during the first week of testing.

In a manner similar to the ACC, when looking at VZV infection as a chronic PHN pain state in the VPM found no between group differences and neither hypothesis 1 nor 2 were supported. There was one overall difference in activity by week, indicating a change in VPM field potential over the course of the experiment. However, in looking at the field potential activity in the VPM during only the first week, a main effect of VZV/MeWo was seen in the delta, theta, and alpha bands. In the delta band, field potential activity was smaller in the VZV group

than in the MeWo group. In the theta and alpha bands, field potential activity was greater in the VZV group than in the MeWo group. In this case, the expectation that there will be greater LFP activity in the rats with VZV infected cells than in rats with MeWo uninfected cells(H1) is partially supported in the VPM.

3.2 Specific Aim 2

For the second specific aim, we endeavored to investigate the relationship between pain behavior and LFP activity in the thalamus and ACC. We expected that the rats in the VZV condition would exhibit more escape-avoidance behavior by spending more time in the light side of the chamber than rats in the MeWo group (H1). We also expected that, once the Gi protein is activated by CNO, the VZV infected rats will exhibit less escape-avoidance behavior than VZV infected rats in the saline group (H2). Finally, rats exhibiting more escape-avoidance behavior were expected to have a higher level of LFP activity than those who spend more time in the dark side of the chamber (H3).

Pain behavior was measured using the Placement Escape Avoidance Paradigm and results were analyzed using repeated measures ANOVA. Between group variables were the VZV/MeWo and CNO/saline groups while the within subject response was based on a percentage of time spent in the dark side of the chamber for each of the three weeks. The escape avoidance behavior was analyzed using two independent groups. In the first group, the behavior was analyzed while simultaneously recording the field potential in the VPM and ACC. This PEAP testing was conducted with the rat outfitted with the entire backpack and recording module, a combined weight of 38 grams. In the second group, the behavior was analyzed independently of field potential testing. This testing was conducted without the LFP recording module and backpack; all other experimental conditions were the same.

3.2.1 With backpack

When the rats outfitted with the backpack and LFP recording module were tested in the PEAP chamber, there were no significant differences in the between subject conditions of VZV/MeWo or CNO/saline relating to the percentage of time spent in the dark side of the chamber. There is a main effect of the within subject measure of week, $F(2,6) = 9.405$, $p = 0.14$, partial $\eta^2 = .758$, a highly robust effect size. Pairwise comparisons of this main effect show that during week one, the percentage of time spent in the dark side of the chamber (M = 90.251 ± 4.529) was significantly ($p = .002$) greater than the time spent in the dark during week three (M = 63.323 ± 5.671). See Figure 13. In this instance, none of the hypotheses regarding specific aim 2 can be supported.

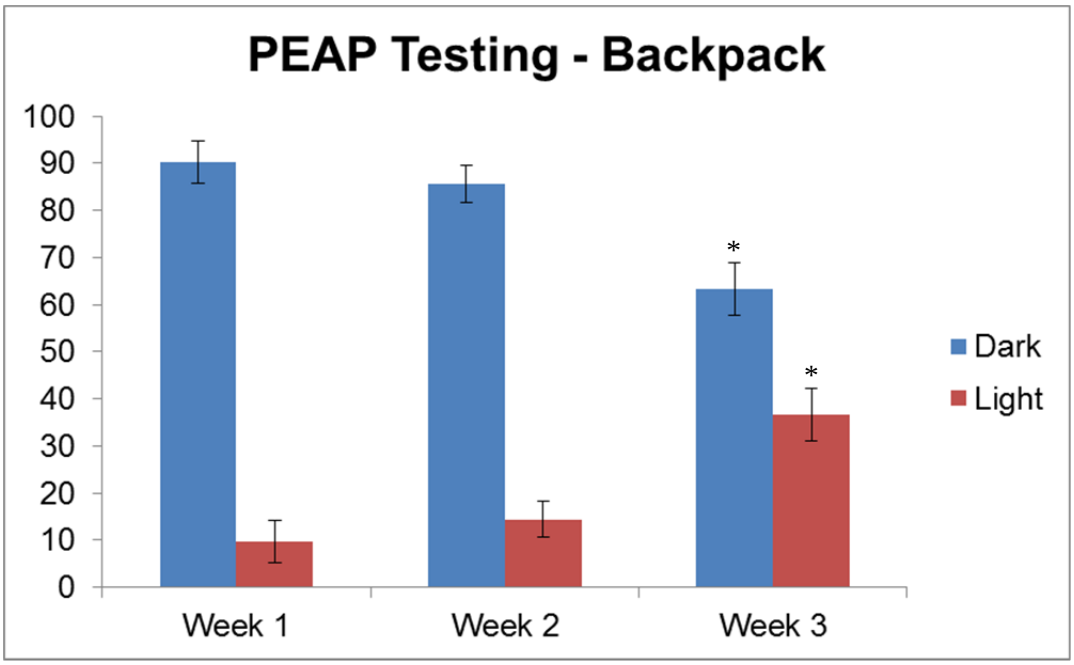

Figure 13. Percentage of time spent in dark side of the PEAP testing chamber compared to the light side of chamber by week across condition.

3.2.2 Without backpack

Rats tested in the PEAP chamber without the backpack and LFP recording module showed a main effect in both between subject groups. There was a main effect of VZV/MeWo, $F(1,14) = 54.82$, $p < 0.001$, partial $\eta^2 = .797$, a strong effect size. Using post hoc tests, it was found that the percentage of time rats infected with MeWo cells spent in the dark side of the chamber was significantly greater (M = 92.99 ± 2.92, $p < 0.001$) than the percentage of time that rats infected with VZV spend in this preferred condition (M = 62.41 ± 2.92). There was also a main effect of CNO/saline, $F(1,14) = 4.859$, $p = 0.045$, partial $\eta^2 = .258$, a large effect size. Post hoc testing showed that rats whose mGPCR was activated through CNO spent more time in the dark side of the chamber (M = 82.25 ± 2.75, $p = 0.045$) than the rats whose mGPCR remained inactivated in the saline group (M = 73.15 ± 3.08), indicating less escape avoidance behavior in the CNO condition. See Figure 14.

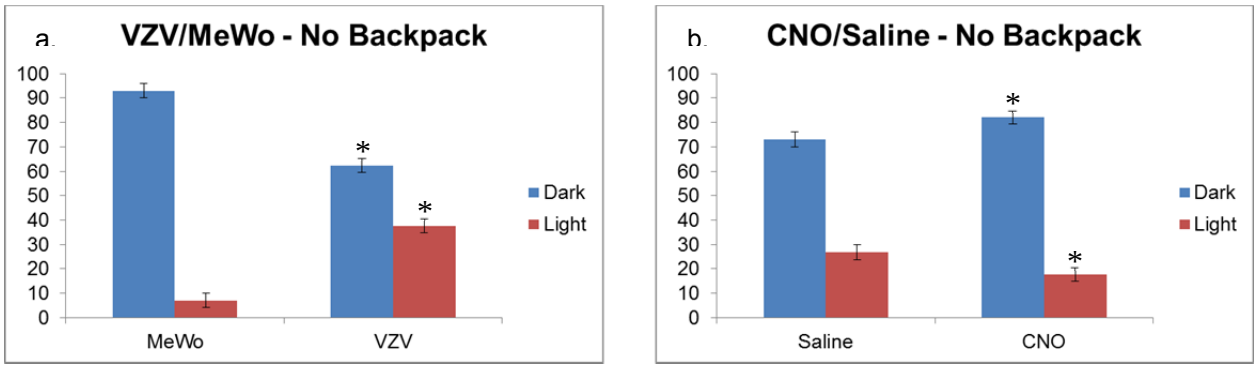

Figure 14. Percentage of time spent in dark side of the PEAP testing chamber compared to the light side of chamber while the rat was not wearing backpack and recording module. (a). VZV compared to MeWo conditions. (b). CNO compared to saline conditions.

Using data from the rats unencumbered by the backpack module, the first hypothesis was supported as the VZV infected cell rats exhibited more escape-avoidance behavior than the

MeWo uninfected cells. The second hypothesis stating that VZV infected rats would exhibit less escape-avoidance behavior when activated with CNO was partially supported in this set of data, as the rats in CNO condition exhibited less escape-avoidance behavior than the rats in the saline group. Unexpectedly this behavior was not limited to VZV infected rats and the CNO group spent more time in the preferred chamber across groups and time. Hypothesis 3, that the rats exhibiting more escape-avoidance behavior would demonstrate higher levels of LFP activity was not supported using this data set as there was no simultaneous LFP recording in this group of rats.

3.3 Coherence

While not a specific aim in this experiment, it may be notable to review the results of waveform correlations between the simultaneous recordings of the thalamus and anterior cingulate cortex over the course of the three week experiment. Coherence is the phase relationship between two separate signals at frequency index. Zero indicates no coherence, or no phase relationship while one indicates a strong coherence, or a constant phase relationship (Kramer, 2013). Coherence was investigated by using Spike2 software to create a waveform correlation comparing the raw electrode data trace between the VPM and ACC for each field potential recording. Significance between conditions at each week was then determined using a t-test to compare the means.

For the first week of testing, the mean correlation between ACC and VPM waveforms was 0.58. There was not a significant change in coherence between the baseline condition ($M = 0.59 \pm 0.055$) and the PEAP testing condition ($M = 0.58 \pm 0.053$) of week 1.

During the second week of testing the mean correlation between the two waveforms was 0.74. In this week there was a significant difference ($p < .001$) in coherence with the baseline condition having a higher level of waveform coherence ($M = 0.85 \pm 0.03$) than the PEAP testing condition ($M = 0.64 \pm 0.05$).

In the final week of testing the correlation between the raw trace waveforms was 0.75 coherence. In this week there was also a significant difference (p = 0.003) in the coherence of the ACC and VPM with the baseline condition (M = 0.83 ± 0.03) once again being more correlated than the PEAP testing condition (M = 0.67 ± 0.03). See Figure 15.

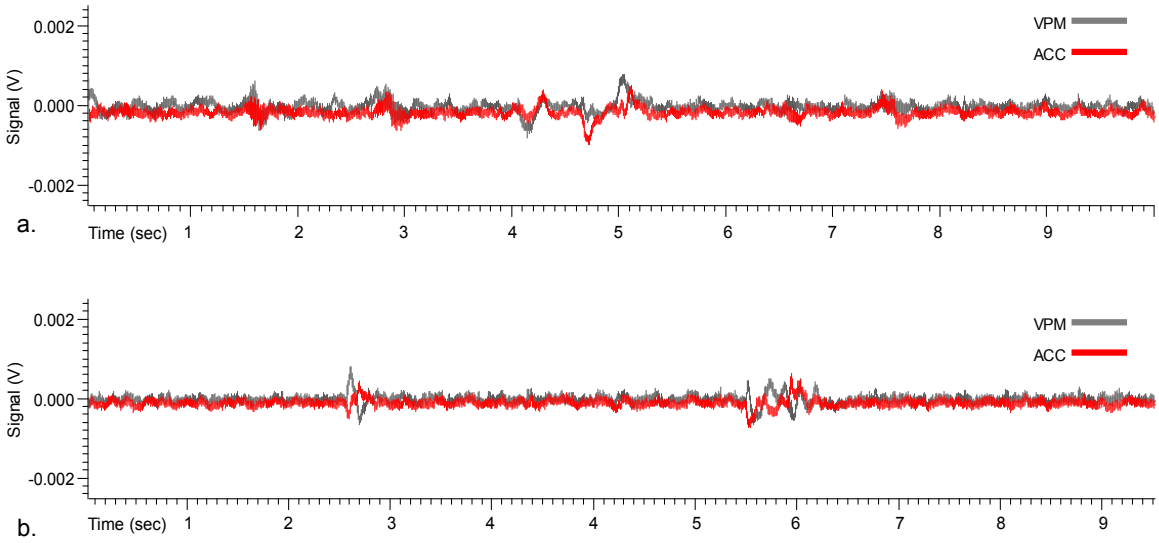

Figure 15. Representative raw traces of LFP recordings of VPM and ACC overlay to show coherence at (a). 0.54 coherence and at (b). 0.74 coherence.

Chapter 4

DISCUSSION

The experience of pain involves both sensory and affective components. In preliminary experiments this lab has shown that cellular modifications to the thalamus, the area most responsible for the sensory component of pain, caused changes in the experience of pain by observing that these modifications decreased pain behavior and also decreased the power of local field potential activity in the thalamus in a rat experiencing acute levels of pain. The goal of this study was to expand on this finding and offer a more complete look at the pain experience by including the affective dimension of pain in a more chronic pain condition, post herpetic neuralgia. It was expected that the same cellular modifications in the thalamus would cause changes in the anterior cingulate cortex, an area largely responsible for the affective dimension of the pain experience, and thereby change the experience of pain unpleasantness. If the same modification, activation of the mGPCR in the thalamus, could reduce both pain sensation and pain unpleasantness in a freely moving rat, it could ultimately lead to new and multidimensional therapies for the reduction in the experience of pain.

4.1 Pain Behavior

As rats are unable to verbalize their experience of pain, the most telling indicator of whether or not they are experiencing painful sensations and corresponding pain unpleasantness is their behavior. The PEAP chamber has been shown to measure behavior of pain unpleasantness (Labuda & Fuchs, 2000). In this study the PEAP testing clearly showed the aversive nature of VZV pain and the analgesic effect of CNO activation. Of significance is the fact that the animals infected with VZV exhibited more escape-avoidance behavior than the animals not infected with VZV. In line with our expectations, the rats in the saline condition also exhibited more escape-avoidance behavior than those rats that experienced the activation of

the mGPCR. While these clear results were shown in the PEAP testing of animals not outfitted with the backpack and recording module, an argument can be made that this is the more accurate outcome.

The animals tested in the PEAP chamber while simultaneously recording their electrophysiological responses were outfitted with a backpack and module that weighed 38 grams. This reflects an 11-13% increase of the body weight of the animals being tested. The only significant change in the group of animals outfitted with the backpack is that they exhibited less escape-avoidance behavior in the first week and more escape-avoidance behavior in the third week. As this change is across all conditions, it seems likely caused by the rat's habituation to the apparatus. Future behavior trials on rats outfitted with the recorded module should include a habituation phase for the animal to become accustomed to the device.

4.2 Local Field Potential

While local field potential 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.

Local field potential recordings are useful to provide measures of general electrical activity in localized brain areas and can give us insight into how different pathways interact with each other when multiple areas are simultaneously recorded. It should be noted, however, that local field potential alone may not provide the most complete picture of electrical activity in the VPM. The thalamic relay nuclei have two distinct patterns of firing, tonic and phasic. Tonic firing is more predictable and was long believed to be the only firing pattern at work in awake animals. However, recent data suggests that phasic, or bursting, mode is also important in ways that are not yet clearly understood yet appear to have markedly different consequences on thalamic behavior. Switching between tonic and phasic firing is irregular and occurs between

every 100 ms to several seconds. Tonic firing is also directly related to EPSP while burst firing, or low threshold spiking, is only indirectly related to EPSP (Sherman, 2001). This dual response mode in the thalamus is integral to how the thalamus processes information, especially how it projects and receives information from cortical areas (Mease et al, 2014). As these changes in firing patterns are not easily noted in LFP recording conditions, a better way to more fully explore events in the thalamus might be to record field potential simultaneously with spiking activity in order to investigate comparisons and any LFP changes due to firing patterns.

4.2.1 LFP Changes Over 3 Weeks

In considering the changes to local field potential that looked at the entire 3 week study, there were no significant changes found based on the experimental group into which the rats were placed, in any of the frequency bands. The changes found were the same across all conditions and only varied by the week in which the animals were tested.

The delta frequency bands in the ACC were higher at the beginning of testing than they were in week three and the gamma frequency bands were lower in week three than in week one for all the animals. As with the PEAP testing in the backpack, this is likely a result of learning and habituation. Only a small number of studies have looked at the low frequency delta band, but oscillations have been linked to cognitive processes such as behavioral inhibition and attention to emotionally salient environmental stimuli (Harmony, 2013; Nacher et al, 2013). With typically small fluctuations, gamma power is most often elevated during working memory and learning (Whittaker, 2011; Jia & Kohn, 2011).

In the VPM gamma frequency bands increased in power as the weeks progressed. As this band has been associated with learning, it may be interesting to investigate this further in relationship with the concept of thalamocortical coherence and feedback from the ACC to the VPM. This will be discussed more fully in relation to coherence.

4.2.2 LFP Changes in Week One Alone

The three week, longitudinal study showed no significant field potential differences between the experimental and control conditions at any frequency band. Further investigation was made into the individual testing weeks to determine if there were between subject differences at any one given time point. It was determined that the first week of testing showed differences in both the ACC and the VPM. In the ACC, the activation of mGPCR through CNO demonstrated less LFP power than the saline condition in the delta, theta, and alpha frequency wavebands.

In the VPM, there were differences between VZV infection and MeWo control conditions in the delta, theta, and alpha frequencies. In the delta frequency, the LFP power was lower in the VZV condition compared to MeWo. In the theta and alpha frequencies, LFP power was higher in the VZV condition.

Theta and alpha frequencies have been researched in terms of long range, integrative processes, especially in top down processing when the frequencies have high synchronization (Palva & Palva, 2013; vonStein & Sarnthein, 2000).

It might be tempting to try to interpret the lack of longitudinal significance for local field potential changes due to VZV/MeWo or CNO/Saline conditions in relation to the between group changes seen throughout the three weeks of pain behavior testing by assuming that VZV reduced in potency over the weeks, as we know the virus does not replicate well outside of humans (Guedon, 2015). However, other studies have shown pain behavior in VZV lasting longer than 21 days (Fleetwood-Walker et al, 1999, Garry et al, 2005, Stinson et al, 2016). We ourselves saw significance in longitudinal pain behavior in the animals that were not recorded for field potential. In this group, while the rats were cannulated and the VPM was infused with AAV8 containing mGPCR, no electrodes were implanted. The electrodes, larger in diameter than the infusion cannula, were implanted into the group in which no behavioral changes were observed due to condition. It is possible that the insertion of the bipolar electrodes caused

tissue damage within the brain. This may have elicited an immune response, limiting the replication of the AAV8 virus and diminishing the amount of mGPCR seen at the cellular level. More likely, as we do see electrophysiological between group effects during week one, any tissue damage would have could have caused scarring and interfered with synaptic activity as the scar tissue developed (Groothuis et al, 2014; Polikov, Tresco, & Reichert, 2005). Histological examination could be done to determine if scar tissue developed near the electrodes. If so, experimentation with electrode size and placement is warranted to reduce scarring in longitudinal studies.

4.3 Thalamocortical Coherence

In the 1981, Bouyer et al proposed (however unlikely) that the thalamic-cortical pathway might act as more of a loop, with projections of the thalamus not only affecting cortical activity, but subsequent changes to cortical layers providing feedback changing how the thalamus processes sensory information (Bouyer et al, 1981; DaSilva et al, 1980). Recent studies have found these ideas to be prescient, thalamocortical feedback has been shown to shape and sharpen receptive fields in the thalamus. This feedback has also been indicated in increasing thalamic responses to nociception (Wang et al, 2007; Briggs & Usrey, 2008).

Given the large effect sizes in the activity change of the delta, beta, and gamma frequency bands as the experiment progressed, regardless of condition, it seems important to look at the strength of VPM-ACC coherence. Changing levels of coherence may indicate a restructuring of the connections between the thalamic and cortical layers (Kramer, 2013). The first week of testing resulted in the lowest thalamocortical coherence, 0.58 with no difference in coherence between the baseline and pain conditions. However, during the second and third week, there is a significant jump in coherence; 0.74 and 0.75 respectively. Notably there is also a change in the waveform coherence between the conditions, with the baseline condition at a high level of coherence (0.85, 0.83) and the pain condition exhibiting lower coherence (0.64,

0.67). During the first week, the rat does not know what to expect in either chamber. Given that the VPM involves the sensory component of pain and the ACC involved the unpleasant component, the level of coherence seen may indeed be that he is in unfamiliar circumstances and outfitted in an uncomfortable device. Notable in this week is that the coherence does not change regardless of condition.

During the second and third weeks, the overall ACC-VPM coherence is much higher, which could indicate a strengthening of the thalamic – cortical connections. See Figure 16. Interesting here is the change in coherence between conditions. This change could also reflect thalamocortical feedback that has been shown to alter thalamic response in a pain condition (Briggs & Usrey, 2008).

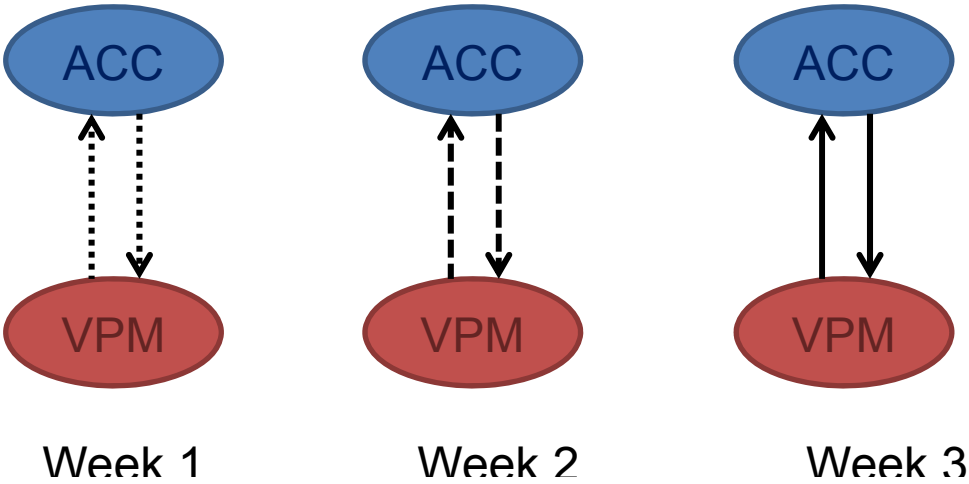

Figure 16. Representation of changing reciprocal relationship between ACC and VPM over time.

Chapter 5

CONCLUSION

While this study did not confirm all expectations, it has offered some interesting insight into several novel methods of inquiry in the pursuit of understanding post herpetic neuralgia as a multidimensional experience of pain and the reciprocal relationship of pain sensations and pain unpleasantness.

This study was able to further validate the novel work of Stinson et al (2016) in developing a rat model of post herpetic neuralgia in the orofacial region, which affects many patients and whose mechanisms are, as of yet, poorly understood. Rats in the VZV condition spent more time attempting to avoid stimulation to their injected whisker pad than did rats that were only injected with MeWo.

Bridging molecular events such as the activation of mGPCR through CNO to animal pain behavior is the first step to understanding the mechanisms involved in this pain experience. It was clear that rats injected with CNO spent less time trying to escape the mechanical nociception, even those in the virus condition. While we were not able to conclusively tie this behavior modification to changes in the local field potential, we have noted potential areas in which we can investigate in the future; refining instruments and technique, especially comparing thalamic field potential to spontaneous firing patterns, and investigating the effect of mGPCR on field potential changes and coherence in connected substrates.

Armed with evidence of behavioral changes in the presence of activated mGPCR, we are ready to continue pursuing the mechanism of how protein modification in the thalamus can act as an analgesic. A major area of future investigation should be into thalamocortical coherence. Recent studies have shown the importance of thalamocortical coherence on the pain experience, especially in the theta and alpha frequencies (Stern et al, 2006; Sarnthein & Jeanmonod, 2008; Walton et al, 2010). We are able to provide evidence of a reciprocal relationship between the ACC and VPM by looking at the changes to the overall levels of

coherence over time. Of future interest may be how the coherence changes specifically in the theta and alpha bands, and also the role of other important areas such as the S1, hippocampus, and amygdala play on the rhythmic/dysrhythmic patterns of thalamocortical coherence. Investigating these changes further might be an important next step to begin to discover the link between molecular and behavioral changes in the pain experience.

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