ELECTROPHYSIOLOGICAL ANALYSIS ASSESSING THE ANALGESIC NATURE OF THE LATERAL HABENULA (LHb)

by

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ABSTRACT

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Local field potential (LFP) is a collective neural signal of all the synaptic activity occurring at a specific area of the brain, thereby offering a unique insight into how the brain functions. The habenula is involved in the pain pathway and decision-making. Located above the thalamus, it has been proposed to function with nucleus accumbens and periacqueductal gray (PAG) along with other regions in a descending pain modulation pathway. The main purpose of the present study was to determine the contribution of LHb to the nociceptive input and the effect of activation on antinociception. One week after implantation of electrode in the LHb in adult Sprague Dawley male rats (n=16), formalin was injected in the right hind paw, LFP recordings were recorded at baseline and post-formalin. Electrical stimulation was delivered to the LHb, and LFP were recorded in these freely moving animals. Animals were also subjected to mechanical and thermal paw withdrawal tests to assess the change of nociception and LFP responses. LFP were analyzed by power spectrum analysis. The results showed that: (1) Behaviorally, significant decrease in paw withdrawal threshold and latency were observed after formalin injections ($p < .05$), indicating increase in nociception.

Interestingly, electrical stimulation of LHb has significantly reversed the phenomena, suggesting an antinociceptive role by LHb. (2) Simultaneously, we observed significant increase for the LFP powers during formalin period ($p < .05$) in response to mechanical and thermal stimuli, which were reduced by electrical stimulation of LHb ($p < .05$). (3) There was a trend of significant increase for all the frequency bands following formalin injection ($p < .05$) comparing to the baseline. The possible explanation is that the increased activity in habenula is due to increased inputs from the lateral hypothalamus and the spinal cord, which are part of the neural circuitry involved in pain transmission. (4) Following LHb electrical stimulation, significant decreases of the LFP power in different frequency bands were also observed ($p < .05$). Since LHb projects further into ventral tegmental area (VTA, the substantia nigra (SNc), dorsal raphe, and PAG, which are important structures in descending modulation of pain, electrical stimulation of habenula may activate the descending inhibitory system to achieve the analgesic effect. In conclusion, formalin-induced inflammatory nociception increases the LFP recordings in the habenula while electrically stimulating this region induce an antinociceptive effect which was also observed via both behavioral and electrophysiological tests.

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Chapter 1
INTRODUCTION

The International Association for the Study of Pain defines pain as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Jarvis & Boyce-Rustay, 2009). Pain is deemed to have a significant role as it helps to alert the organism towards the presence of any prevalent danger, however this very mechanism fails to serve this very purpose when dealing with chronic pain. According to Gaskin and Richard, 2012, the total cost of pain in the USA is $560 to $635 billion annually. The incremental cost that the health care department went under due to pain ranged from $261 to $300 billion and the total cost of loss of productivity, resulting from reduced sleep, reduced quality of life and reduced social interactions, due to pain ranged from $299 to $334 billion making research focused on pain of utmost importance (McCarberg & Billington, 2006).

Pain is one the primary reason why physician care is sought after. Pain is an extremely complex paradigm and most of the theory related to pain has been derived from rodent behavioral analysis that heavily relied on the animal's sensory organs, hence expanding the knowledge related to pain and its pathways is necessary and should not be just restricted to behavioral analysis. A combination of sensory, emotional as well as various evaluative components together formulate into pain (Melzack & Casey, 1968). As aforementioned pain is a relative and everyone has different degrees of pain tolerance, regardless of which presence of pain affects the psychological and as well as emotional state of an individual resulting in a significant health ailment. Continued emotional and psychological distress often leads to

depression, fear and anxiety (Gatchel, Peng, Peters, Fuchs, & Turk, 2007). The affect resulting from pain has been defined as an interpretation of the unwanted and unpleasant stimuli (Craig, 2003), hence there is a need for research focusing on the treatment of pain as well as comprehending the effects of emotional and psychological distress on the patients.

Animal models of pain have been imperative for studying how pain is processed in the nervous system and have been widely used for research for decades (Craig A.D.,2004). Animal models of pain are primarily applied in rodents. Pain models in rodents consist of application of stimulation to the nervous system that provides the researcher with an opportunity to study the subsequent processing in the brain. There are many rodent models that mimic clinical pain conditions (Barrot, 2012; Gregory et al., 2013; Le Bars, Gozariu, & Cadden, 2001; Wang & Wang, 2003).

For the purposes of this experiment we will be using the inflammatory model of pain. Inflammation can be evoked by variety of chemical agents such as carrageenan (Pratt, Fuchs & Saluka, 2013), Capsaicin (Sluka K.A., 2002), CFA (Cobos et al., 2012) and formalin (Dubuisson & Dennis, 1977). We will be using formalin to induce inflammatory state in the animal hind paw.

1.1 Complex Nature of Pain

Traditionally it was viewed that pain is only dominated by sensory processing until the 1960 (Gatchel, Peng, Peters, Fuchs, & Turk, 2007), however there was a shift in paradigm around the year 1965 where Melzack and Wall, introduced the gate control theory. This ushered in the modern era of pain research dealing with both psychological as well as physiological components.

The pain processes have been studied and there are many factors that should be considered when dealing with the history as well as the pre-clinical assessment. All the noxious stimuli ascending from the sensory organs via the sensory neurons travel through the dorsal horn of the spinal cord; there are several projections that arise from the thalamus ascending towards the somatosensory cortex. Projections from the VPL (ventral posterolateral nucleus), VPM (ventral posteromedial nucleus) extend into the cortical layers of the postcentral gyrus. Parallel projections from the posterior medial nucleus as well as VPN (ventral posterior nucleus) innervates S1. VPN also projects into the postcentral gyrus conveying sensory information (touch and pain). The thalamocortical pathway has parallel connections to the S1 and S2 that help process nociceptive as well as non-nociceptive information (Craig A.D., 2004). A recent novel study by Uhelski, Davis, & Fuchs, 2012, had found out that processing of pain affect via the limbic system is functionally different from the process of sensory information and even in the absence of any information pertaining to the location as well as the intensity of the stimuli, noxious stimuli can still be perceived as unpleasant, this just reinforces the extremely complex nature of pain and pain processing.

1.2 Quantification of Pain

Normally pain “affect” is qualitative in nature however this can be read and interpreted quantitatively in the laboratory. There are numerous tests utilized to quantify pain within the laboratory setting. For example, measuring the escape and avoidance behavior in animals is often used as a representative measure for the pain affect. Stimulating the painful areas often motivates these behaviors in animals; followed by some form of motivation to

end the pain is provided, which is then measured (LaBuda & Fuchs, 2000). One of the best examples of this is the conditioned place avoidance (CPA) that examines how when an unconditioned stimulus administered to a specific area at a specific time, induces avoidance behavior in the same animal later.

There are other avenues of acquiring neuronal information, LFP (local field potential), single cell dorsal horn recording, EEG and ECOG to name a few. Dorsal horn neurons receive nociceptive as well as non-nociceptive input from the primary afferent neurons innervating skin and tissues. This information is further relayed to several areas of the brain, including habenula, hypothalamus and the frontal cortex (Guan Y et al., 2006). A fine tip tungsten microelectrode is advanced using a micro-positioner, placement of the microelectrode on a single neuron is confirmed by an audio monitor and then a real-time data acquisition software is used to measure the action potential waveform.

Another electrophysiological monitoring system that places electrodes directly on the exposed area of the brain to record the electrical activity is Electrocorticography (ECoG). This is another invasive procedure that mostly requires surgery, though it can be performed extra-operatively as well (Chernecky & Berge, 2008). A noninvasive option that records the electrical activity of the brain as well is known as Electroencephalography (EEG). Electrodes are fixed on a cap that is placed along the scalp. These electrodes measure the voltage fluctuations in the brain caused by ionic exchange (Chernecky & Berge, 2008)

A technique that has become popular over the past 10 years is local field potential (LFP) recording. LFP is a collective neural signal usually

obtained by passing it through a low pass filter (low-pass frequency ranging from 100-300 Hz). This collective neural signal comprising of the extracellular neuronal electrical potential is recording utilizing electrodes implanted intracranially (Mazzoni, Logothetis and Panzeri, 2012). The local field potential spectrum consists of different frequency bands, Delta 0-4 Hz, Theta 4-8 Hz, Alpha 8-12 Hz, Beta 13-29 Hz, and Gamma 30-100 Hz. These can be separated into the individual frequencies and further analyzed. LFP was neglected for a few decades mainly because the research was focused on obtaining isolated action potential from individual neurons, mainly using in-vivo techniques, however, there has been a renewed interest in studying cortical function using LFP recordings to understand the dynamics and functional capabilities of neuronal circuitry under various experimental conditions. Recent experiments have used LFP recording in tandem with behavior analysis to support their hypotheses (Mazzoni, Logothetis and Panzeri, 2012).

There are also other behavioral tests present to try and quantify nociception, for example the Plantar test for mice also known as Hargreaves' Method. Hargreaves' method is a nociceptive assay that involves using a high intensity light (infrared) beam, aimed at the hind paw of the animal. Paw withdrawal is observed when optimal heat stimulus is achieved. Using a photoelectric-sensitive device commercially available, the withdrawal response gets measured in real time. The latency of the paw withdrawal is measured at different time intervals after injecting an inflammatory agent (for e.g. carrageenan) (Allen and Yaksh, 2004).

Another test for quantifying nociception is the Von Frey Mechanical Paw Withdrawal Test. Von Frey filaments applying varied force are used. The animals are placed on a wire mesh like floor inside the plexiglass cage and stimuli is applied to the plantar surface of the hind limb (paw). If a lack of response is observed, then a higher value force stimulus is applied but in case of a response a lower force stimulus is applied. Mechanical paw withdrawal threshold scores (MPWT) are calculated using a formula that utilizes the pattern of the response, the force of the initial as well as the last response was observe. Lower scores indicate sensitivity to nociception whereas higher scores indicate reduced sensitivity to the nociception.

For the purposes of this experiment I will be concentrating on utilizing LFP for electrophysiological analysis and MPWT and Thermal Paw Withdrawal test for behavioral data.

Chapter 2
Habenular Complex

The two major pathways connecting the limbic forebrain to the midbrain and the hindbrain are the DCC (dorsal diencephalic conduction) and the MFB (medial forebrain bundle). These two pathways appear to share sources of afferent inputs as well as efferent targets and there is an overlap in their physiology and function (Bianco & Wilson, 2009). The DCC has three major components, the habenula the stria medullaris (SM) through which inputs from the forebrain arrive at the habenula and the Fasciculus Retroflexus (FR), which is the main efferent pathway from the habenula towards the midbrain and hindbrain. While the MFB consists of the Olfactory Bulb, Periamygdaloid Region, Septal nuclei and the Ventral Tegmental Area (VTA) (Bianco & Wilson, 2009). Many forebrain areas are innervated by the Dopamine (DA) neurons projecting from the ventral tegmental area (VTA) modulating behavior and cognitive functions. There are also efferent innervations form the habenula into the VTA, inhibiting the dopaminergic VTA activity (Bianco & Wilson, 2009). Thus, we can observe that the habenular nuclei is an important structure connecting the forebrain to the mid and hindbrain, and hence has gained popularity in neuroscience research in the last two decades.

The habenular connectivity is extremely complex and is observed across all species, however it is larger compared to the brain in subhuman mammals (Boulos, Darcq, & Kieffer, 2017). Figure 1 illustrates the position while Figure 2 exhibits some of the afferent as well as efferent connections of the habenula.

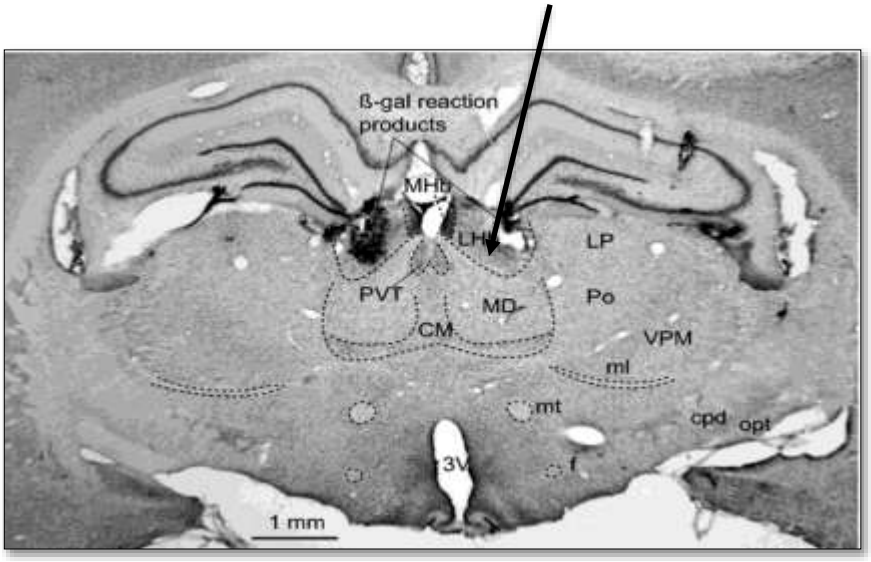

Figure 1 Location of the Lateral Habenula.The location of the rat lateral habenula (LHb) show by the arrow with the lateral located medial habenula (MHb). The other structures shown are the PVT (paraventricular nucleus), mediodorsal thalamus (MD), centrum medianum, (CM), ventral posteromedial nucleus (VPM). Adapted without permission from (http://physreports.physiology.org/content/physreports/3/2/e12297/F1.large.jpg)

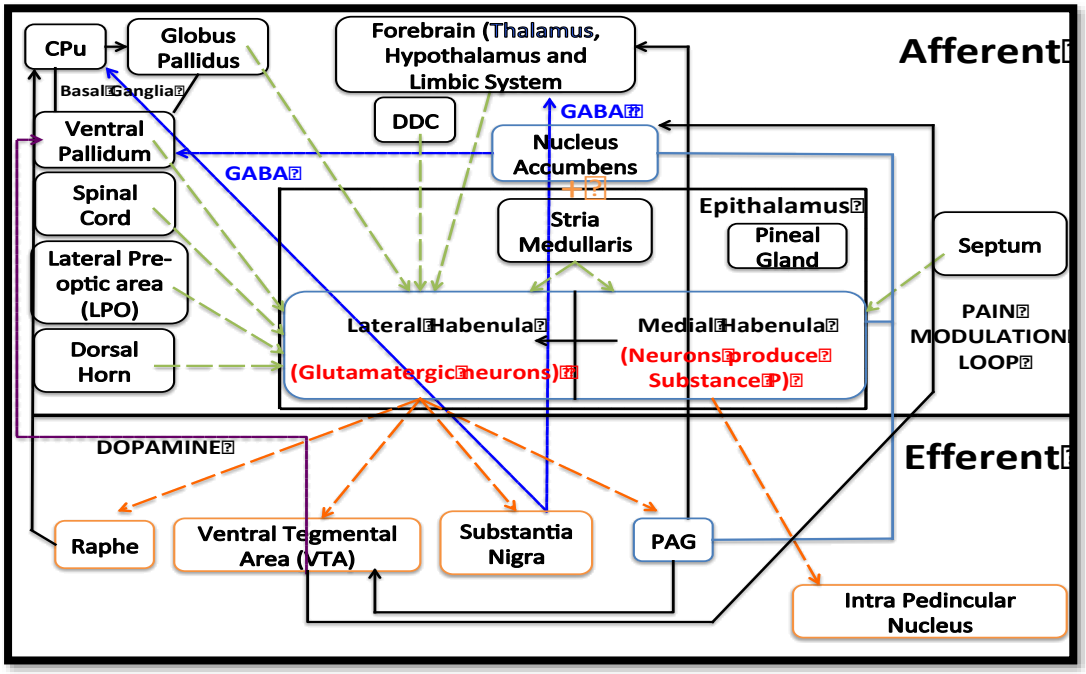

Figure 2 Afferent and Efferent connections of the habenula. The green dashed lines represent the afferent connections towards the lateral and medial habenula while the orange dashed lines are the efferent connections from the habenular complex. The purple dashed lines represent the regions having reciprocal connections with the habenular complex releasing GABA while the black dashed lines represent dopamine releasing brain areas.

Located above the thalamus, the habenula has been proposed to function with nucleus accumbens and periacqueductal gray (PAG) along with other regions in a descending pain modulation pathway (Shelton, Becerra, & Borsook, 2012). Habenula has also been known to be evolved along with the pineal gland with which it maintains reciprocal connections. The habenula is made of two nuclei (medial and lateral) along with a habenular commissure. The habenula also receives afferent connections from the limbic system and the basal ganglia, through the stria medullaris (Figure 2). The habenula with the stria medullaris and the pineal gland together form a structure known as the epithalamus.

Habenular nuclear complex is responsible for a wide variety of functions ranging from sleep-wake cycle, homeostasis, pain and stress processing and is also involved in reproductive behavior, addiction, depression as well as avoidance learning (Andres, Von During, & Veh, 1999) (Hikosaka O., 2010).

Habenula has been postulated to be an important structure for the REM sleep. It has also been hypothesized that habenula synthesizes melatonin just like the pineal gland and may be responsible for generation as well as maintaining the state of hibernation (Valjakka et al., 1998).

Habenula also has a role to play in homeostasis (Zhang & Gao, 2016), punishment avoidance (Dafny & Qiao, 1990), addiction (Velasquez, Molfese & Salas, 2016), as well reward based decision making (Lecourtier and Kelly, 2005) relies on the lateral habenula’s dopamine inhibiting activity in the VTA and SNc, both of which are involved in dopamine release. Habenular lesions have induced motor hyperactivity in rats and this may be due to the inactivity

of the lateral habenula in inhibiting dopamine neurons (Lee and Huang, 1988). The lateral habenula (LHb) has also been reported to be involved in avoidance learning, related any aversive stimuli mainly under stressful conditions (Thorton & Bradbury, 1989). In another experiment in 1990 by Thorton, Bradbury, and Davies, animals with habenular lesions exhibited lack of avoidance learning, and they also redundantly repeated the high stress task they were forcefully subjected to. In a third experiment in 1991, Thorton and Davies also observed that habenular lesions affected the ability of the animals to vary their strategies in response to different stress conditions. It was also observed that the same type of animals with habenular lesions implemented non-adaptive strategies when subjected to operant schedules, in which a response pattern was essential for gaining optimal reinforcement (Haack et al., 2014). During Morris water test, animals with habenular lesions exhibited diminished ability to escape the maze, cementing the importance of habenula in decision-making and adaptive strategy, especially under stress conditions (Lecourtier and Kelly, 2005).

Stress related activation of the lateral habenula has been postulated due to its afferent inputs from the limbic system (Borca and medial frontal cortex) as well as from the dopamine neurons from the VTA. A typical physiological response to stress is suppression of the motor activity and is carried out by the lateral habenula's dopamine inhibiting activity in the VTA and SNc (Hikosaka O., 2010).

Some experiments have also reported sex differences, i.e., habenular stimulations produce different effects in male and female rats (Terenzi, Guimaraes, & Prado, 1990). Stimulation of the habenula in male rats

produced immediate analgesic affects that dispelled after 15 minutes (Terenzi, Guimaraes, & Prado, 1990), whereas in female rats the analgesic effects were slowly developed over a period of 60-80 minutes and lasted for 3 hours and longer (Terenzi & Prado, 1990). One possible explanation of this is the involvement of habenular complex in the estrous cycle in female rats (Kobayashi et al., 2013).

Dopamine hypoactivity along with abnormal circadian rhythm is mainly responsible for depression. The lateral habenula is known to innervate VTA and SNc, regions responsible for the release of dopamine and direct connections between the habenula and the pineal gland have been postulated due to its evolution with the pineal gland (responsible for circadian rhythm). Hence the habenula has been reported to be involved in depression.

There has been a lot of research carried out to understand the neural substrates of pain with the aim to gain insight into the pain pathway. The habenula has been known to be involved in the pain pathway as it has afferent inputs from the lamina I of the dorsal horn, the trigeminal nucleus as well as the hypothalamus (which is the main gatekeeper of the pain pathway) (Craig, 2004, Shelton, Becerra, & Borsook, 2012, Hikosaka O., 2010). The medial habenula receives afferent inputs from the limbic brain regions that are directly or indirectly innervated by the cerebral cortex (substantia innominata, and diagonal band, parts of the extended amygdala, lateral hypothalamic as well as the lateral preoptic areas, ventral pallidum and the nucleus of the stria terminalis) (Craig, 2004). The lateral habenula is primarily innervated by the basal ganglia, particularly by the globus pallidus (known as entopeduncular nucleus in rodents) via the striatum. Through these connections and parallel

circuits, extensive information is processed by the cerebral cortex ultimately reaching the LHb (Becerra, & Borsook, 2011). Hence lateral habenula has been regarded as the point of convergence for the limbic and basal ganglia circuitry and could very well play a key role in pain processing.

The lateral habenula (LHb) has efferent projections into the ventral tegmental area (VTA), substantia nigra pars compacta (SNc), involved in dopamine release and innervates the median (MRN) and dorsal raphe nucleus (DRN), that release serotonin (Figure 2) (Takase et al., 2004). Dopamine is mainly responsible for reward processing while serotonin is an important neurotransmitter mainly involved in modulation of pain, mood regulation, sleep and appetite. The lateral habenula also has efferent connections into the PAG (periaqueductal gray) which is regarded as the primary control center for descending modulation of pain. Recent studies have shown that the rostromedial tegmental nucleus (RMTg) (releasing GABA) principally mediates the efferent connections of the LHb (Shelton, Becerra, & Borsook, 2012). Lateral Habenula also aids in decision-making process as studies have suggested a possible link between suppressing motor behavior and the habenula (Shelton, Becerra, & Borsook, 2012). Animals with habenular lesions often exhibited rash, hyperactive and distractible behavior, one of the possible reasons for this is its connections of the LHb to dopamine neurons in the SNc and the VTA (Lee and Huang, 1988). The medial habenula (MHb) efferently connects to the interpeduncular nucleus (IPN), which has been known to project to MRN and DRN (median and dorsal raphe nuclei) in addition to other areas, hence it can be postulated that the MHb controls the release of serotonin (Takase et al., 2004).

There are lots of mu-opioids receptors present within the habenula and morphine injections into this area have been documented to produce analgesic effects (Cohen & Melzack, 1985). In another experiment carried out in 1987 by Mahieux and Benabid, it was demonstrated that electrical stimulations to the habenula produced analgesic effects, however during the tail flick test, this effect was reversed, mainly by administering naloxone (opioid antagonist). Similar analgesic results were observed when the habenula was electrically stimulated during formalin tests, leading to reduction of observable pain behavior usually associated with formalin in rats, for e.g. licking of the paw, paw biting and paw elevation (Cohen & Melzack, 1985). There is also a direct correlation between the frequency of electrical stimulations and the time the analgesic effect is observed, i.e. animals stimulated more frequently experience extended periods of analgesia (Cohen & Melzack, 1985). Recent experiments have indicated the role of habenula in depression (Lawson et al., 2016) and drug addiction (Velasquez, Molfese & Salas, 2014).

Thus, the role of the habenular complex in processing pain is extremely complex and is dependent on a variety of factors (Fuchs & Cox, 1993). Habenula has not only a significant role to play in modulating the pain pathway but is also involved in a variety of functions as we have seen earlier, making it a very interesting region to study. Recent experiments have observed that the lateral habenular complex is larger on the left side of the brain for both males and females and this is not true for the medial habenula (Ahumada-Galleguillos et al., 2016). Due to this reason, the lateral habenula on the left side will be the region of interest for this experiment.

Chapter 3
PRELIMINARY STUDY

The focus of the future experiment was acquiring LFP readings from the habenula, and the only way to be certain, that optimal data will be acquired for all future experiments, is by establishing the functionality of the recording module and learning the complex and sensitive stereotaxic animal surgery, hence a preliminary study was executed focusing on thalamus/VPM (ventral posteromedial nucleus) and the trigeminal ganglion. The **ultimate goal** of this experiment was to lay the foundation for future studies pertaining to bilateral local field potential recordings and to prove the recording functionality of the recording module.

To achieve this, VPM recordings in anesthetized animals during variable trigeminal nerve stimulation in the jaw were carried out to establish a relationship between stimulation and VPM. This helped determining the local field potential in the VPM and how it relays nociceptive stimuli as well as modulation of those stimuli. Bilateral recordings were executed by implanting two recording electrodes in the VPM in an anesthetized animal, during variable trigeminal nerve stimulation in the jaw. The electrical stimulation was given at different frequencies ranging from (0.2 Hz – 50 Hz) and the electrical stimulation intensity ranged from 0.5 V – 20 V resp. The **hypothesis** of the experiment was that differences would be observed in the contralateral and ipsilateral recordings of the VPM with the contralateral side exhibiting higher readings thus establishing the functionality of the recording module. The **Specific Aim** was to observe differences in the contralateral and ipsilateral recordings of the VPM with the contralateral side exhibiting higher readings

when the trigeminal ganglion is electrically stimulated. The purpose of the experiment

The **purpose** of this experiment was to establish the functionality of the recording module and to apply the techniques of stereotaxic surgery and local field potential recordings for future experiments.

Adult Sprague Dawley male rats aged 4-6 months old were taken at random from the University of Texas at Arlington vivarium. Rats were kept on a 12-hour light/dark cycle, with testing occurring during the light cycle from 7:30 a.m. to 7:30 p.m. Subjects had access to food and water ad libitum. Subjects were housed in cages of 2-4 until after electrode implantation; all procedures had the approval of the UTA Institutional Care and Use Committee and followed the ethical guidelines for pain experiments in animals (Donoghue and Kadereit, 1992). Before the recording electrodes were implanted, holes were drilled at the following co-ordinates, -3.5 mm (Bregma), 3mm (lateral) and 6.4 mm depth, under isoflourane anesthesia. An additional burr hole was created in the skull for the placement of a separate screw attached to a wire acting as the ground and reference. Syringes were used to target the trigeminal ganglion and stimulating crocodile clips were attached to these syringes to deliver the required electrical stimulation.

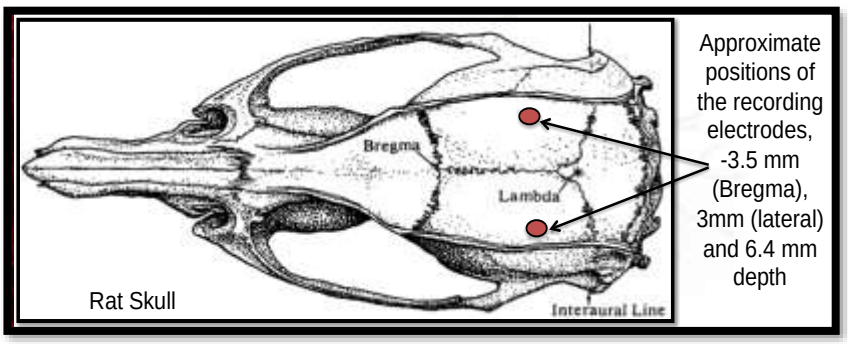

Figure 3 Approximate positions of the electrodes, preliminary study

The data acquisition and analysis were carried out as per Zuo et al., 2012 and Senapati et al., 2005. In short, the raw local field potential raw traces/raw data was recorded using MC_Rack data acquisition software. The recorded files were further imported into Spike 2 data analysis software. The specific time when the stimulations began, and end were prerecorded and using this time specific 10-second bins were selected in the raw trace for obtaining the power spectrums. A histogram was also obtained for each of the power spectrums ranging from 0-100 Hertz. The following frequency bands, (Delta 0-4 Hz, Theta 4-8 Hz, Alpha 8-12 Hz, Beta 13-29 Hz, and Gamma 30-100 Hz) were separated in the excel file. The mean of power at each of the aforementioned frequency was computed in excel and then graphed with their standard error of means. The same data was also imported into SPSS was further analysis. The analysis between the different frequency bands was conducted in SPSS using ANOVA followed by LSD post hoc tests. This helped determine if there were differences between the two hemispheres.

Our results showed that there was a significant difference in the local field potential recording, between the contralateral and ipsilateral side, for Delta band F (1, 7) = 13.68, p = .008, Wilk's Λ = 0.339, partial η^2 = .66 however no significant differences were observed for theta band (p = .450), Alpha band (p =.187), Beta band (p = .230) and Gamma band (p = .410). From these results, it was concluded that the recording LFP in the VPM could play a key role in identifying nociceptive signals.

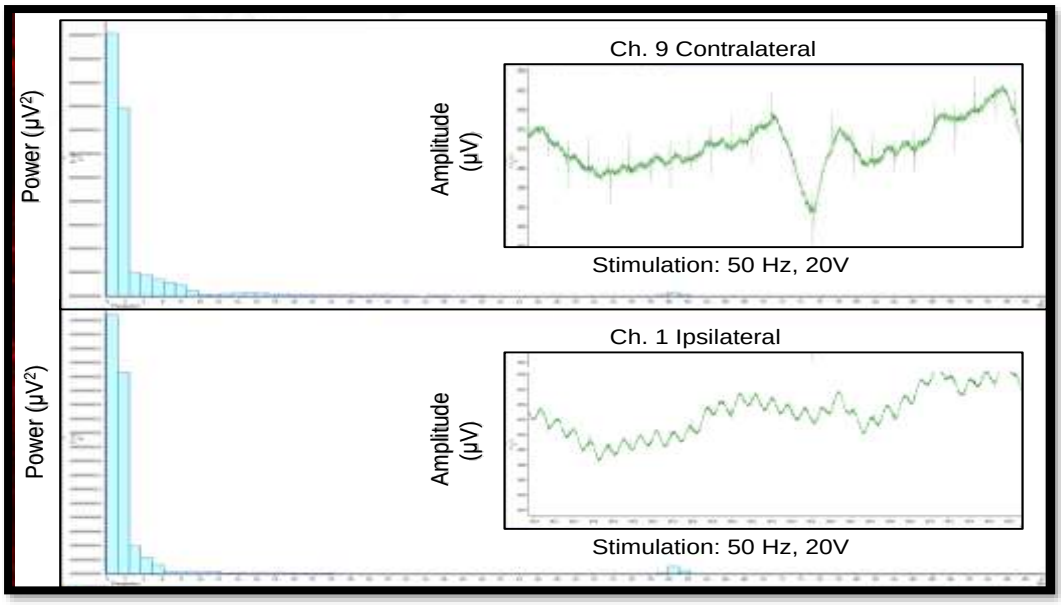

Figure 4 Raw Traces and Power Spectrum, Preliminary Study. Raw trace and power spectrums recording of the electrical stimulation, channel 9 (Contralateral) vs. channel 1 (Ipsilateral)

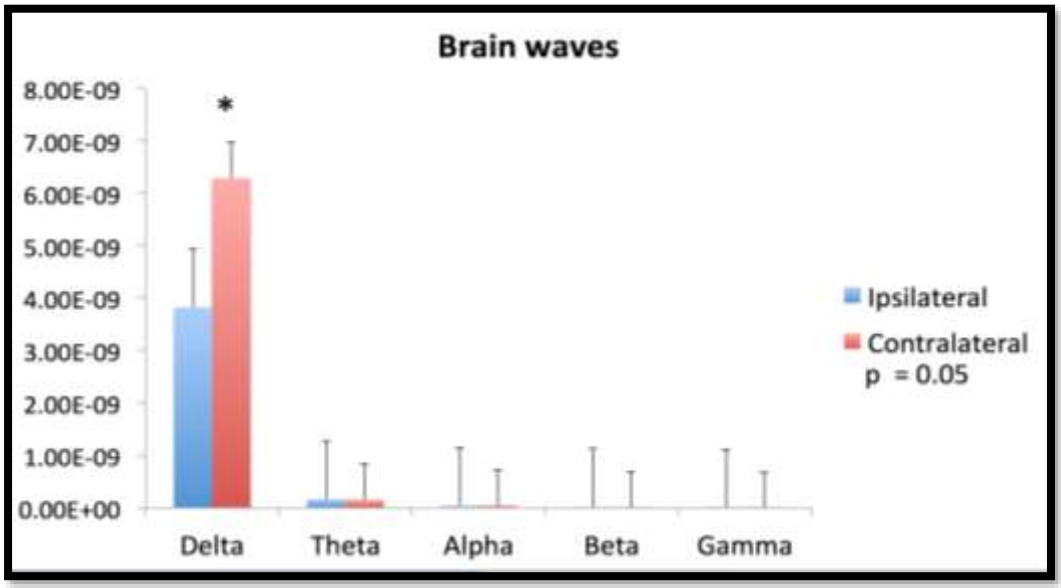

Figure 5 Differences between Contralateral and Ipsilateral sides, Preliminary Study. Bar graph representing the differences between contralateral and ipsilateral sides after electrical stimulation of the trigeminal ganglion with significant differences seen only in delta wave.

Chapter 4

Aims and Methodology

4.1 Specific Aims for Thesis Project

From the preclinical data and the literature review it was quite evident that habenula is an important region associated with pain and analgesia. Previous experiments have also established afferent inputs to the habenula from the lamina I of the dorsal horn as well as the trigeminal nucleus (Craig, 2003). There are also inputs to the habenula from the hypothalamus (a region involved with processing pain) (Goto, Canteras, Burns and Swanson, 2005). As noted earlier administering morphine lateral habenula have been known to produce analgesic effects. The **main purpose** of this present study will test the hypothesis of LHb's (lateral habenula) contribution to the affective element of pain using electrophysiological analysis. The **hypothesis** of the experiment will be that the local field potential readings of the habenula after formalin injections will be higher as compared to the local field potential readings recorded during the baseline readings and stimulating the habenula will produce analgesic effects reverting these readings back to normal, almost matching the baseline.

Specific Aim 1: To determine the electrophysiological activity in the habenula in response to formalin induced pain.

Specific Aim 2: To determine the antinociceptive effect of electrical stimulation of the lateral habenula.

The **rationale** for the experiment is as follows, as observed in the preliminary study stimulating the trigeminal ganglion increases the activity in the VPM, also previous studies (Fanselow & Nicolelis, 1999) have proved that stimulating the foot increases the activity in the VPM and the habenula being

another important region in the pain pathway it is hypothesized that the habenular activity will increase after injecting the foot with an inflammatory agent. As aforementioned the habenula has efferent connections to the VTA along with PAG (Shelton, Becerra & Borsook, 2012) (Gatchel, Peng, Peters, Fuchs, & Turk, 2007) and is also responsible for release of neurotransmitter such as GABA, all of which are known for their inhibitory activity. It has also been observed from previous experiments that electrical stimulation of the LHb inhibits spontaneous firing of VTA dopamine neurons (Hikosaka O., 2010) and the dorsal raphe nucleus serotonin neurons (Hikosaka O., 2010). Thus, the LHb is known to form a connection between the cortex and the midbrain that results in inhibition of several midbrain nuclei. Hence it is hypothesized that stimulating the habenula will result in decreased activity due to the analgesic effect.

4.2 Methods and Materials

4.2.1. Animal Selection

16 Adult Sprague Dawley male rats aged 4-6 months old were taken at random from the University of Texas at Arlington vivarium. Rats were kept on a 12-hour light/dark cycle, with testing occurring during the light cycle from 7:30 a.m. to 7:30 p.m. Subjects had access to food and water and libitum. Subjects were housed in cages of 2-4 until after electrode implantation, all procedures have the approval of the UTA Institutional Care and Use Committee and will follow the ethical guidelines for pain experiments in animals.

4.2.2 Electrode Implantation

The recording electrodes (81MS2021SPCE MS303-1-B-SPC-ELECT SS 2C TW .010in Plastic One) were implanted for collecting local field at baseline, formalin and post-formalin electrical stimulation (Table 1). Under isoflourane anesthesia, the electrode was placed as follows, LHb [anteroposterior (AP), −3.8 from bregma; mediolateral (ML), 0.8; dorsoventral (DV), 4.5 from dura] (Paxinos & Watson, 2007) (Figure 6). As aforementioned, owing to the larger habenular volume on the left side, the electrodes were implanted on the left side. An additional burr holes were created in the skull for the placement of separate anchor screws (Anchor Screw: 8L010121201F SCREW 0-80X1-16 1212 080 X .062 (diameter) Plastic One) attached to hold the dental cement and had wires connected to serve as ground and reference. After electrode implantation, the rats were kept in recovery for a week. During recording connectors (Connectors: 305-305 5CM TO 100CM NO SPRING TT2 C 50 CM PLASTICS ONE) are used to link the electrodes to the wireless recording module.

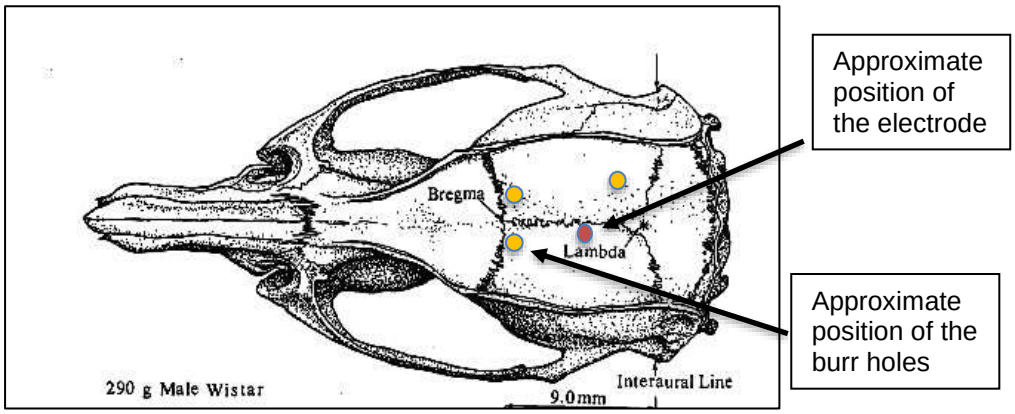

Figure 6 Approximate position of the electrodes, thesis project. The (red circle) where the electrode was implanted, and the additional burr holes (orange circle) were created screws. The two burr holes near the bregma had screws with wires that served as reference and ground electrodes.

4.2.3. Inflammatory pain model

To test the hypothesis of the experiment that the local field potential readings of the habenula after formalin injections will be higher as compared to the local field potential readings recorded during the baseline readings and that stimulating the habenula will produce analgesic effects reverting these readings back to normal, almost matching the baseline, the animals were administered with 3% formalin (0.5 ml) in the right hind paw. After injecting formalin, the LFP was recorded immediately for spontaneous activity and responses to peripheral mechanical and thermal stimulation. The left foot served as control.

4.2.4 Mechanical paw withdrawal threshold test (MPWT)

To test the MPWT, Von Frey filaments of different forces were used (Aesthesio® Precision Tactile Sensory Evaluator Kit containing the series of Von Frey filaments). The animals were placed in a plexiglass chamber seated on top of a mesh floor. The animals were carried from the colony room and placed in the plexiglass chamber for at least 15 minutes prior to testing to get them habituated to the unfamiliar environment. The Von Frey filaments ranging from 3.85 to 251.34 mN were used to poke to the plantar surface of the right and left paw till the filament bends. The up and down method as described by Dixon, 1980 will be utilized. Three trials were performed on both the paws and an average value was calculated using the formula 50% g threshold = $(10^{(X_f+k*\delta)})/10{,}000$ (Chaplan et al., 1994), where X_f = log value of the final Von Frey hair, k = tabular values of the positive and negative responses (XOXOX pattern) and δ = mean difference in log units. Each trial was initiated with the lowest force being applied to both the left and right paws

and depending on the response the next force applied will be lower or higher. The first three trials were carried out before implantations as part of acclimatizing the animal and then the next set of trials were carried out 7 days after implantation as baseline. Another set of trials were carried out after injecting the animal with formalin and finally one more after stimulating the habenula. Lower scores indicate the presence of hypersensitivity.

4.2.5 Thermal Paw withdrawal test (Planar/Hargreaves' test)

Thermal sensitivity test was executed using the Planar or Hargreaves' test of paw withdrawal (Hargreaves et al., 1988). The rats were placed in a plexiglass box with a metal mesh floor. The rats were placed in the box 30 minutes prior to the experiment to habituate the rats to the box. A mobile source of heat light (Ugo Basile, Comerio VA, Italy) was placed directly under the hind paw of the rat. The paw withdrawal latency period (measured in seconds) was calculated as the time source of light turns on (bulb used will be a 50 W, intensity was being adjusted to around 7 and distance of the lamp to paw was around 40 mm) and the hind paw was withdrawn. Paw withdrawal was tested on both the right paw where formalin is injected and the left-paw as control. While running the trial the maximum time the source of light was turned on was 30 seconds. The first trials were carried out before implantations and then the next set of trials were carried out 7 days after implantation. Another set of trials were carried out after injecting the animal with formalin and finally one more after electrically stimulating the habenula

4.2.6 Electrical Stimulation

45 minutes after formalin injection, the recording module was switched with a stimulatory module. Previous deep brain stimulation (Friedman et al., 2011) as well as habenular stimulation (Ilango et al., 2013) studies had indicated that low frequency (10-15 Hz) stimulation increased the activity while higher frequency (100 Hz) stimulation had no effect at all and both the studies had stimulated the target area for 15 minutes, hence the electrical stimulation was given to the animal as follows the train duration (200 ms), intensity (1V), pulse width of 0.5 ms for 5 seconds at 15 Hz. The parameters for electrical stimulation were selected based on previous publication from Peng lab (Li et al., 2016) and based on preliminary data, where no aversive response was exhibited by the animal to the similar stimulation parameters.

4.2.7 Euthanasia

After recording, animals were euthanized with carbon dioxide gas following the guidelines of the American Veterinary Medical Association's guidelines for euthanizing rodents (AVMA Panel on Euthanasia, 2007).

4.2.8 Histology

Once the animals were euthanized with CO2 after the end of each experiment, their brains were extracted and stored in 10% formalin solution for at least 48 hours, further transferring them to a 30% solution of sucrose. Once the brains will sink to the bottom of the tube they will be sliced and sectioned at 80 µm thickness and then stained using thionine. These sectioned and stained slices will be observed under a light microscope to confirm the location of the tip of the electrode. Placement of the tip of the electrode was verified independently by another experimenter.

4.2.9 Statistics and Analysis

The raw local field potential raw traces/raw data were recorded using MC_Rack data acquisition software. The recorded files were further imported into Spike 2 data analysis software. The specific time when the stimulations began, and end will be prerecorded and using this time specific 10-second bins will be selected in the raw trace for obtaining the power spectrums. A fast Fourier transformation was used to produce the power spectrum for each time bins using an FFT block size of 8192 and Hanning window. A histogram will also be obtained for each of the power spectrums ranging from 0-100 Hertz for both baseline readings and post injection readings. Each of the power spectrums were also saved as a text file containing the numerical values of both the amplitudes and the recorded voltage at that frequency. This data was later imported into an excel file and the same procedure was applied for all the rats. The following frequency bands, (Delta 0-4 Hz, Theta 4-8 Hz, Alpha 8-12 Hz, Beta 13-29 Hz, and Gamma 30-100 Hz) were separated into an excel file. The mean of power at each of the frequency will be computed in excel (for both the baseline and the post injection) and then graphed with their standard error of means. The same data will also be imported into SPSS for further analysis. The analysis between the different frequency bands was conducted in SPSS using within subjects ANOVA followed by LSD post hoc tests. This helped determine if there were differences between the baseline LFP readings before injecting formalin injection and then after injecting it. Similar SPSS statistical analysis was used to determine the differences between the baseline and after stimulation recordings for the mechanical paw withdrawal tests while student sample T-tests were used to analyze the

thermal paw withdrawal tests. All data are shown in mean ± standard error of mean. Significant difference is determined by $p < 0.05$.

4.2.10 Summary of the methodology

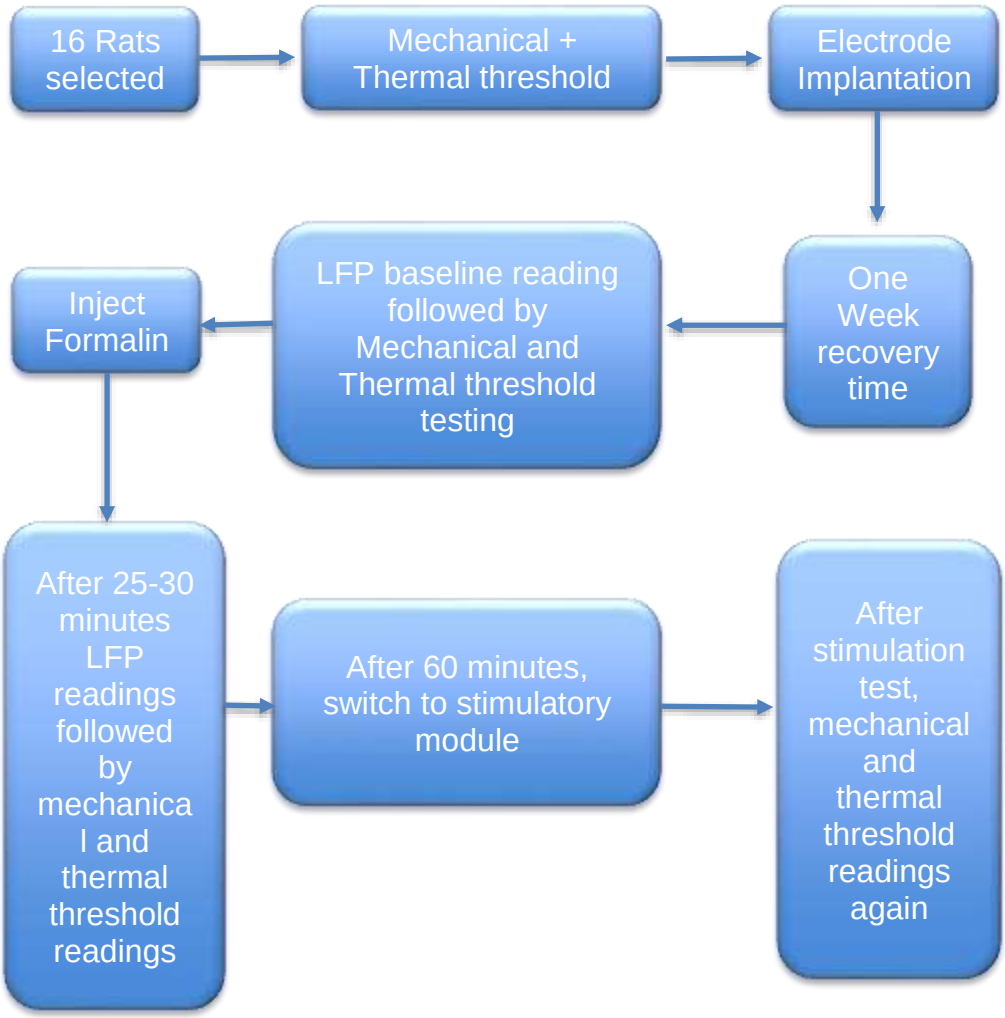

Table 1: Summary of Methodology

Chapter 5
Results

The main purpose of the present study was to determine the contribution of LHb to the nociceptive input and the effect of activation on antinociception.

5.1 Histology

Brains were stained mainly to observe the locate and confirm the placement of the electrode at the desired co-ordinates as seen below: The electrode is falling in the region of LHb ranging from -3.3 to -4.3 bregma as show in figure 7

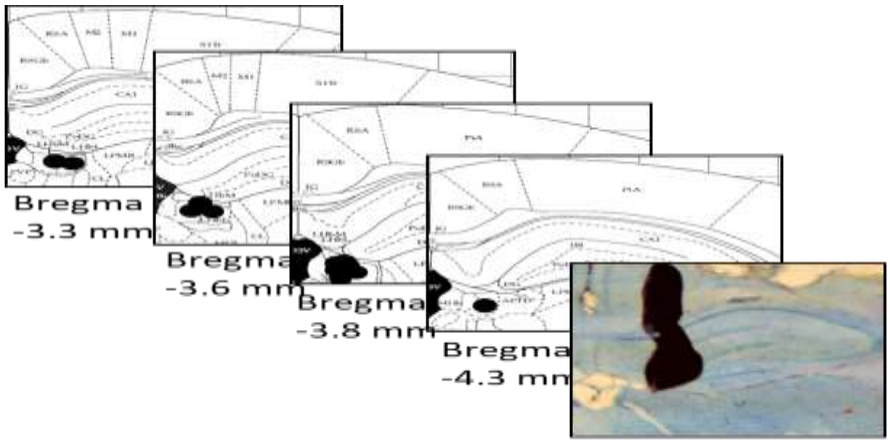

Figure 7 Histology. Schematic representation of the localization of the electrodes' tips (black dots) on different coronal slices anterior to the bregma (modified from Paxinos & Waston, 1998) and an actual histology slide on the right.

5.2 Habenular Stimulation reduces formalin induced thermal and mechanical nociception

The results for the thermal test showed a significant decrease in paw withdrawal threshold and latency was observed after formalin injections, (formalin left - 9.2 seconds, formalin right – 5.9 seconds) indicating increase in nociception. Interestingly, electrical stimulation of LHb has significantly reversed the phenomena (post stimulation left – 9.3 seconds, post stimulation

right – 9.4 seconds), suggesting an antinociceptive role by LHb (Figure 8). Repeated measures ANOVA using SPSS revealed a significant main effect of paw withdrawal threshold, F (5, 75) = 117.14, p < .05. A Bonferroni post hoc test revealed a difference between the paw withdrawal threshold for the right leg (site of formalin injection) (M = 6.10, SE = .10), p < .05 and formalin left (M = 8.99, SE = .11), p < .05, post stimulation right (M = 8.99, SE = .19), p < .05 and post stimulation left (M = 9.11, SE = .19), p < .05.

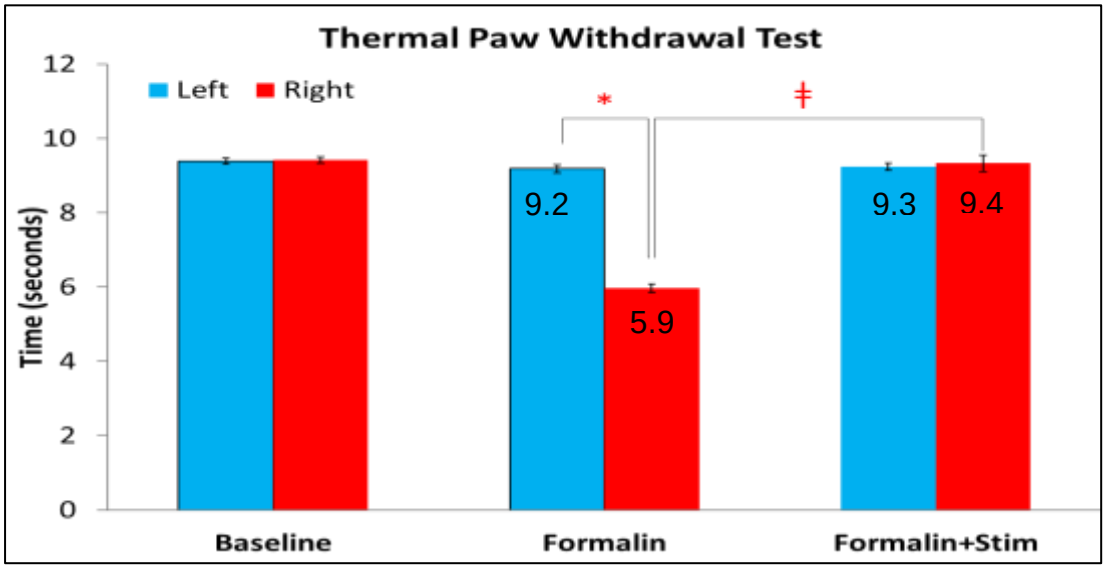

Figure 8 Thermal Paw Withdrawal Test: Significant decrease (p < .05) in paw withdrawal threshold (*) and latency were observed after formalin injections. Interestingly, electrical stimulation of LHb has significantly reversed (p < .05) this effect (‡)

For the mechanical paw withdrawal tests, the results showed that, a, significant decrease in paw withdrawal threshold and latency after formalin injections, (formalin left – 399.1 nN, formalin right – 224.23 nN) indicating increase in nociception. Interestingly, electrical stimulation of LHb has significantly reversed the phenomena (post stimulation left – 401.7 mN, post stimulation right – 385.26 mN), suggesting an antinociceptive role by LHb (Figure 9). Repeated measures ANOVA using SPSS revealed a significant

main effect of paw withdrawal threshold, F (5, 75) = 158.46, p < .05. A Bonferroni post hoc test revealed a difference between the paw withdrawal threshold for the right leg (site of formalin injection) (M = 224.48, SE = 9.87), p < .05 and formalin left (M = 399.48, SE = 5.51), p < .05, post stimulation right (M = 385.20, SE = .19), p < .05 and post stimulation left (M = 401.70, SE = 8.36), p < .05.

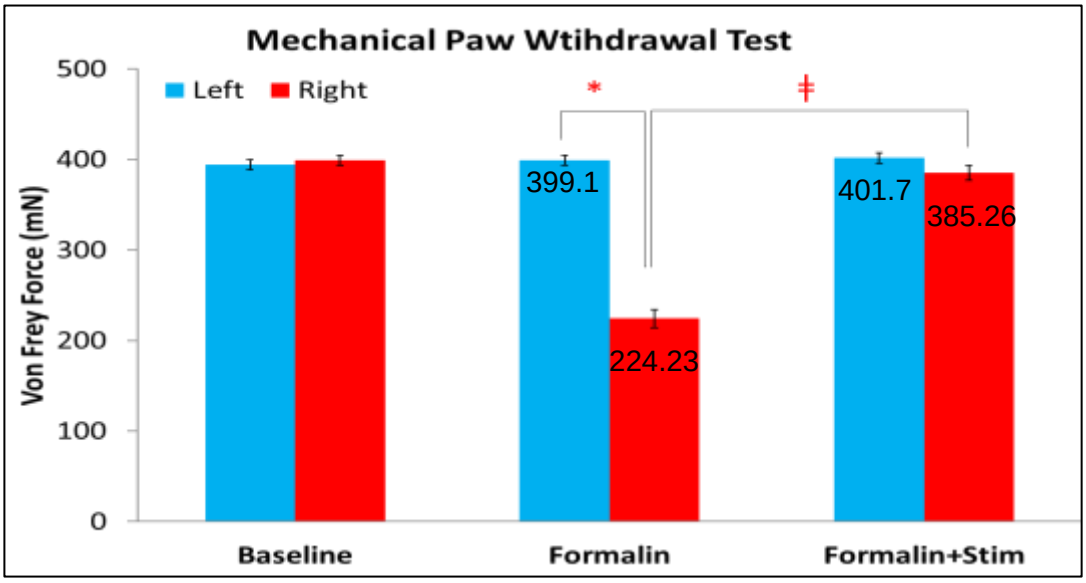

Figure 9 Mechanical Paw Withdrawal Test. Significant decrease (p < .05) in paw withdrawal threshold and latency were observed after formalin injections (*). Interestingly, electrical stimulation of LHb has significantly reversed (p < .05) this effect (ǂ)

5.3 Spontaneous Formalin Response

It was observed that there was a trend of significant increase for all the frequency bands following formalin injection comparing to the baseline. A repeated measures ANOVA using SPSS reveled a significant difference between the baseline, formalin and post formalin electrical stimulation results, for the **Delta wave** (Fig.10A), F (2,47) = 5.48, p < .05, Post hoc tests using the Bonferroni correction revealed significant difference between baseline (M = 3.10E-10, SE = 7.55E-11), p < .05 and formalin and between formalin (M =

2.15E-06, *SE* = 9.19E-07), $p < .05$ and post electrical stimulation (*M* =4.81E-10, *SE* = 1.30E-10), $p < .05$. However, no significant differences were observed in between the baseline and post electrical stimulation data. Similarly, a repeated measures ANOVA using SPSS reveled a significant difference between the baseline, formalin and post formalin electrical stimulation results, for the **Theta wave** (Fig.10B), $F(2,47) = 5.57$, $p < .001$, Post hoc tests using the Bonferroni correction revealed significant difference between baseline (*M* =2.25E-10, *SE* = 4.82E-11), $p < .05$ and formalin and between formalin (*M* = 3.68E-07, *SE* = 1.50E-07), $p < .05$ and post electrical stimulation (*M* =2.83E-09, *SE* = 2.39E-09), $p < .05$. No significant differences were observed in between the baseline and post electrical stimulation data. A repeated measures ANOVA using SPSS reveled a significant difference between the baseline, formalin and post formalin electrical stimulation results, for the **Alpha wave** (Fig.10C), $F(2,47) = 7.8$, $p < .05$, Post hoc tests using the Bonferroni correction revealed significant difference between baseline (*M* =1.63E-10, *SE* = 7.43E-11), $p < .05$ and formalin and between formalin (*M* = 1.34E-07, *SE* = 4.77E-08), $p < .05$ and post electrical stimulation (*M* =9.38E-10, *SE* = 8.25E-10), $p < .05$. No significant differences were observed again in between the baseline and post electrical stimulation data. Significant differences were also observed between baseline, formalin and post formalin electrical stimulation results, for the **Beta wave** (Fig.10D), $F(2,47) = 6.33$, $p < .05$, Post hoc tests using the Bonferroni correction revealed significant difference between baseline (*M* = 3.64E-11, *SE* = 9.21E-12), $p < .05$ and formalin and between formalin (*M* = 2.32E-08, *SE* = 9.18E-09), $p < .05$ and post electrical stimulation (*M* = 4.56E-11, *SE* = 1.90E-11) , $p < .05$. No

significant differences were observed in between the baseline and post electrical stimulation data. Lastly the **Gamma wave** (Fig.10E) also had significant differences between the baseline, formalin response and the post electrical stimulation, F (2,47) = 5.87, p < .05. Post hoc tests using the Bonferroni correction revealed significant difference between baseline (M = 3.64E-11, SE = 9.21E-12), p < .05 and formalin and between formalin (M = 2.32E-08, SE = 9.18E-09), p < .05 and post electrical stimulation (M = 2.34E-11, SE = 9.12E-12), p < .05. No significant differences were observed in between the baseline and post electrical stimulation data.

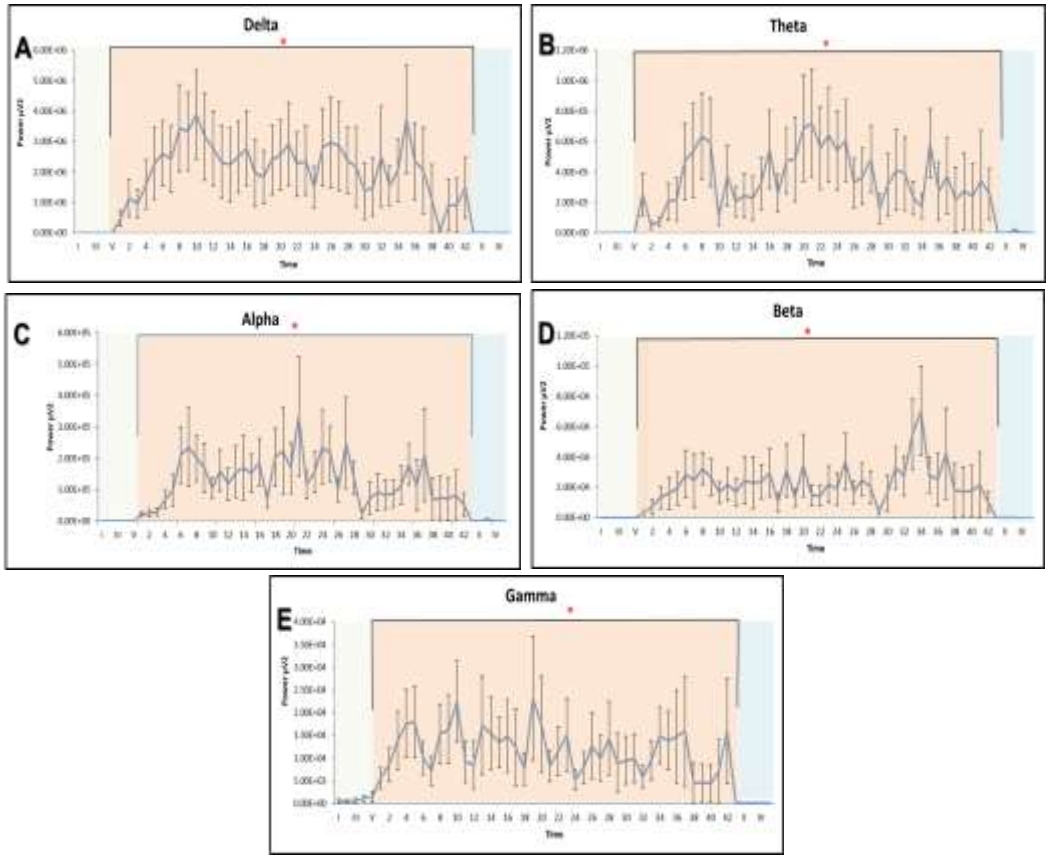

Figure 10 LFP activity changes. Delta (A), theta (B), alpha (C), beta (D), gamma (E) during formalin response. Significant differences (p < .05) were observed between baseline (□), formalin (□), and post stimulation (□) of LHb.

5.4 Increase LFP response to formalin induced thermal hypersensitivity is suppressed by electrical stimulation

There were six conditions (Baseline Left, Baseline Right, Formalin Left, Formalin Right, Post stimulation Left, Post stimulation Right) for the thermal (Hargreaves) testing, from the electrophysiological data we observed significant increase for the LFP powers during formalin period in response to the thermal stimuli, which were reduced by electrical stimulation of LHb. The right foot where the formalin was injected was compared to the rest using a repeated measures ANOVA using SPSS followed by a Bonferroni correction. The ANOVA revealed significant difference between the formalin right foot readings and between the rest of the conditions, F (5,95) = 2.48, $p < .05$ for the **Delta wave** (Figure11A). Post hoc tests using the Bonferroni correction revealed significant difference between the rest of the experimental conditions and the site where the formalin was injected i.e. the right foot ($p < .05$). For the Theta wave the ANOVA revealed significant difference between the formalin right foot readings and between the rest of the conditions, F (5,95) = 4.88, $p < .05$ for the **Theta wave**(Figure11B). Post hoc tests using the Bonferroni correction also revealed significant difference between the rest of the experimental conditions and the site where the formalin was injected i.e. the right foot ($p < .05$) and no significant difference was observed between the other conditions). For the **Alpha wave** (Figure11C), the ANOVA revealed significant difference between the formalin right foot readings and between the rest of the conditions, F (5,95) = 8.42, $p < .05$ for the Delta wave. Post hoc tests using the Bonferroni correction also revealed significant difference between the rest of the experimental conditions and the site where the

formalin was injected i.e. the right foot ($p < .05$) and no significant difference was observed between the other conditions. The ANOVA revealed significant difference between the formalin right foot readings and between the rest of the conditions, F (5,95) = 9.42, $p < .05$ for the **Beta wave**(Figure11D). Post hoc tests using the Bonferroni correction revealed significant difference between the rest of the experimental conditions and the site where the formalin was injected i.e. the right foot ($p < .05$) but no significant difference was observed between the other conditions. A significant difference was also observed for the **Gamma wave** (Figure11E), F (5,95) = 7.45, $p < .05$. Post hoc tests using the Bonferroni correction revealed significant difference between the rest of the experimental conditions and the site where the formalin was injected i.e. the right foot ($p < .05$) but no significant difference was observed between the other conditions.

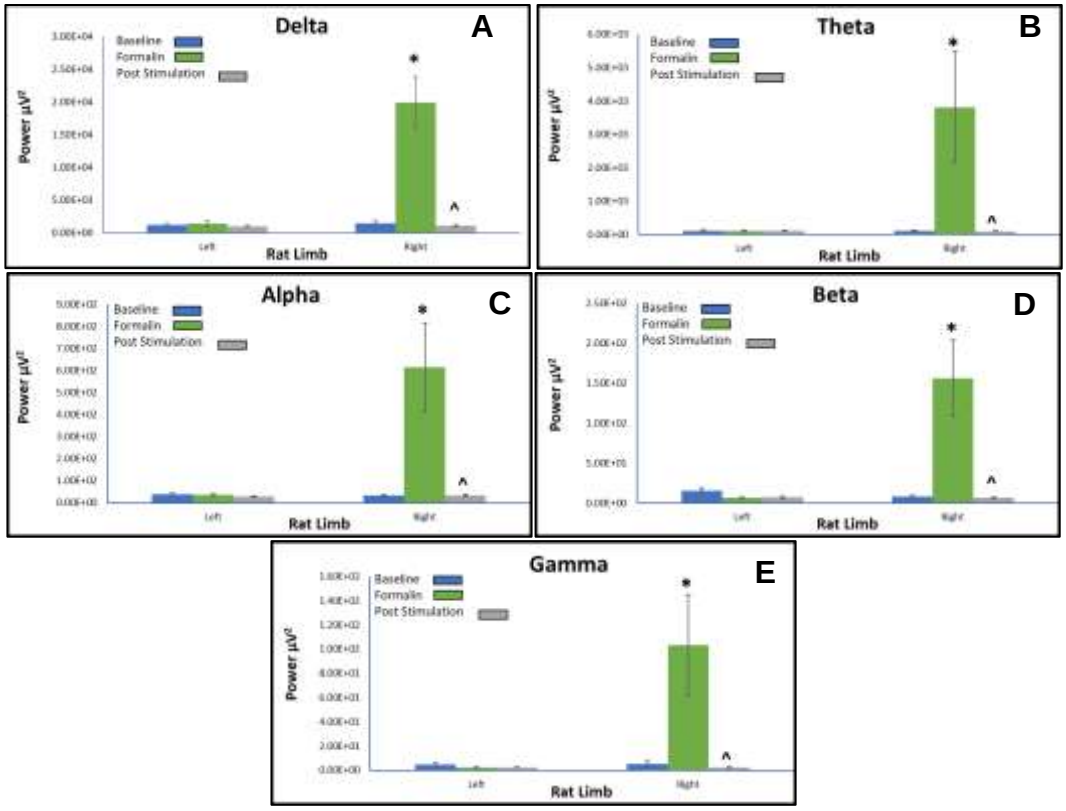

Figure 11 LFP activity Mechanical Paw Test. LFP activity changes in delta, theta, alpha, beta, gamma during formalin response. Significant differences ($p < .05$) were observed between baseline, post stimulation and formalin response for the left and right foot

5.5 Increase LFP response to formalin induced mechanical hypersensitivity is suppressed by electrical stimulation

To test the MPWT, Von Frey filaments of different forces were used. Aesthesio® Precision Tactile Sensory Evaluator Kit containing the series of Von Frey filaments was commercially purchased and the formalin response was observed in filaments numbers 4,8, 15 and 26 only and from the electrophysiological data we observed significant increase for the LFP powers during formalin period in response to the mechanical stimuli, which were reduced by electrical stimulation of LHb. Repeated measures ANOVA using SPSS followed by a Bonferroni correction revealed significant difference between the formalin and the baseline and post stimulation readings, F (11, 517) = 8.16, $p < .05$ for the **Delta wave** (Figure12A). Similar significant differences were observed for **Theta** (Figure 12B), F (11, 517) = 7.91, $p < .005$, **Alpha** (Figure 12C), F (11, 517) = 6.56, $p < .05$, **Beta** (Figure12D), F (11, 517) = 3.19, $p < .05$ and **Gamma** (Figure12E), F (11, 517) = 5.60, $p < .05$. No significant differences were observed between the baseline and post stimulation baseline readings.

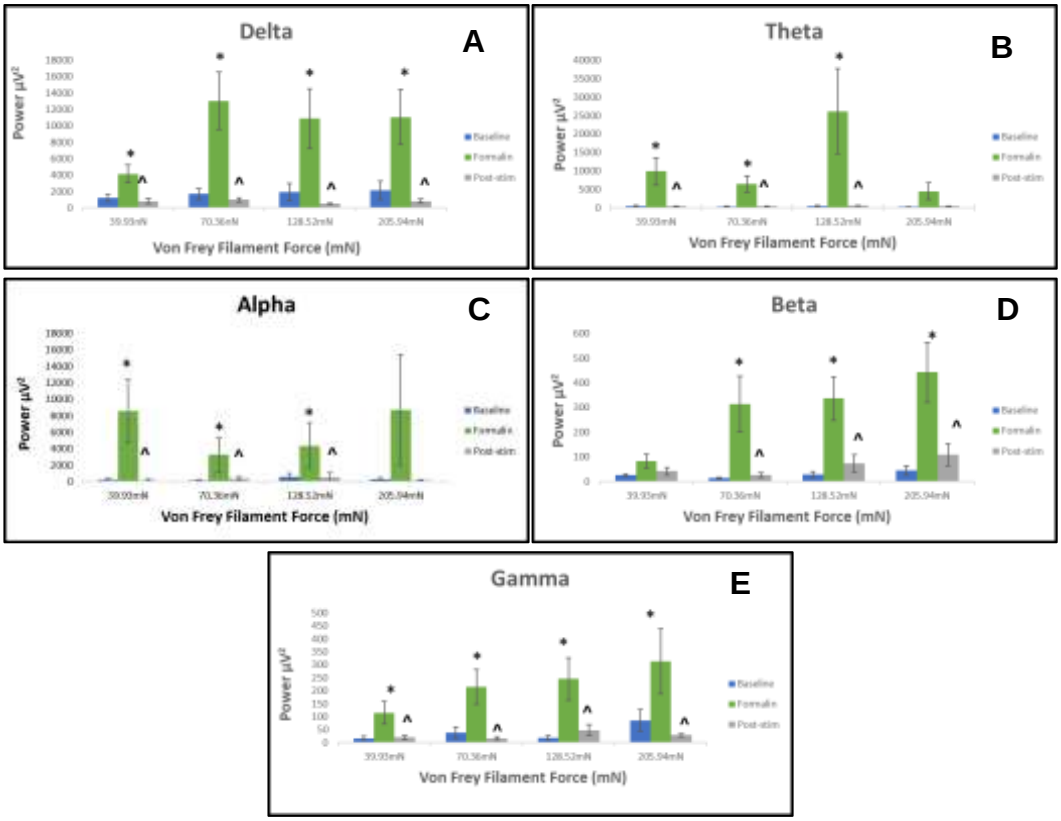

Figure 12: LFP activity Mechanical Paw Test. LFP activity changes in delta, theta, alpha, beta, gamma during formalin response. Significant differences ($p < .05$) were observed between baseline, post stimulation and formalin response.

Chapter 6
Discussion

We found increased LHb activity in response to formalin that got suppressed post electrical stimulation. Previous experiments by Cohen and Melzack, 1986 had observed something similar. However, in our experiment the behavioral analysis was also accompanied by the local field potential data. Thus, the role of the lateral habenula (LHb), in nociception and analgesia could be postulated to be an important one. A key study was conducted by Matsumoto & Hikosaka, 2007 in rhesus monkeys had described the habenula as a “brain nucleus” that gets activated during failure to obtain rewards, anticipating aversive responses and suppressing motor activity. Research in habenular function has since then attracted a lot of attention both in neuroscience as well as clinical research. The lateral habenula (LHb) controls the raphe nuclei, and a recent study has also investigated its participation in regulating pain-associated depression (Li et al., 2017). The role of habenula in drug addiction has also been investigated (Velasquez, Molfese, & Salas, 2014) and habenular dysfunctions have been linked to, schizophrenia, as well (Boulos, Darcq & Kieffer, 2017). Thus, the role of the habenula is quite complex and diverse.

As discussed before the habenula is a centrally located structure connecting the forebrain, hindbrain and ventral midbrain. It is also primarily responsible for regulating dopamine and serotonin levels, along with emotional and sensory processing (Boulos, Darcq & Kieffer, 2017). The results showed that: (1) Behaviorally, significant decrease in paw withdrawal threshold and latency were observed after formalin injections ($p < .05$), indicating increase in nociception. (2) There was also a trend of significant

increase for all the frequency bands following formalin injection ($p < .05$) comparing to the baseline, (3) also observed during the mechanical and thermal stimuli test. The reason behind this observation can be explained as follows, preclinical data has implicated habenulas involvement in modulating pain as well as analgesia (Shelton, Becerra, & Borsook, 2012). The habenula receives afferent inputs originating from the lamina I of the dorsal horn as well as from the trigeminal nucleus (Craig A.D., 2004). Another structure that is involved processing pain is the hypothalamus which is known to have direct inputs into the lateral habenula (Goto et al., 2005). The other structures that have been reported to be involved in pain processing pathways are midbrain central gray and serotoninergic raphe nuclei both of which have connections with the lateral habenula. The raphe nucleus is involved in the pain perception through serotoninergic, opioidergic and GABAergic system (Shelton, Becerra, & Borsook, 2012).

Interestingly, electrical stimulation of LHb has significantly reversed the phenomena, suggesting an antinociceptive role by LHb. The nociceptive action of the habenula can be explained as follows, the lateral habenula has efferent projections into the VTA and SNc, two structures that have dopaminergic neurons that are involved in reward and aversion and are mainly inhibited after an aversive stimulus. The lateral habenula also projects into the raphe nucleus, that releases serotonin, which is an important neurotransmitter mainly involved in modulation of pain. Previous studies have indicated that the PAG, habenula and the nucleus accumbens might constitute a unidirectional pain modulatory loop system (Shelton, Becerra, & Borsook, 2012). Thus, these midbrain projections, into the PAG and raphe

nucleus are important in habenula mediated analgesia. The PAG has already an established role in the descending pain modulation while the dorsal raphe directly innervates the spinal cord via the raphe magnus in the medulla. Both of these structures contribute to the serotonergic pain modulation pathway (Shelton, Becerra, & Borsook, 2012). Both dopaminergic as well as serotonergic pain modulation systems have an important part in the pain processing, including modulation and reward and electrical stimulation of habenula may activate the descending inhibitory system to achieve the analgesic effect.

In conclusion, based on our findings we can postulate that the ascending pain signals not only reach traditional pathways like ACC, S1 and thalamus but also activate the habenula, which in turn triggers the descending pathway to have a close circuit loop for endogenous antinociceptive effect. Thus, it can be observed that habenula is involved in pain transmission and electrical activation of the LHb could induce antinociceptive effect.

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