THE PAIN IN THE PLEASURE: ELECTROPHYSIOLOGICAL ANALYSIS OF THE TAIL REGION OF THE VENTRAL TEGMENTAL AREA (tVTA).

by

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Dedication

Finally, I would love to dedicate this dissertation to a handful of people. My parents, without whom I would not be here, they have always given me the freedom to do anything I want. I also want to dedicate this to my three dogs, Mr. Wuu, Riot and Khaos, for being the stress relief I needed during this period. I love you all and I am blessed to have you in my life.

Abstract

THE PAIN IN THE PLEASURE: ELECTROPHYSIOLOGICAL ANALYSIS OF THE TAIL REGION OF THE VENTRAL TEGMENTAL AREA (tVTA).

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The mesolimbic (midbrain) reward system is well documented to be involved with the analgesic effects of opioids, which involves the ventral tegmental area (VTA) causing release of dopamine (DA). The dopamine neurons from the ventral tegmental area (VTA) directly innervate the nucleus accumbens (NAc) and the prefrontal cortex via D1 DA Receptors. GABAergic inhibition of VTA DA system occurs regularly, but in 1990s Johnson and North showed the administration of opioids led to hyperpolarization of GABA interneurons in the VTA removing this inhibition, further leading to the “disinhibition model”. However, more recent studies have defined a new structure heavily influencing this DA system i.e. the tail of the ventral tegmental area (tVTA). The tVTA sends dense GABA projections to VTA. tVTA neurological connections have been postulated to play an influential part not only in the mesocorticolimbic pathway but also in the nigrostriatal pathway Research has also shown that neurons from the tVTA overwhelmingly synapse on the tyrosine hydroxylase positive neurons within the VTA as well as the substantia nigra and electrically stimulation of the tVTA suppresses the DA neuron firing. Previous studies have proposed that tVTA may play an important role conveying information about noxious stimuli to the VTA DA neurons and

mediate the appropriate response. There are however some fundamental questions still unanswered and unaddressed. Previous studies have indicated that these inhibitory responses are driven by the GABAergic transmission onto the DA neurons, hence it's important to observe opioid-induced alterations of local field potentials (LFPs), especially post noxious stimuli (formalin) and the involvement of dopamine (DA) system in this process recorded simultaneously from the four brain regions, tVTA, VTA, NAc and ACC. Here, I present data demonstrating that administering formalin significantly increased the LFP activity in the tVTA., An increase in activity within the VTA and ACC was also observed following formalin injection with all the waves exhibiting increased activity expect the gamma. Morphine injections led to significant increase in activity in the NAc, however very low to no activity was observed within this region following a noxious stimulus. Thus, it could very well be postulated that tVTA-VTA-NAc-ACC complex neurocircuitry could very well play any important role in relaying nociceptive information.

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Chapter 1

1.1 Introduction

Pain is extremely complex in nature and physical injury is just one the reasons an individual can experience it (Parchure & Peng, 2020). Multiple factors play a significant role in the pathogenesis of pain. 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).

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.

It is of vital importance that the patients experience pain relief, especially after surgical procedures. Adequate pain relief is often deemed to be an important measurement of patient satisfaction and it also helps prevent any postsurgical chronic pain. The most commonly prescribed therapeutic intervention for acute as well as chronic pain (postoperative or otherwise) are opioids. Some examples of commonly available/prescribed opioids are morphine, fentanyl, methadone, buprenorphine, codeine, hydrocodone and oxycodone. Opioids not only occur naturally but they are synthesized on a regular basis using a chart that helps calculate equivalent amount of dose between the different analgesics. This helps to easily switch between different agents and routes of administration (Parchure & Peng, 2020).

Over prescription of opioids while avoiding the use of alternative interventions have led to an opioid crisis in the USA, the total annual cost out of which has been estimated to be around $500 billion. Given the prevalence of chronic pain and its often-disabling effects, it is not surprising that opioid analgesics are now the most commonly prescribed class of medications in the United States (Compton & Volkow, 2006). In 2014 alone, U.S. retail pharmacies dispensed 245 million prescriptions for opioid pain relievers, of these prescriptions, 65% were for short-term therapy (<3 weeks),6 but 3 to 4% of the adult population (9.6 million to 11.5 million persons) were prescribed longer-term opioid therapy (Volkow & McLellan, 2016b). Although opioids rapidly relieve acute nociceptive signals their effectiveness in treatment of chronic pain is still unclear. According to the CDC a 10-day opioid treatment can

also lead to addiction hence opioid use for acute pain is also heavily associated with increased risk of long-term opioid use (Shah, Hayes, & Martin, 2017).

The use of opioids for pain management has significantly increased in the past decade leading to the increased deaths due to over dosage (Compton & Volkow, 2006). Hence there is a significant need for therapeutic interventions that balance treating pain, while minimizing risks for opioid abuse. The present screening strategy for potential opioid abuse includes assessment of premorbid and comorbid substance abuse; assessment of aberrant drug-related behaviors; risk factor stratification; and utilization of opioid assessment screening tools and is deemed ineffective at the present, hence research relating to opioids and its role in nociception is warranted.

The pharmacological effect of opioids is mediated via opioid receptors which are the G-protein coupled receptors located both pre and post synaptically. These act by directly inhibiting the cell signaling by reducing the excitability as well as neurotransmitter release. These receptors are distributed throughout the central nervous system (CNS) and to a lesser extent in the periphery. These receptors have been characterized to play important roles in antinociception, sedation as well as drug reward (Mansour, Khachaturian, Lewis, Akil, & Watson, 1987). Once an opioid agonist binds to the G-protein coupled receptor, the α subunit of the G-protein, intracellularly exchanges its bound guanosine diphosphate (GDP) molecule with the guanosine triphosphate (GTP). G protein coupled receptors are made up of two complexes α-GTP and βγ complex (see figure 1). The GDP to GTP exchange causes the α-GTP complex to break away from the βγ complex, making them free to interact with other target proteins. The α-GTP complex interacts with adenylate cyclase, which in turn causes a reduction

in the intracellular levels of the cAMP (cyclic adenosine monophosphate) levels. Cyclic adenosine monophosphate is a secondary messenger required in a lot of biological process including intracellular signal transduction (Kitchen, Slowe, Matthes, & Kieffer, 1997). These complexes also interact with several ion channels, causing increase in activation of potassium channels and inhibiting calcium conductance. The overall effect of all these processes is reduced cAMP levels and a hyperpolarized cell (as seen in figure1). In the case of neuronal cells this means reduced neurotransmitter release and relay of information (Pathan & Williams, 2012).

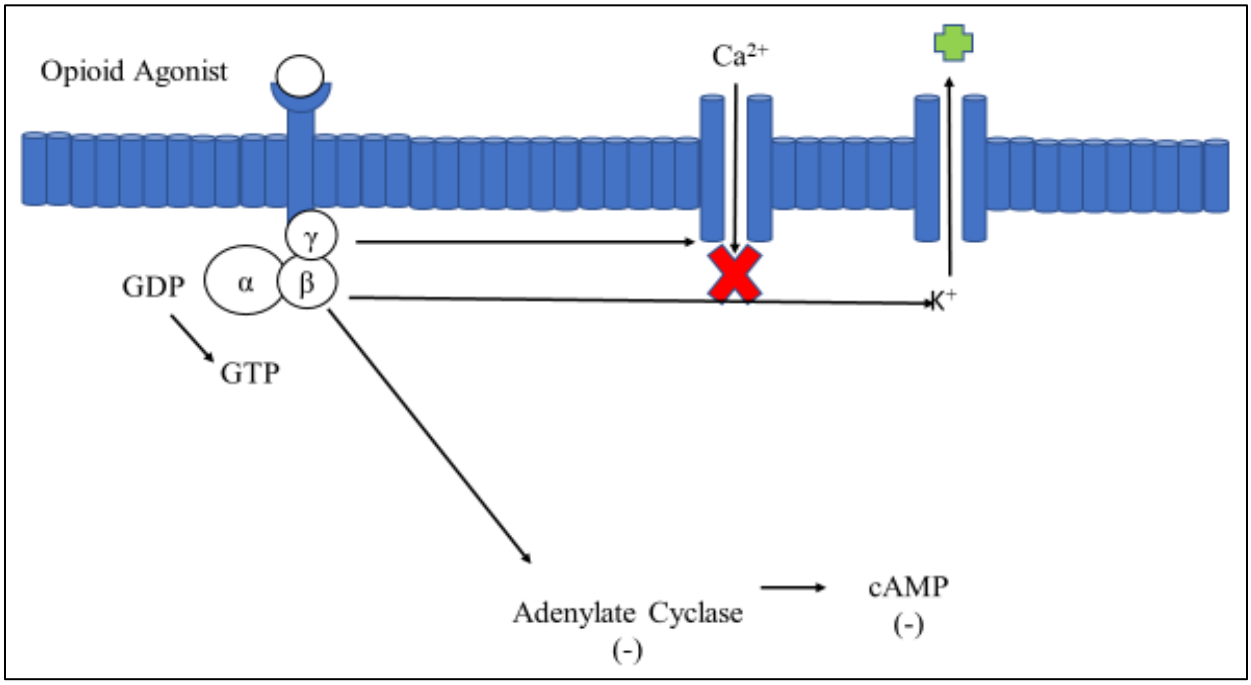


Figure 1: Opioid G-protein-coupled mechanism of action. Binding of the opioid agonist to the receptor causes the α subunit of the G-protein, intracellularly exchanges its bound guanosine diphosphate (GDP) molecule with the guanosine triphosphate (GTP). This separates the two complexes α-GTP and βγ complex. The α-GTP complex interacts with adenylate cyclase causing the intercellular levels of cAMP to drop. These complexes also interact with ion channels, inhibiting the flow of Calcium and upregulating the flow of potassium causing the cell to hyperpolarize, thereby reducing conductance and relay of information (Parchure & Peng, 2020).

1.2 Opioid Receptors

Analgesic and rewarding effects of opioids are mediated by actions at opioid receptors throughout the nervous system. Opioid receptors are seven-transmembrane G-protein-coupled receptors (Synder & Pasternak, 2003).

Receptor	CNS location	Effects	Specific effects
mu (μ) (MOR)	Cerebral cortex, thalamus, periaqueductal gray, and rostral ventromedial	Analgesia, euphoria, constipation, respiratory depression, physical dependence	Reward reinforcements (hedonic and Incentive)
delta (δ) (DOR)	Basal ganglia (pontine nucleus, amygdala)	Analgesia, anxiolysis	
k (κ) (KOR)	Hypothalamus, and periaqueductal gray	Analgesia, diuresis, dysphoria	Anti-reward

Table 1. Opioid Receptors, Subtypes, CNS Location, Effects, and Specific Effects

There are three major types of opioid receptors that mediate analgesic effects, namely, mu (μ) (MOR), delta (δ) (DOR) and k (κ) (KOR). These are also further subdivided into different subtypes depending on duration and onset as rapid-onset, short-acting and long-acting (Al-Hasani & Bruchas, 2011). The following table (table 1) describes the location of these opioid

receptors in the CNS (central nervous system) location, their effects as well as specific effects (Law & Loh, 2013) (Pathan & Williams, 2012).

The MOR were first to be discovered as they are the primarily responsible for generating euphoria and they are also essential for stimulating the reward system (Contet, Kieffer, & Befort, 2004). Animal studies have also indicated the involvement of MORs in social attachment and anhedonia (Cinque et al., 2012). Continuous opioid abuse leads to tolerance, which leads to increased craving for more opiates at the expense of the natural reward system in the body. This causes the reward system homeostasis to be compromised (Der-Avakian & Markou, 2012).

The KORs have been observed to produce anti-reward effects thus leading to dysphoria (Wee & Koob, 2010). Another study observed that KORs were associated with decrease in social play in rats (Trezza, Damsteegt, Achterberg, & Vanderschuren, 2011). Another study has also observed that prolonged exposure to drugs of abuse, enhances KOR function, via corticotropin-releasing factor (CRF) signaling, this also leads to relapse among individuals (Van'T Veer, Yano, Carroll, Cohen, & Carlezon, 2012). Stress induced by long-term drug exposure has also been observed to produce a depressant effect, hence administering a KOR antagonist is used to treat depressive disorder (Knoll & Carlezon, 2010). Thus, it can be concluded that KOR has anti-reward effect via the process of addiction and has an effect opposite of that of MORs. While being addicted or developing addiction, the increased stressors leads to enhanced KOR functions, leading to dysphoria during withdrawal and abstinence stages increasing the chances of relapse (Wang, 2019).

The DORs have been known to reduce anxiety and reduce depression (Roberts et al., 2006). Studies have also indicated the role of DOR in alcohol consumption but the exact role of DORs in other drugs of abuse is still being investigated (Wang, 2019).

1.3 Neuronal circuits underlying opioid action

The classical model of opioid reward has established the mesolimbic DAergic reward pathway. This pathway originates in DA-containing cell bodies of the VTA that project to terminal sites in the forebrain including the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), amygdala (Darcq & Kieffer, 2018) (see figure 2). Dopamine neurons within the VTA are known to play an important role in reward processing and addiction. Research has shown that addictive properties of opiates such as morphine is heavily dependent on the μ opioid receptor (MOR) activation. Over the past decade, increasing evidence has accumulated to support a modification of the classic disinhibition model that suggests morphine exciting the VTA-DA neurons by disinhibiting surrounding GABAergic neurons (Johnson and North, 1992). Along with the GABAergic modulation of the VTA dopamine neurons, glutamatergic inputs from the variety of brain areas also affect the VTA DA neurons. This glutamatergic influence switches the pacemaker like neuronal activity to a higher frequency bursts causing increase DA release in the target region. Research has shown that VTA glutamate neurons play an important role in morphine rewarding behavior. Research has also observed that endogenous opioid transmission is required within the ACC for nociceptive relief and this effect is also known to activate the NAc DA signaling (Navratilova et al., 2015). Another rodent study had detected

increased G-protein activation, in the perigenual and midcingulate region of the ACC after opioid administration (L. J. Vogt, Sim-Selley, Childers, Wiley, & Vogt, 2001). Other studies have also confirmed that importance of MORs in the ACC for activating the opioid relief (Wang et al., 2020).

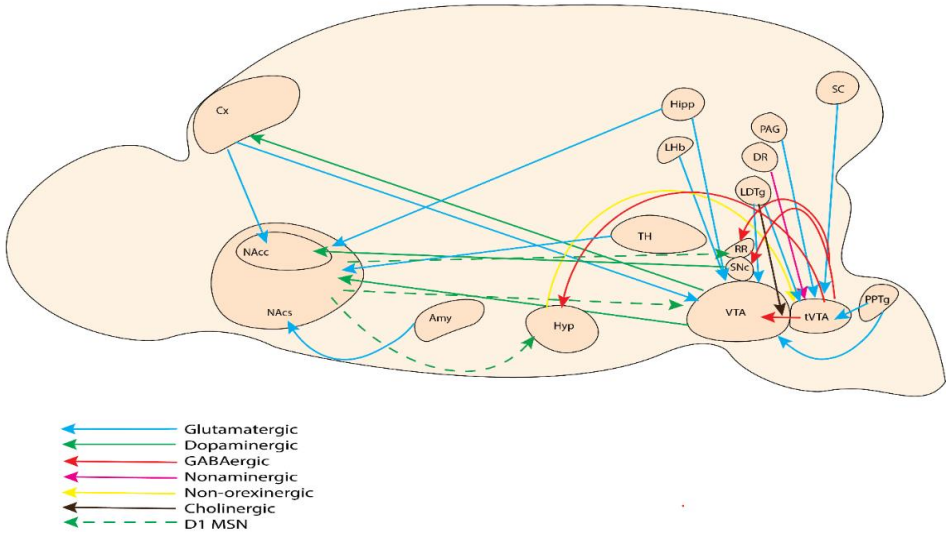


Figure 2. The afferent and efferent connections of the mesocorticolimbic pathway. The tVTA receives afferents from a broad range of cerebral structures. The main afferents arise from the frontal cortex (Cx), lateral habenula (LHb), hypothalamus (Hyp), superior colliculus (SC), periaqueductal gray (PAG), dorsal raphe (DR), laterodorsal tegmentum (LDTg), and pedunculopontine tegmental nucleus (PPTg). The tVTA efferents (in red) are more restricted and preferentially target midbrain dopamine nuclei: ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and to a lesser extent the retrorubral field (RR). The tVTA also heavily projects to the lateral hypothalamus (LH).

1.4 Revisiting the Disinhibition Model Paradigm

Recently it was reported that tVTA/RMTg send a dense network of MOR expressing GABAergic neurons towards the dopamine neurons in the VTA, which has challenged the original notion of the disinhibition model (Barrot et al., 2012; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009; Kaufling et al., 2010). The rostromedial tegmental nucleus (RMTg), or the tail

region of the VTA (tVTA) was discovered using activity of early response genes namely cFos or ΔFosB after administering psychostimulants (Hope et al., 1994) (Kaufling et al., 2010) (Barrot et al., 2012) (Jhou et al., 2009). This area is caudal to the VTA and dorsal to the interpeduncular nucleus. In another experiment mRNA expression for GAD67, which is an enzyme required for GABA synthesis, tVTA neurons were determined to be GABAergic. Strong immunoreactivity to the μ-opioid receptor was also detected. The study also established that this region received inputs mainly form the lateral habenula (LHb) and send a dense efferent projection into the VTA and the SNc (Kaufling et al., 2010) (Barrot et al., 2012) (Jhou et al., 2009). Noxious/Aversive stimuli has been known to increase the LHb activity followed by decreased tonic firing of dopamine neuron. It was already known that LHb neurons are glutamatergic, hence there had to be a GABAergic inhibitory neuron that resulted in the inhibition of dopamine neurons. Hence tracing studies along with immunolabeling studies resulted in detecting neurons in the RMTg and a small population of VTA interneurons that have been shown to exhibit inhibitory effect on the dopamine neurons (Balcita-Pedicino, Omelchenko, Bell, & Sesack, 2011). Thus, it was postulated that the afferents arising from the LHb to RMTg and possibly LHb to VTA interneurons could possibly have a huge impact on dopamine firing. Also tVTA lesions have been known to reduce passive fear response (Jhou et al., 2009). This behavior is also dependent on the activity of the neurons in the amygdala, periaqueductal gray, and septum, which project into the tVTA. In another experiment animals self-administering the MOR agonist, EM1 (endomorphin-1) into RMTg exhibited conditioned place preference (Jhou et al., 2009). Since this region is still being explored, its neuronal afferents and efferents are still being

characterized, hence it is also important to compare the strength of opioid modulation of this pathway especially post noxious stimuli.

1.4.1 tVTA funneling the DA system?

Studies over the years have found around 8 dopamine pathways. The four major ones are 1) Mesolimbic (VTA-NAc), Mesocortical (VTA-prefrontal cortex), Nigrostriatal (SNc-caudate putamen and tuberoinfundibular (lateral hypothalamus-pituitary gland). The minor one's project from VTA towards amygdala, hippocampus, cingulate cortex, and olfactory bulb. The three pathways that are involved with ascending projections are mesolimbic, mesocortical, and nigrostriatal pathways. There are other descending pathways as well such as the A11 nucleus in the posterior hypothalamus projecting to the brainstem and spinal cord involved with motor control and pain modulation. There are also other dopaminergic pathways that can send direct inhibitory connections to the target areas. Thus, it is evident that dopamine plays an important role not only in drug abuse and motor control but also pain modulation. Hence some of the brain areas included in the dopamine pathways were of interest in this experiment, namely tVTA/VTA, NAc and ACC.

Research has shown that tVTA afferents are widely distributed and arise from the medullary, pontine, and mesencephalic reticular formation, dorsal raphe, PAG, substantia nigra, LHb, zona incerta, hypothalamic areas, preoptic region, parts of the extended amygdala, lateral septum, and frontal cortex (Kaufling et al., 2010) (see figure 2).

tVTA efferents on the other hands are pretty restricted, outputs mainly targeting the lateral hypothalamus, the brainstem efferents being the VTA and the SNc and mildly innervating the retrorubral field as well (Barrot et al., 2012; Jhou et al., 2009; Kaufling et al., 2010). According to research, nearly 80% of the synapses from the tVTA targeting the VTA dopamine neurons produce contact via the dendrites (Balcita-Pedicino et al., 2011), thought other studies have also observed the contact on the cell body itself (Kaufling et al., 2010). Thus, based on research it could be postulated that tVTA acts like a funneling station, combining external and internal information, and then further sending this information out towards the dopamine systems. Experiments have already proven that tVTA plays an important role in the LHb-tVTA-VTA pathway and the tVTA-VTA-NAc complex neurocircuitry.

Direct experimental evidence suggests that the tVTA is involved in a lateral habenula/tVTA/VTA pathway (Balcita-Pedicino et al., 2011) and a tVTA/VTA/NAc complex neurocircuitry (Brinschwitz et al., 2010). Within the tVTA almost 55% of synapses from the lateral habenula are located on GABA positive dendrites, while in the VTA most inputs from GABAergic tVTA synapse on dendrites from dopamine cells (Bourdy & Barrot, 2012). Thus it has been proposed that the tVTA receives glutamatergic projections form the lateral habenula further innervating the VTA dopamine neurons, thus converting the excitatory signal into an inhibitory one (Brinschwitz et al., 2010). The dopamine neurons that project to the NAc from the VTA are opposed by the tVTA, indicating the impact it has on the mesolimbic system. tVTA also innervates the SNc, hence it is postulated that it will also have an impact on the nigrostriatal system. Hence further tracing as well as electrophysiological analysis is required to truly understand how tVTA fits into the brain circuitry. There have also been very limited

amount of studies looking at the tVTA and its involvement in the noxious stimuli. Hence this study will look at the mesolimbic reward pathway under the influence of a noxious stimuli followed by administration of opioids. Particularly the involvement of tVTA (tail region of the ventral tegmental area), along with VTA (ventral tegmental area), NAcs (Nucleus accumbens shell) and the Anterior Cingulate Cortex (Cg1/Cg2).

1.5 Local Field Potential and Nociceptive Studies

Numerous techniques are involved when trying to understand the brain mechanism, in the aforementioned regions (tVTA, VTA, NAc and ACC) namely, recording stimulated brain areas (anesthetized and freely moving animals), lesion studies, biomarkers of activity and evaluating tissues samples post-mortem. However, any real time local field potential (LFP) activity from freely moving animals has not been extensively investigated and this provides a good opportunity to study the activity in these areas as it is taking place. LFP is an extremely useful tool for understanding the brain activity using electrophysiology and has picked up momentum for use in studies in recent years (Einevoll, Kayser, Logothetis, & Panzeri, 2013). LFP is measured by implanting electrodes into the tissue and recording low frequency waves 0 to ~100 Hz. The measured activity directly reflects the sum of neuronal activity nearest to the recording electrode (Buzsáki, 2004, 2009; Mazzoni, Logothetis, & Panzeri, 2012).

Neuronal membrane consists of a bilipid layer that facilitates the movement of ions in and out of the cell membrane (Bedard, Kröger & Destexhe, 2004). A neuronal input causes the bidirectional movement of ions that basically results in the formation of excitatory post-

synaptic potential (EPSPs) and inhibitory post-synaptic potentials (IPSPs) (Pipa, 2006). In theory the spikes are designated as EPSPs and the dips represents the IPSPs (Pipa, 2006). The general consensus regarding LFP readings was that the recorded neural activity was primarily the result of the excitatory inputs, however research has shown that inhibitory inputs also results in recorded activity (Oren & Paulsen, 2010). Along with this "high" frequency activity, "low" frequency activity such as glial cell fluctuations, non-synaptic calcium spikes, somatodendritic afterpotentials also gets recorded (Buzsáki et al., 2012). Hence it is safe to assume that LFP includes a wide range of neuronal activity surrounding the electrode than measuring a single action potential (Buzsáki, 2009).

1.5.1 Robust nature of LFP

Now it is easy to argue that other action potentials could easily influence the overall LFP measurement, however research has found that this effect is negligible. The action potentials classified as "high frequency" are subjected to diminution over space (Buzsáki, 2009), this means that the action potentials that occur next to the electrode get recorded (Bedard, Kröger & Destexhe, 2004). Research has also observed that the extracellular media, consisting of the tissue and the fluid is successful in creating a "low-pass" filter ranging from 100-300 Hz that further helps reduce the activity of "high frequency" action potentials (Bedard, Kröger & Destexhe, 2004, Mazzoni et al., 2012). If the researcher is interested in studying a single action potential that is achieved using a high-pass filter (frequency > 500 or 600 Hz) (Waldert, Lemon, & Kraskov, 2013).

Even though this system of recording could be considered to be quite robust it does not come with its shortcomings. It is a scientific consensus that the LFP activity is the average of the neuronal activity within a specific area (nearest to the recording electrode), the actual area included in this analysis has been questioned and challenged (Kajikawa & Schroeder, 2011) as the reach of the recording electrode is dependent on a variety of factors, including the cellular morphology in the area being recorded (Kajikawa & Schroeder, 2011), the material of the recording electrode (Csicsvari et al., 2003; Zheng et al., 2012). Depending on the electrode used the spatial average recording can range up from 200-400 micrometers to 5mm (Kajikawa & Schroeder, 2011) (Katzner et al., 2009).

1.5.2 Uses in nociceptive studies

Research advancements has allowed to record LFP activity in freely moving animals (Roy & Wang, 2012)(Zhou et al., 2012)(Zuo et al., 2012)(Farajidavar, Hagains, Peng, & Chiao, 2012). As mentioned before simultaneously recording tVTA, VTA, NAc and ACC in freely moving animals has not been attempted hence using LFP in my experiment will help to confirm if these regions are somehow involved in nociceptive processing in real time, fill the gap in research regarding the these regions involvement in pain and how it can change over time when under effect of morphine.

1.5.3 Animal Models of Pain for LFP

Animal models of pain have been extensively used to study and record nociceptive responses using LFP. There are numerous models of animal pain namely inflammatory (Harris Bozer et al., 2016), cancer (Hidaka et al., 2011), postoperative (Brennan, Vandermeulen, & Gebhart, 1996), visceral (Ness & Gebhart, 1990) and neuropathic (Dalziel et al., 2004). For the purposes of this experiment we used an inflammatory model, specifically, a spontaneous (Acute) inflammatory effect using formalin injection. The first breakthrough relating to formalin studies was in 1977, when two scientists Dubuisson and Dennis were the first to introduce the formalin test (Dubuisson & Dennis, 1977). They injected diluted formalin subcutaneously into the animal resulting in acute/temporary pain while requiring no restrains on the animal. This is good since any errors that may arise due to the animal being restricted are eliminated. It has been determined over time that 5% formalin produces a robust flinching response in the animal. The formaldehyde solution work by activating the transient receptor potential subfamily A member 1 (TRPA1) (McNamara et al., 2007). TRPA1 has an excitatory calcium channel that is normally activated by elements present in garlic and mustard and is expressed by nociceptors present on C-fibers (McNamara et al., 2007). Administering formalin normally produces a biphasic response with the first 10 minutes involving an initial burst of nociception signaling involving paw licking, lifting as a behavioral response. There is a decline in the response following this which then comes back around 30-60-minute mark that involves magnification of the afferents resulting from sensitization (Coderre, Vaccarino, & Melzack, 1990; McNamara et al., 2007).

Chapter 2

The Brain Regions of Interest

2.1 tVTA (tail region of the ventral tegmental area)

The tVTA has been observed to be located towards the posterior end of the VTA (Ventral Tegmental Area) and has a dense network of GABAergic (gamma-aminobutyric acid) neurons (Jhou et al., 2009b; Kaufling et al., 2009). These GABAergic neurons have been known to produce and inhibitory effect on the midbrain dopaminergic (DA) neurons. First identified in rodents by Perrotti et al., 2005, tVTA has also been observed as an important region in several species including mice and primates (Kaufling et al., 2009). There are strong glutamatergic projections from the lateral habenula (LHb) that innervate this region (Hong et al., 2011) and also has efferents that innervate dopamine neurons of the VTA and substantia nigra pars compacta (SNc) (Jhou et al., 2009; Kaufling et al., 2010). The inhibitory influence exhibited by tVTA over the dopamine midbrain neurons has also been confirmed by electrophysiological studies in rats where tVTA inhibition facilitated via opioids increased the firing rate of the VTA dopamine neurons (Jalabert et al., 2011) whereas electrically stimulating the tVTA actually decreased the activity of the midbrain dopamine neurons (Lecca et al., 2012; Bourdy et al., 2014). Lateral habenula as well as the midbrain DA neurons have been observed to play an important role in aversive stimuli processing and predicting reward outcomes resp. (Matsumoto and Hikosaka, 2007). So being an intermediatory structure and connecting LHb to

the midbrain dopamine (DA) neurons, tVTA could possibly play an important role in reward and aversive processing. This could have strong implications when dealing with substance abuse cases, and psychiatric diseases.

2.1.1 Cellular, synaptic and electrophysiological profile

The tail region of the ventral tegmental area is a heterogenous population of neurons, vast majority of which are GABAergic (Jhou et al., 2009), eliciting Fos expression in response to any aversive stimuli as well as psychostimulants (Perrotti et al., 2005; Kaufling et al., 2009). Dense GABAergic efferents from the tVTA have been observed to innervate VTA and SNc, (Jhou et al., 2009b; Balcita-Pedicino et al., 2011), thus exerting a strong inhibitory effect on the midbrain dopaminergic neurons (Lecca et al., 2012). Electron microscopy has affirmed that most of the tVTA axons connecting the VTA are unmyelinated in nature and are comprised of boutons that appear to be contactless with the surrounding dendrites (Balcita-Pedicino et al., 2011). Additional analysis has revealed that the mean firing rate of the tVTA neurons lies somewhere between 11 and 18 Hz, while the average total time of the action potential is approximately around 1ms ms (Jhou et al., 2009a; Jalabert et al., 2011; Lecca et al., 2011). It has also been observed that the neurons in the “core” region of the tVTA have different electrophysiological properties as compared to the “shell” region. The mean firing rate of the core neurons is around 20 Hz while the shell region is lower around 11 Hz, thus confirming the differences in functional properties of both (Jhou et al., 2009). Further research is still required

to understand the functional properties of the non-GABAergic neurons within the tVTA to better understand the whole structure.

2.1.2 Afferents and efferents of the tVTA

Studies involving the tVTA have been carried out in various species such as mice and monkey (Hong et al., 2011; Wasserman et al., 2013), however a proper anatomical in depth analysis involving its boundaries as well as its neuro-connectivity has been carried out in only rats (Jhou et al., 2009b; Kaufling et al., 2009). Retrograde and anterograde tracing has shown a wide distribution of tVTA afferents

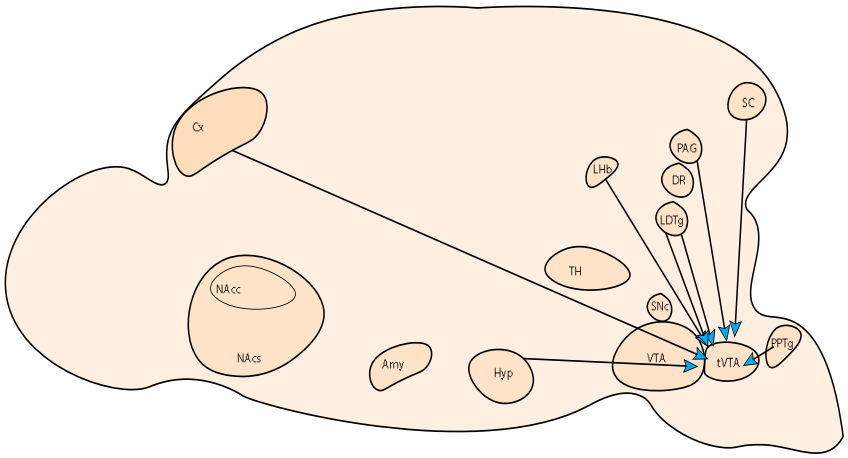


Figure 3. tVTA afferents, the tVTA receives afferents from a broad range of cerebral structures. The main afferents arise from the frontal cortex (Cx), lateral habenula (LHb), hypothalamus (Hyp), superior colliculus (SC), periaqueductal gray (PAG), dorsal raphe (DR), laterodorsal tegmentum (LDTg), and pedunculopontine tegmental nucleus (PPTg).

Afferents to the lateral and medial tVTA arise from the lateral and medial habenula respectively (Jhou et al., 2009b). Studies have also determined that around 35% of LHb axons connecting to tVTA are myelinated, while the remaining, approximately 65% are unmyelinated having no direct contacts on dendrites (Balcita-Pedicino et al., 2011). The afferents originating from LHb are postulated to have glutamatergic effect on tVTA since most of the LHb neurons contain the vesicular glutamate transporter 2 (VGlut2) (Brinschwitz et al., 2010; Root et al., 2014). Studies have also observed afferents to the tVTA from VTA/SNc complex which have been implicated in a feedback loop countering the significant tVTA efferents to these regions (Jhou et al., 2009). Strong afferents have been found to originate from prelimbic and infralimbic cortex, lateral hypothalamus (LH), superior colliculus (SC), periaqueductal gray (PAG), interpeduncular nucleus (IP), and dorsal raphe (DR). Moderately strength afferent connections have also been found from cingulate cortex, nucleus accumbens (NAc), bed nucleus of the stria terminalis, pedunculopontine nucleus (PPTg) and the septum (Kaufling et al., 2009; Yetnikoff et al., 2015).

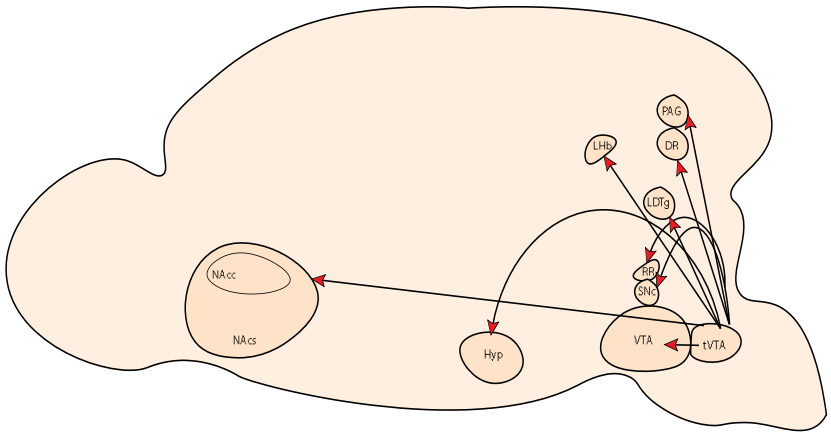


Figure 4. tVTA efferents, the tVTA efferents (in red) are more restricted and preferentially target midbrain dopamine nuclei: ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and to a lesser extent the retrorubral field (RR). The tVTA also heavily projects to the lateral hypothalamus (LH), nucleus accumbens (NAc), superior colliculus (SC), periaqueductal gray (PAG), dorsal raphe (DR).

The tVTA efferents have been observed to have a very focused spread primarily targeting the dopaminergic neurons of the midbrain. The tVTA outputs to the forebrain are quite restricted with the main efferent targeting the lateral hypothalamus (Bourdy and Barrot, 2012). There are also important efferents to the DA neuronal cell bodies of the VTA/SNc complex (Jhou et al., 2009; Kaufling et al., 2010). A topographical study of the tVTA efferents to VTA have shown strong connections between lateral parts of the tVTA and laterally placed neurons within the VTA, whereas medial neurons from the tVTA sent efferents towards the centrally located neurons within the VTA (Jhou et al., 2009). 80% of the synapses between the tVTA axons and the DA neurons within the VTA have preferential connections via dendrites (Bourdy and Barrot, 2012). Other efferent targets from tVTA include ventral pallidum (VP), nucleus accumbens (NAc) lateral habenula (LHb), lateral hypothalamus (LH), periaqueductal area (PAG), laterodorsal tegmental nucleus and pontine reticular nucleus (Kaufling et al., 2010a).

Studies have suggested that tVTA is involved in a lateral habenula-tVTA-VTA pathway as well as a tVTA-VTA-NAc complex neurocircuitry (Jhou et al., 2009). Lateral habenular efferents innervate the tVTA neurons that project to the VTA (Jhou et al., 2009). 55% of the synapses from the lateral habenula connect with the GABA +ve dendrites in the VTA (Bourdy and Barrot, 2012). In a lateral habenula-tVTA-VTA pathway, it has been proposed that the glutamatergic excitatory signals from the LHb is converted by the tVTA into an inhibitory

signal affecting the VTA dopamine neurons (Jhou et al., 2009). Studies have also shown tVTA axons juxtaposed VTA dopamine neurons that further project to the NAc (Kaufling et al., 2010; Bourdy and Barrot, 2012) thus confirming the influence of tVTA on the mesolimbic system. As previously stated, since the tVTA also projects to the SNc, there is a good possibility of its involvement in the nigrostriatal system and the basal ganglia circuitry.

2.1.3 tVTA as a dopaminergic control center

Studies have shown that tVTA could very well be responsible for the regulating the activity of the dopamine neurons (Jhou et al., 2009; Kaufling et al., 2010). This observation has also been confirmed by electrophysiological studies in rats, where inhibiting tVTA increased the dopamine cell activity whereas tVTA stimulation decreased it (Lecca et al., 2012). In vivo studies also observed the same phenomenon where electrical stimulations of the tVTA invoked inhibitory post-synaptic currents (IPSCs) inhibiting VTA dopamine neurons (Matusi and Williams, 2011). One possible technical concern relating to electrophysiological studies in the tVTA is the presence of high density of fibers but optogenetic studies involving the stimulation of tVTA neurons have been known to evoke IPSCs in the VTA dopaminergic neurons, confirming the inhibitory effect of tVTA via the $GABA_A$ receptors (Matusi and Williams, 2011; Bourdy and Barrot, 2012). Using the accelerator/brake paradigm proposed by Arvid Carlsson (for regulation of the monoaminergic brainstem neurons), tVTA could be considered as an important GABA brake center for dopaminergic systems (Carlsson et al., 2001). This concept could potentially be extended to the PFC that projects to both tVTA and VTA and the lateral habenula that has glutamatergic afferents to the aforementioned structures (Bourdy and Barrot, 2012). Thus, the

dopamine activity would be dependent on the glutamatergic “accelerator” and the GABAergic “brake”, fine tuning the firing of DA neurons.

2.1.4 tVTA in aversive responses

tVTA receives inputs from brain structures that are known for their role in aversive responses, namely, ACC (anterior cingulate cortex), septum, PAG (periaqueductal area), extended amygdala and the LHb (lateral habenula) (Matsumoto and Hikosaka, 2009), so there is a good possibility that tVTA might be involved in nociceptive stimuli pathways. Studies have shown that endomorphin-1 injections to the tVTA have reduced the nociceptive response in the second formalin phase in rats (Jhou et al., 2009). This could be due to dopamine dependent analgesic effect or due to the brainstem projects of the tVTA. Food restrictions as well as electric foot shocks have also been shown to increase the c-Fos expression in the tVTA (Jhou et al., 2012). It was also observed that these foot shocks also increased the overall activity of tVTA neurons. In mice, studies have observed that acute unpredictable electric shocks increased the excitatory signals form the LHb to the tVTA (Stamatakis and Stuber, 2012). Lesions to the tVTA have shown to produce reduced behavioral responses (freezing) to a conditional auditory tone (Jhou et al., 2012). In mice optogenetic stimulation of the lateral habenula terminals that are present within the tVTA induced active, passive as well as conditioned behavioral avoidance of the stimulation (Stamatakis and Stuber, 2012). Thus, it will be of great interest to study effect of nociceptive information relay to and from the tVTA and not to look at tVTA as only a DA activity modulator.

2.1.5 tVTA and drugs of abuse

When dealing with drugs of abuse and tVTA the Fos responsiveness is mainly observed. FosB/ΔFosB and c-Fos are induced within the tVTA when exposed to acute or chronic cocaine. The FosB levels were observed 3h post administration of cocaine and the levels remained elevated in the system for over 4 days (Kaufling et al., 2010). Fos proteins were also observed in the tVTA post exposure to amphetamine drugs (Perrotti et al., 2005; Kaufling et al., 2010). The psychostimulant induced Fos and other gene activity within the tVTA could be attributed to DA system as administering a DA reuptake inhibitor (GBR-12909) increased Fos-like immunoreactivity within the tVTA (Perrotti et al., 2005). However, this increase in activity is not limited to psychostimulants with addictive properties, administration of non-addictive psychostimulants such as methylphenidate hydrochloride used in treatment of ADHD induced the same effect (Kaufling et al., 2010). Administration of drugs such as morphine, ethanol, THC (Δ9-tetrahydrocannbinol) solution failed to produce any Fos like activity within the tVTA drugs (Perrotti et al., 2005; Kaufling et al., 2010). In another experiment administration of acute morphine in anesthetized rats decreased the firing rate of tVTA neurons (Jalabert et al., 2011). Thus, through a disinhibition process tVTA may act as an important target for certain drugs of abuse such as opioids and cannabinoids.

2.2 VTA (ventral tegmental area)

Located on the floor on the midbrain, the VTA (ventral tegmental area) has long been considered to be an influential region when dealing with nociceptive processing. Previously named as the "ventral tegmental nucleus" (Tsai, 1925), further research defined it as the "ventral tegmental area" mainly because of its heterogeneity. The neural makeup of the VTA was first suggested by Johnson and North, 1992, where they described the two main types of neuron: primary and secondary. This differentiation was based around electrophysiological and pharmacological studies. Basically, neurons that had a longer duration of action potentials (APs) were postulated to be DA neurons while the ones with shorter APs were postulated to be GABAergic. Combining studies till date has made it somewhat possible to predict the complexity of the VTA neuronal population with some accuracy. The split being approximately 60% are dopaminergic, around 25% are GABAergic, and only a very small population 2-15% are glutamatergic (Ungless and Grace, 2012; Yamaguchi et al., 2011). There is also a small population of combinatorial neurons that have been observed via electrophysiological studies (Morales and Margolis, 2017). The DA neuronal activity is regulated by inputs form different regions of the brain in tandem with the local GABAergic and glutamatergic neurons. In addition to being locally connected, the VTA GABAergic and VTA glutamatergic neurons innervate various other structures via long range projections, which we will discuss in the next few sections to better understand the importance the structure plays in decision making, negative and positive reinforcement, aversion and the one that is of the most interest in this paper, nociception.

2.2.1 Cellular, synaptic and electrophysiological profile

There have been numerous studies carried out for several decades trying to understand the neuronal diversity of the VTA. Recent findings have suggested that subset of DA neurons that share similar properties tend to be concentrated together within the subregions of the VTA (Morales and Margolis, 2017). Scientific consensus regarding the compartmentalization of the VTA has not reached yet, however, the latest consensus suggests VTA has 5 subregions that contain the "A-10" group of dopamine neurons. As previously stated, the largest population of the neurons present in the VTA are the dopamine neurons. Dopamine is produced when tyrosine hydroxylase (TH) converts l tyrosine to l DOPA (3,4 dihydroxyphenylalanine), which is then further converted into dopamine by aromatic-l amino-acid decarboxylase. VTA DA neurons have been typically known to target and innervate a single region, with the different subpopulations innervating NAc, amygdala, globus pallidus and LHb (Swanson, 1982; Beier et al., 2015). DA neurons have also been identified based on electrophysiological properties, that include a long action potential (triphasic), a low firing rate and the occurrence of the I_h current (Olivia and Wanat, 2016). The medial aspect of VTA has dopamine neurons that display the I_h current but do no express TH. It is wise to take into account that the action potential and the I_h current are not the best at confirming the dopamine content of any neuron, however the electrophysiological analysis does help predict where the VTA dopamine projections end up.

The second largest subpopulation within the VTA is GABAergic neurons that are primarily identified via GAD (decarboxylase) and glutamic acid (Nair-Roberts et al., 2008). The GABAergic neurons have been observed to directly influence the activity of VTA dopamine

neuron, while also further projecting to the ventral pallidum (VP), lateral hypothalamus (LH), lateral habenula (LHb), nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Tan et al., 2012). A recent study also identified dopamine neurons as a potential source of GABA within the VTA through an aldehyde dehydrogenase-mediated pathway (Kim et al., 2015). VTA and substantia nigra have been known to bundle up GABA and transport them using the vesicular transport vis vesicles. This basically means that GABA could potentially be released along with dopamine to produce the desired effects on the medium spiny neurons within the NAc.

The third subpopulation of neurons present are the glutamatergic (displaying the VGluT2 [vesicular glutamate transporter 2] marker). These neurons have been known to be located on the medial aspect of the VTA, mainly innervating PFC, VP, LHb and amygdala (Aransay et al., 2015). There also exists a smaller subset of VGlut2+ve neurons that also express TH. Projecting to PFC and ventral striatum these particular neurons have been known to release both dopamine and glutamate (Chuhma et al., 2014). It was believed that VTA was solely comprised of dopamine and GABA neurons, however, recent research has quantified the presence of combinatorial neurons that release dopamine-GABA, dopamine-glutamate, and glutamate-GABA (Morales and Margolis, 2017).

2.2.2 Afferents and efferents of the VTA

A combination of electron and light microscopy, researchers have been able to accurately predict connectivity of most of the human brain in general. Similar approaches were used when quantifying the connections of the VTA, providing a comprehensive picture of what exists. It has been observed that VTA neurons receive excitatory and inhibitory connections

from a variety of structures. Many studies have characterized the sources from which the glutamatergic or GABAergic synapse on the dopamine and GABA VTA neurons (Morales and Margolis, 2017) however there are no studies (at least I did not come across) that highlight glutamatergic and GABAergic inputs onto the VTA glutamate neurons. Glutamatergic inputs innervate the VTA dopamine neurons from PFC, mesopontine tegmentum nucleus, lateral habenula (LHb), subthalamic nuclei and periaqueductal area (PAG) (Omelchenko and Sesack, 2010). Also, there are significant glutamatergic inputs innervating the non-dopaminergic neurons form the LHb, PAG, PFC (Omelchenko and Sesack, 2010). GABA inputs to the VTA are quite extensive, with the RMTg (rostromedial mesopontine tegmental nucleus) or the tVTA synapsing with almost 80% of the VTA dopamine neurons (Balcita-Pedicino et al., 2011). The GABAergic inputs from the tVTA still remain as the largest source of GABAergic projection into the VTA. There is also evidence that GABAergic projections also arise from the NAc via the medium spiny neurons onto the non-DA VTA cells (Xia et al., 2011). Being inhibitory in nature these inputs have been known to disrupt the VTA neuronal activity. Studies have also indicated that there is a complex region-specificity where the glutamatergic inputs innervate the VTA, this just makes fully understanding the region even more convoluted. There are also serotonergic inputs onto the VTA form the dorsal raphe nucleus and the medial raphe nucleus (Adell and Artigas, 2004).

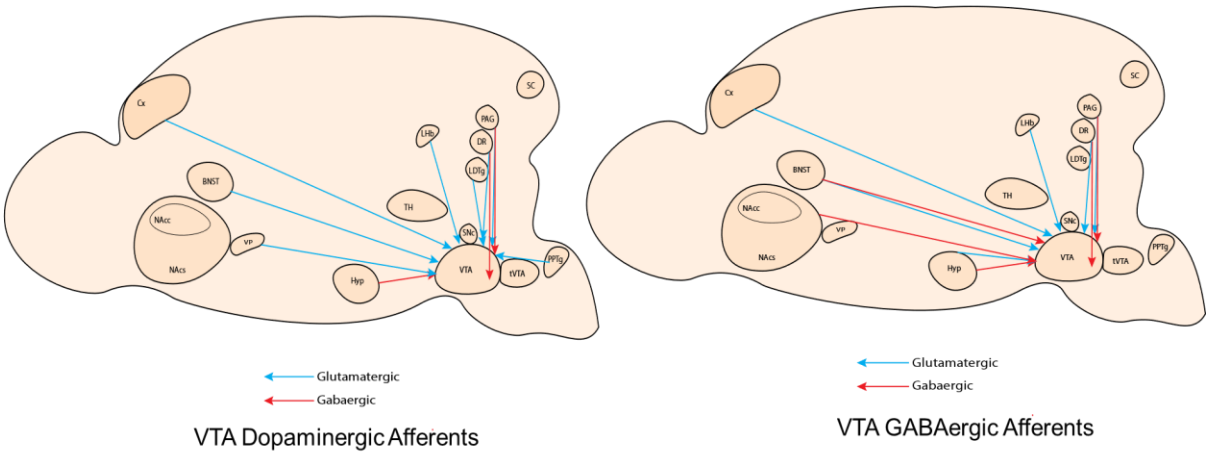


Figure 5. VTA afferents, GABAergic (in red) and Glutamatergic (blue) arrive from ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and to a lesser extent the retrorubral field (RR). The tVTA also heavily projects to the lateral hypothalamus (LH), nucleus accumbens (NAc), periaqueductal gray (PAG), dorsal raphe (DR), cortex (Cx), lateral habenula (LHb), bed nucleus of the stria terminalis (BNST). Ventral pallidum (VP) and laterodorsal tegmentum (LDTg),

One of the major efferent targets from VTA is PFC. The “mesocortical” pathway is thought to consists primarily of GABA neurons (60%) but studies have shown that it also contains a significant amount of dopamine neurons (25%) (Yamaguchi et al., 2011). It has been hypothesized that this pathway might be responsible for combining the nociceptive stimuli with the cognitive information, which then are relayed back to the NAc to make behavioral decisions. The other important pathway is the “mesolimbic” pathway that involves the DA and the non-DA efferents from the VTA towards the limbic system. There are important areas that are involved in this pathway that are important for nociceptive processing, including its emotional and motivational aspect of it. The regions involved are amygdala, hypothalamus, and the NAc. Another important research with regards to the efferents from the VTA deal with its projections to the NAc, as it innervates differently based on the core and shell region, which in turn, alters the overall role it plays in pain and reward.

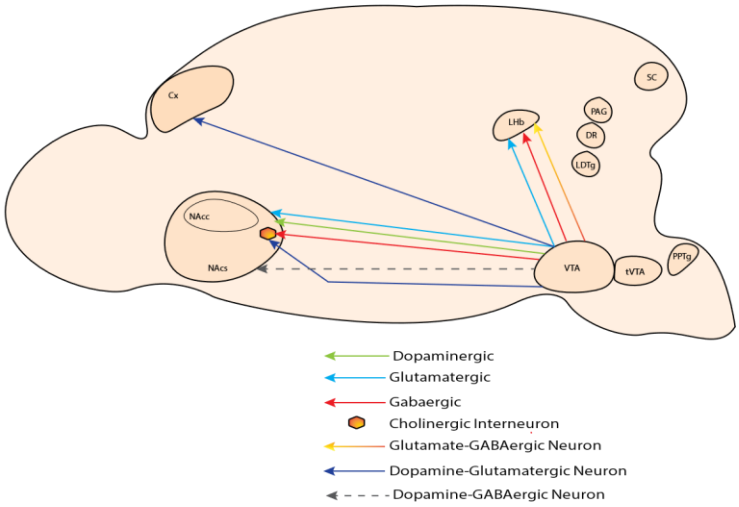


Figure 6. VTA efferents, VTA sends out dopaminergic, glutamatergic, GABAergic, cholinergic, and combinatorial neuronal efferents towards lateral habenula (LHb), nucleus accumbens (NAc) and the cortex (Cx).

2.2.3 VTA in aversive responses

The role of VTA in the reward system has been extensively studied but recent studies have also examined its role in nociception processing. Research has shown that acute noxious stimulations tend to produce phasic responses in the VTA DA neurons (Brischoux et al., 2009; Cohen et al., 2012). Elevated dopamine levels were observed in the NAc and the medial prefrontal cortex after receiving aversive stimuli which were confirmed using fast scan cyclic voltammetry (FSCV) and micro-dialysis (Bassareo et al., 2002; Budygin et al., 2012). Experiments with direct electrical stimulation of the dopamine neurons within the VTA and also administration of selective agonist leading to the activation of D2 (dopamine D2) receptors in the nucleus accumbens, produced antinociceptive effects in rodent models of pain (Sotres-Bayón et al., 2001). On the other hand, administering D2 receptor antagonists in the nucleus accumbens caused increase in nociceptive sensitivity, suggesting the important role VTA plays

in pain processing and also the dampening role of the dopaminergic signaling (Sotres-Bayón et al., 2001; Wood, 2008). Measuring D2 receptor binding levels is often tested as a direct response to nociception in humans (Hagelberg et al., 2002), and also patients suffering from Parkinson's disease (death of dopamine neurons) develop some form of chronic pain (Silva et al., 2008). It is also observed that there is activation of DA neurons and release of dopamine during nociception, and the enhanced activity likely exerts an inhibitory influence on the levels of nociception (Morales and Margolis, 2017). Hence form the above studies it could very well be postulated that VTA could play an important role in nociception processing.

2.2.4 VTA and drugs of abuse

Drugs of abuse along with stress have been known to alter the synaptic plasticity within the ventral tegmental area (VTA) (Niehaus et al., 2010). It is postulated that such modifications are the main factors behind various aspects of addiction. Excitatory as well as the inhibitory synapses on the DA neurons are adept at undergoing long-term changes. One study has found that morphine could possibly disinhibit glutamatergic inputs present on the DA neurons in the VTA, which in turn could possibly trigger the pre-synaptic glutamate release (Yang et al., 2020). Another electrophysiological study observed that acute morphine administration increased the local field potential frequency band within the VTA (Soleimani et al., 2018). But another study observed that the firing rate of the VTA DA neurons was higher in morphine dependent rats as compared to naïve animals with acute morphine injections (Georges et al., 2006). Another study observing the long-term potentiation of drug effects on VTA found out that administering

morphine decreased the midbrain inhibitory synapse activity to express LTP_{GABA} (Niehaus et al., 2010).

2.3 NAc (Nucleus Accumbens)

The nucleus accumbens (NAc) is recognized to be highly important structure within the ventral striatum since its involved with motivation as well as emotional processes. Research has also proven its involvement in limbi-motor interface, and it is also considered to be a major target for certain psychoactive drugs (Salgado and Kaplitt, 2015). Studies have postulated and studied its involvement in variety of neurological diseases ranging from depression, OCD (obsessive compulsive disorder), anxiety, Parkinson's, Alzheimer's, and Huntington's. Nucleus accumbens has a wide array of neuronal connections which has led to the increased research in the recent times. Targeted regularly for potential therapeutic treatments of physiological and psychological disorders, one therapeutic aspect that is overlooked is nociception.

2.3.1 Cellular, synaptic and electrophysiological profile

The main cell type (90-95%) in the nucleus accumbens are the medium spiny neurons (MSN), GABAergic in nature. These are further subdivided into two subpopulations based on the dopamine receptor (D1 or D2) as well as their neuropeptide expression (Gerfen and Surmeier, 2011). The dopamine receptors on the medium spiny neurons are G-protein coupled receptors with opposing effects on the intracellular signaling cascades. This is the main reason

behind the variable response the receptor shows towards dopamine, thus giving the different cell populations within the NAc with different physiological properties (Gerfen and Surmeier, 2011). The D2 dopamine receptor tend to couple with $G\alpha_{i/o}$ proteins, which then inhibits adenyl cyclase and cAMP production. This in turn causes opposing effects on the intracellular signaling along with the gene expression (Beaulieu and Gainetdinov, 2011). Studies have also revealed a role for D1 MSN in regulating behavior induced by psychostimulants in a more positive way whereas D2 MSN does this the exact opposite way (Park et al., 2013).

The second biggest concentration of cells in the NAc are the interneurons (5-10%). They are further subdivided into several classes based on the protein profile they express. (Kawaguchi et al., 1995). The three main subpopulations of interneurons are as follows, the ones that express parvalbumin, the ones that express somatastatin, neuropeptide Y along with neuronal nitric oxide synthase at the same time and lastly the ones that express calretinin (Tepper et al., 2010). The fourth subdivision is the class of neuron expressing choline acetyltransferase also known as cholinergic neurons.

2.3.2 Afferents and efferents of the NAc

Studies have shown that the NAc could very well be involved in nociceptive processing based on its connections to the other brain structures that are well known for nociceptive modulations. There are dopaminergic afferent projecting from the VTA (ventral tegmental area) and substantia nigra, while it receives glutamatergic projections from the amygdala, thalamus, PFC (pre frontal cortex), hippocampus and the prelimbic cortex (Gupta and Young, 2018; Li et

al., 2018). The nucleus accumbens is divided into a shell and core region and both have very distinct connections, the core region receives afferents from the dorsal prelimbic cortex, perirhinal cortices, substantia nigra and the anterior cingulate cortex (ACC) (Salgado and Kaplitt, 2015; Li et al., 2018). While the shell receives inputs from the basolateral amygdala, lateral hypothalamus, and the ventromedial prefrontal cortex (Salgado and Kaplitt, 2015; Li et al., 2018). There is an exception where both regions receive inputs from the ventral tegmental area (VTA) but the lateral region of the VTA targets the core while the medial region of VTA targets the shell (Li et al., 2018). Studies using retrograde labeling have also found connections from the spinal cord innervating directly the NAc, opening the door to postulate the involvement of this region in nociceptive relay (Gear and Levine, 2011; Salgado and Kaplitt, 2015).

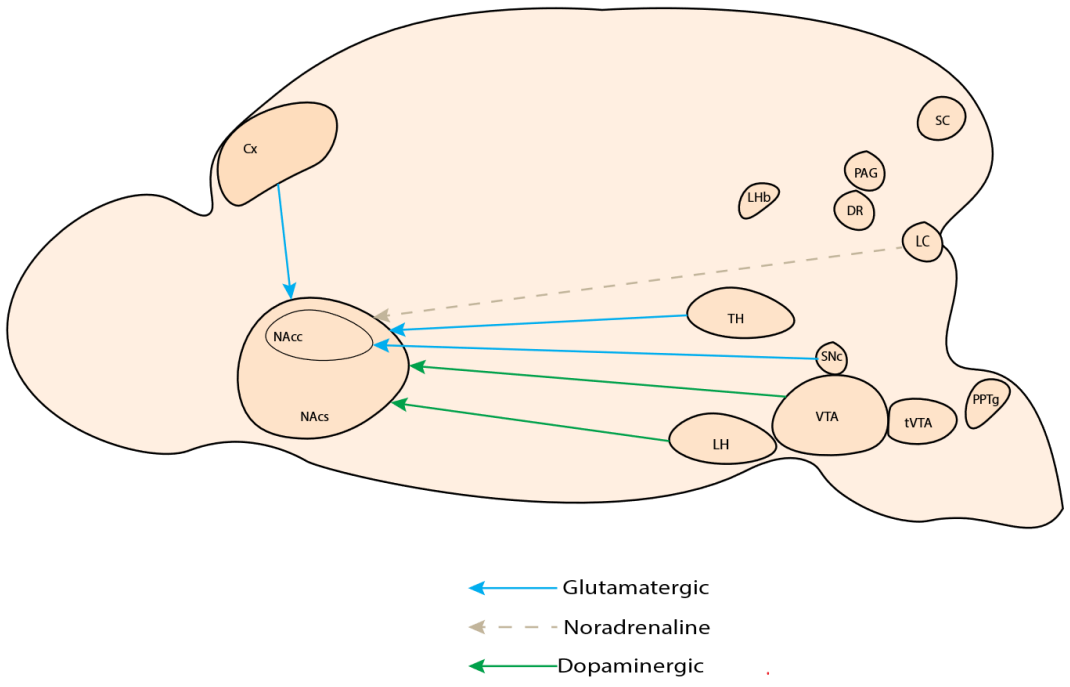


Fig. 7 NAc afferents, Dopaminergic (green) and Glutamatergic (blue) and noradrenergic (dash arrow) arrive from ventral tegmental area (VTA), locus coeruleus (LC), lateral hypothalamus (LH), substantia nigra pars compacta (SNc), cortex (Cx), lateral habenula (LHb) and Thalamus (TH).

Efferents from the NAc regularly target the regions within the diencephalon and pallidal complex such as the stria terminalis, lateral hypothalamus, substantia nigra, nucleus mediodorsalis thalami and the globus pallidus (Bálint et al., 2011; Salgado and Kaplitt, 2015). The core region of the NAc is known to project towards the lateral hypothalamus, amygdala, and the ventral pallidum (Bálint et al., 2011; Salgado and Kaplitt, 2015). Rodent studies have also revealed that both the shell and core project towards the entopeduncular nucleus, which is equivalent to the globus pallidus in humans (Harris and Peng, 2020).

There are also studies that have specifically looked at pain processing and NAc, since there are direct connections to the ACC, PFC, amygdala, thalamus, somatosensory cortex and the spinal cord input (Bálint et al., 2011; Gear and Levine, 2011; Salgado and Kaplitt, 2015; Li et al., 2018), this could actually force researchers to look at the NAc as more than a region associated with pleasure.

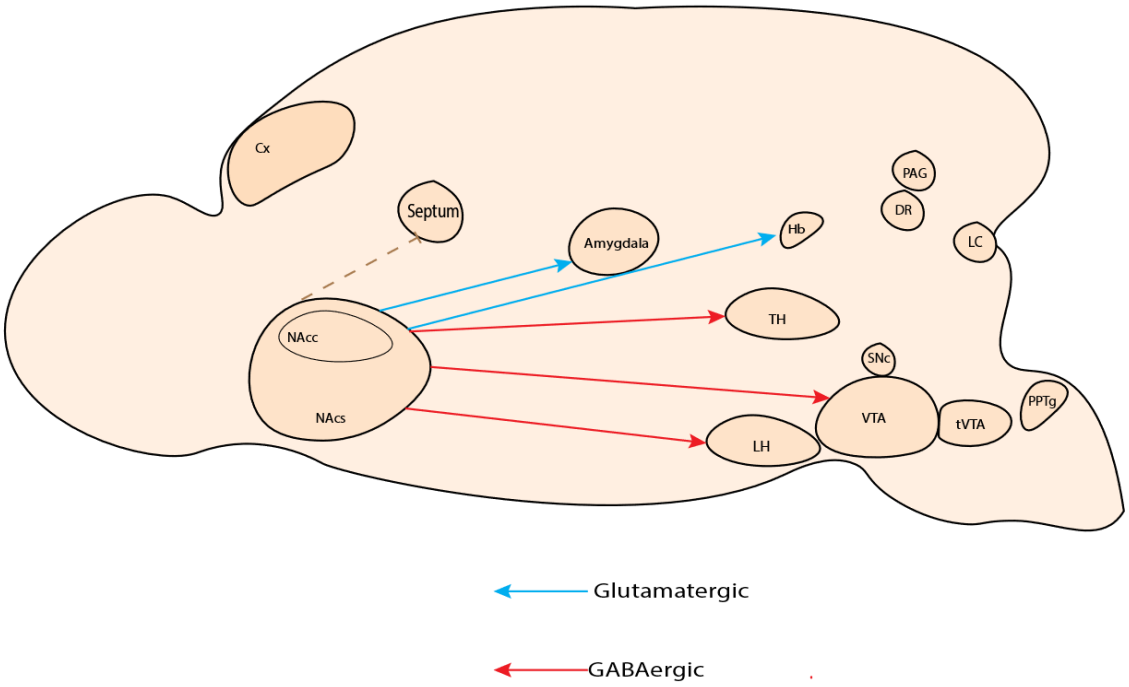


Fig. 8 NAc efferents, GABAergic (Red) and Glutamatergic (blue) projections innervate the from ventral tegmental area (VTA), habenula (Hb), lateral hypothalamus (LH), lateral habenula (LHb), Thalamus (TH), Amygdala, and Septum.

2.3.4 NAc in aversive responses

Studies have proven that lateral habenula receives projections from nucleus accumbens as well as the medial frontal cortex (Bianco and Wilson, 2009). The aforementioned structures have been well documented to be involved in nociceptive processing, so it could be possible that NAc could very well be involved as well along with being part of the pleasure pathway. NAc also sends efferents to the amygdala and also to the ventral pallidum, two regions that have been studies to be heavily involved as nociceptive modulators (Kato et al., 2016). Studies have also shown that both the core and shell region have feedback loops with regions of the brain that are directly involved with processing nociception, which could very well point towards the

fact that NAc receives afferents and projects efferents to regions in the pain pathway (Seminowicz et al., 2019; Harris and Peng, 2020).

2.3.5 NAc and drugs of abuse

Opiates have been long known to reduce nociceptive sensations by mainly mimicking the anti-nociceptive effect of endorphins. Research has shown that opiates primarily target medulla, midbrain and the pons to produce these antinociceptive effects (Erfanparast et al., 2018). Earlier studies mainly identified PAG, locus coeruleus and nucleus raphe magnus as the primary brain regions affected by morphine however recent studies have also identified the same antinociceptive effect exhibited by NAc increasing the neuronal activity (Hong et al., 2017; Yoshida et al., 2019). Another study by Corkrum et al., 2019 also observed the involvement of "astrocytes-neuron signaling mechanism" using calcium imaging as well as whole-cell patch clamp electrophysiological analysis in brain slices. These could be the reasons behind the increased neuronal activity in the NAc.

Studies have also observed that ethanol has no effect on the dopamine uptake however in chronic ethanol treatment there was enhanced dopamine uptake (Budygin et al. 2007). FSCV studies pertaining to nicotine, cocaine both increased the amplitude as well as the frequency of the spontaneous phasic DA release within the NAc (Wu et al. 2001).

2.4 ACC (Anterior Cingulate Cortex)

The anterior cingulate cortex (ACC) is located on the frontal aspect of the cingulate cortex, that is known to form a large region around the corpus callosum in the mammalian brain. Research dealing with both human and animal ACC has demonstrated that it plays an important role when dealing with pain, fear, anxiety, sexual arousal, memory as well as learning (Neugebauer et al., 2009). Studies have also observed spinal nociceptive tail flick reflex, memory and aversive learning when subjected to chemical or electrical stimulation of the ACC (Calejesan et al., 2000; Tang et al., 2005). It is a well-known fact that pain is multifaceted in nature and research over the years has provided crucial information about the prominent brain areas involved with nociception, that include, ACC, insular cortex, primary somatosensory cortex (S1), secondary somatosensory cortex (S2) and the prefrontal cortex (PFC) (Bliss et al., 2016). MRI studies have also observed ACC activation during neuropathic and visceral nociceptive conditions such as IBS (irritable bowel syndrome) (Bliss et al., 2016). ACC involvement has also been observed in emotional and psychological pain; hence it can be of interest to look at this region for negative effects related to pain.

2.4.1 Cellular, synaptic and electrophysiological profile

The anterior cingulate cortex is a part of the thalamo-limibic-cortical system, which means that it receives inputs from the thalamus while sending out connections towards the

hippocampus, hypothalamus and the amygdala (Zhuo et al., 2007). The anterior cingulate cortex is made up of several layers numbered as I, II, III, V and VI. Small local interneurons make up the layer I and numerous projected fibers, from the central nuclei, pass through this first layer as well. Pyramidal cells make up later II and III, and these receive inputs from the thalamus and project further into the deeper layers (Jun Wu et al., 2009). Projections from layers II and III and the thalamus innervate the pyramidal neurons in layer V and sends out projections further into the subcortical structures (Wang and Shyu, 2004). Within the laters II-VI there are larger number of interneurons (Zhuo et al., 2007). It is postulated that neurons within the ACC form excitatory and inhibitory connections, but direct evidence relating to the connections between pyramidal and interneurons has not been concretely established. Research has also observed that pyramidal cells have larger somata of around 15-30 µM while the interneurons range from 10-15 µM (Jun Wu et al., 2009). The resting membrane potential for both pyramidal and interneuron was also determined to be at –71.3 mV and –67.8 mV respectively (Jun Wu et al., 2009).

2.4.2 Afferents and efferents of the ACC

The prefrontal cortex of rats is divided into three regions and ACC makes one of the divisions, others being agranular insular and orbitofrontal areas (Zilles & Wree, 1995). Zilles and Wary, 1995, defined the three subregions of the ACC as Cg1, Cg2, Cg3, however Paxinos G., 1998 defined Cg3 as prelimbic cortex (PrL). The medial prefrontal cortex, along with the anterior Cg1 and the Cg3 project o the medial nucleus accumbens (NAc) along with the

amygdala. Cg1 and Cg2 are known to project to the mediodorsal nucleus of the thalamus (MDL), caudate, amygdala, while the posterior cingulate cortex projects to the anteroventral and the anterodorsal nuclei, hippocampus, visual cortex and the mediodorsal striatum (Paxinos G., 1998; Zilles & Wree, 1995). The ACC shares reciprocal connections with the prefrontal cortex along with the basolateral amygdala and also has connects (directly and indirectly) with ventral striatum. Along with its projections to the mediodorsal striatum, it also projects to the core and rostral pole of the nucleus accumbens. The ACC receives a major dopaminergic afferent from the VTA (Zilles & Wree, 1995) and sends a major efferent to the ventral striatum, which is part of the "limbic loop" of the basal ganglia, which is projected back to the ACC and the medial pre frontal cortex via the ventral pallidum. These connections from the basis of the theory that both the medial prefrontal cortex and the ACC are the primary regions whose information content is highly influenced by nucleus accumbens.

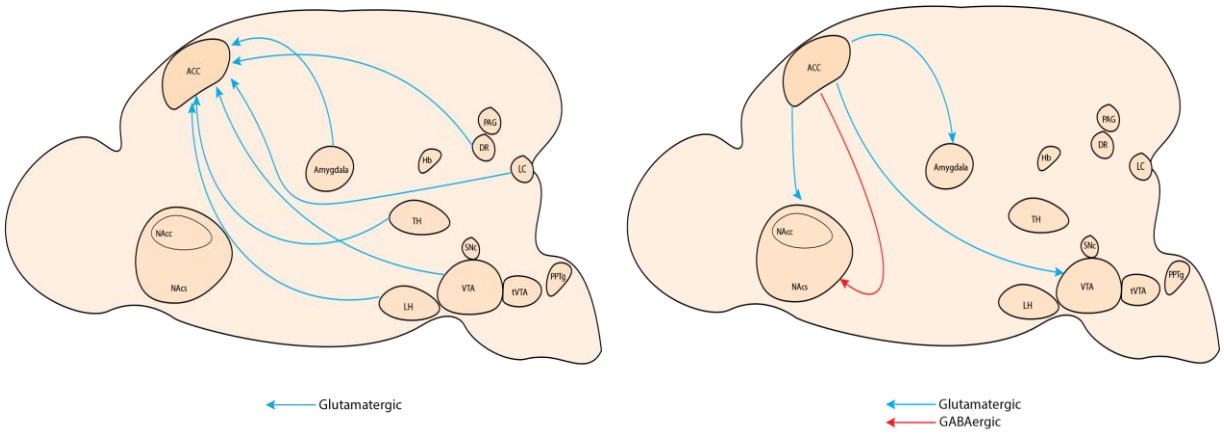


Fig. 9 ACC afferents and efferents. Glutamatergic (blue) arrive from ventral tegmental area (VTA), locus coeruleus (LC), lateral hypothalamus (LH), cortex (Cx), Dorsal raphe nucleus (DR) and Thalamus (TH), and Amygdala. ACC projects glutamatergic connections back to the VTA, Amygdala and also to the Nucleus Accumbens which is a GABAergic efferent.

2.4.3 ACC in aversive responses

Research has observed that nociceptive information from the visceral and somatic areas is passed on to the ACC via three major projections. The first projection comes from the thalamus, specifically the ACC received this nociceptive afferent from the medial thalamus, which is turn received those signals from the spinal projections (spinothalamic tract) (Shyu and Vogt, 2009). Electrophysiological analysis of the spino-thalamic tract proved the functional aspect of the aforementioned connection pathway (Yang, et al. 2006). A recent study it was observed that the neurons within the mediodorsal thalamus directly innervate and stimulate the parvalbumin +ve interneurons located in the dorsal ACC. Its postulated that these neurons then further inhibit the pyramidal neurons within the layers II and III (Delevich et al., 2015). The second nociceptive projection into the ACC is through the amygdala, which includes the central nucleus receiving this nociceptive information via the parabrachial area (Ma and Peschanski, 1988). The third source of nociceptive afferent to the ACC is from the cortical area such as S1 and insular cortex.

Pyramidal cell within layer V of the ACC have been known to project to the hypothalamus and the PAG, the later of the two is known to be involved in descending spinal sensory transmission. Studies have also revealed a direct projection from the ACC pyramidal cells to the dorsal horn and the spinal cord (Chen et al., 2014). Research has also shown connections between ACC and the amygdala, which is known to play an important role in fear and anxiety (Tovote and Luthi, 2015). In another mammalian study directly stimulating the ACC produced vocalization which could have been an indicator of nociception and fear. It was

observed that the connections between ACC and motor cortex were possibly involved (Devinsky et al., 1995). ACC also projects to the locus coeruleus, a region that is known for its involvement in thermal nociceptive thresholds in mice (Jones and Cohen, 2005). Another reason why ACC might be involved in nociceptive information transmission is due to the cholinergic, dopaminergic, adrenergic as well as serotonergic connections it receives from the subcortical regions (Chandler et al., 2015).

2.4.4 ACC and drugs of abuse

Previous research has proven the importance of ACC in motivations, cognitive and evaluative functions (Navratilova and Porreca, 2014) along with nociception (Vogt B.A., 2005). In another study, ACC activated using glutamatergic inputs have shown to elicit pain-like responses in naïve rats (Johansen and Fields, 2004). Another study investigated the effect of opioid on the three sub regions of ACC and the RVM (rostral ventromedial medulla). Morphine was cranially microinjected to produce analgesic responses in RVM to thermal and mechanical noxious stimuli in injured rats, however similar morphine microinjections into the ACC had no significant effect on the thermal or mechanical responses and was responsible for reducing nociception only in one of the three ACC sites. Thus, it was postulated that systemically administered opioids acted preferentially within the ACC, helping to alleviate the emotional quality of pain without affecting the nociceptive threshold (Bingel et al., 2011; Navratilova et al., 2015).

ACC has long known to be involved with various cognitive functions such as conflict monitoring, arousal, feedback, error processing, attention control and prediction errors

(Vazquez et al., 2019). In a study by Vazquez et al., 2019 showed that administration of cocaine heavily diminished neural signals associated with attention control within the ACC and the neural firing patterns and behavioral changes were directly related with the amount of cocaine self-administration by the rats. The overall result of the diminished attention is the impact it has on other neuronal processing specifically decision making. Studies have also established correlation between risky behavior and abnormalities in decision making with nicotine addiction, with the dorsal anterior cingulate cortex (dACC), insula, thalamus and striatum as the main brain regions involved in it (Wei et al., 2016; Hong et al., 2009). Acute administration of nicotine did not show any significant effect on the aforementioned neuronal circuits, however it did enhance the cingulate-neocortical neuronal connections that are not primarily associated with the addiction, but have been postulated to play an important role in enhancing nicotine's cognitive properties (Hong et al., 2009).

2.5 Overall Importance

Thus, research has also shown that tVTA is involved in a LHb-tVTA-VTA pathway and also a tVTA-VTA-NAc (nucleus accumbens) pathway. The tVTA is basically acting as a relay between the LHb and the VTA/SNc complex. In the tVTA over 55% of the synapses from the LHb are present on the GABAergic dendrites and in the VTA most of the synapses from the tVTA are GABAergic in nature ending on dopaminergic dendrites (Jhou et al., 2009; Kaufling et al., 2010). The tVTA has been proposed to convert the glutamatergic excitatory signal from the lateral habenula into a inhibitory one exerted on the VTA DA neurons. It has also been observed that the axons from the tVTA are placed in proximity with the VTA dopamine neurons that further

project to the NAc. This in fact supports the paradigm that tVTA could possibly influence the mesolimbic system. The tVTA has also been known to project to SNc, thus possibly establishing some influence over the nigrostriatal system as well. Fos expression studies have also shown that tVTA belongs to a circuitry that connects it with the cortex as well. Thus possibly exerting a control or at least acting as relaying station for the mesocorticolimbic as well as the nigrostriatal pathway, it can be postulated that tVTA acts a hub converging an array of signals from various brain structures and funneling them towards the dopaminergic systems and other forebrain regions. Though there have been studies trying to understand the neurocircuitry of tVTA-VTA-NAc and cortex, using LFP to get real time information with regards to how these regions respond simultaneously to noxious stimuli or to a combination of opioid and a noxious stimuli has been not been seen yet. Thus, the present study aimed at understanding how tVTA might be involved in nociception. Since the tVTA acts as a hub it was of great interest to observe the activities of the other structures such as the VTA, NAc and the ACC.

Chapter 3

3.1 Aims and Rationale

From the preclinical data and the literature review it was quite evident that mesolimbic reward pathway exhibited a trend under the influence of a noxious stimuli followed by administration of opioids, while recording from four areas simultaneously. The **hypothesis** of the experiment was that differences would be observed in the reward circuitry during noxious as well as during morphine administration. The effect opiates have on the spike activity of the mesolimbic system have been observed, however there have been limited studies that focus on collecting data simultaneously from four brain areas. Thus, the overall **Aim** of the study was observing opioid-induced alterations of local field potentials (LFPs), especially post noxious stimuli and the involvement of dopamine (DA) system in this process recorded simultaneously from the four brain regions.

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

Specific Aim 2: To determine the antinociceptive effect of morphine in these four areas as measured by the LFP activity.

The **rationale** behind the experiment is as follows, the tVTA is a GABAergic nucleus discovered in 2009 that has been observed to act as a major inhibitory input to the VTA, heavily influencing the dopaminergic activity (Barrot, 2014; Jhou et al., 2009). As stated, earlier tVTA neurological connections have been postulated to play an influential part not only in the mesocorticolimbic pathway but also in the nigrostriatal pathway (Barrot, 2014; Rotllant et al., 2010). Research has

also shown that neurons from the tVTA overwhelmingly synapse on the tyrosine hydroxylase (TH) positive neurons within the VTA as well as the substantia nigra (Y. Li et al., 2016) and electrically stimulating the tVTA suppresses the DA neuron firing (Balcita-Pedicino et al., 2011). Studies in the past have proposed that tVTA may play an important role conveying information about the noxious stimuli to the VTA dopamine neurons and mediate the appropriate response (Barrot et al., 2012; Jhou et al., 2009).

There are however some fundamental questions still unanswered and unaddressed. Previous studies have tested tVTA response to a limited range of aversive stimuli, such as footshocks, aripuffs and their predictive cues (S. Hong, Jhou, Smith, Saleem, & Hikosaka, 2011; Jhou et al., 2009). One recent study was an exception where they examined 11 distinct aversive stimuli apart from the ones mentioned before however only 3 of the 11 increased the tVTA expression of c-FOS which is a gene used as a proxy to confirm neuronal firing (H. Li, Pullmann, Cho, Eid, & Jhou, 2019). This does raise questions about generalization of tVTA to various aversive stimuli and the effect on the noxious stimuli if an aversive stimulus is introduced. There are also recent studies that have observed the complex nature of DA response to aversive stimuli, which often prominently inhibitory in nature (Matsumoto, Tian, Uchida, & Watabe-Uchida, 2016; Tian et al., 2016). Previous studies have indicated that these inhibitory responses are driven by the GABAergic transmission onto the dopamine neurons, hence it's important to observe opioid-induced alterations of local field potentials (LFPs), especially post noxious stimuli (formalin) and the involvement of dopamine (DA) system in this process recorded simultaneously from the four brain regions.

Chapter 4

Methods and Materials

4.1 Animal Selection

Thirty-six 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 and were tested 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 followed the ethical guidelines for pain experiments in animals. The rats were split into four groups. The first group had 6 animals as control injected with saline. Group two was the pure formalin group that had 12 animals administered with formalin. Group 3 had 12 animals as well administered with formalin and morphine. While the last group, Group 4 had 6 animals, all administered with pure morphine injections.

4.2 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, and at post morphine and formalin injections. Under isoflourane anesthesia, the electrode was placed at the following coordinates,

Region	ML (mm)	AP (mm)	DV (mm)	D^{o} (degrees)
VTA	2.1	-5.4	8.5	10
tVTA	1.32	-7	7.6	10
NAcs	2.0	+2	7.8	15
Cg1 Cg2	1.96	+0.6	3.6	15

Table 2. Stereotaxic Coordinates. Table represents the four different regions with their stereotaxic coordinates and the angles at which the electrodes were implanted.

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 that held 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) were used to link the electrodes to the wireless recording module.

4.3 Inflammatory pain model

To test the hypothesis that the local field potential readings of the areas after formalin injections will be significantly different as compared to the baseline, the animals were administered with 3% formalin (0.5 ml) in the left hind paw. After injecting formalin, the LFP was recorded immediately for spontaneous activity for an hour.

4.4 Morphine Administration

Morphine (Sigma-Aldrich, USA) was dissolved in saline (10 mg/kg) and was administered intraperitoneally in two groups. Group 3 which was the formalin + morphine group. The morphine was administered at the same time as formalin around the 10-minute mark. Group 4 was the pure morphine group, in which morphine was also administered at the 10-minute mark. To compensate for group 3 that had two drugs being administered at the same time, group 4 rats were administered with saline (as a formalin substitute) and group 2 the pure formalin group had saline administered to compensate for the morphine.

4.5 Locomotion test

Locomotion of rats was measured by an automated video tracking system. Rats were placed into a big tub on the ground. A video camera was mounted at the top of the test chamber, which was connected to a computer to record locomotion of the rats. The video recordings were analyzed using behavioral analysis software (EthoVision XT11.5, Noldus Information Technology, The Netherlands). Locomotion was measured as the total distance travelled in cms and then converted into feet for ease. This test was carried out pre-and post-surgery to confirm that no changes have been introduced in the animal's ability to move due to the surgery.

4.6 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).

4.7 Histology

Once the animals are 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 sink to the bottom of the tube they were sliced and sectioned at 80 µm thickness and then stained using thionine. These sectioned and stained slices were 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.

4.8 Statistics and Analysis

For data acquisition, the raw local field potential raw traces/raw data were recorded using custom designed data acquisition software. The recorded files were saved as text files and further imported into MATLAB (Copyright 2017 The MathWorks, Inc). A custom code programmed by the graduate students in Dr. Peng's lab was used to split the raw data text file into five-minute time bins. A histogram was obtained for each of the power spectrums ranging from 0-100 Hertz for the following frequency bands, (Delta 1-3 Hz, Theta 3-8 Hz, Alpha 8-13 Hz, Beta 13-30 Hz, and Gamma 30-100 Hz) were separated in the excel file.

The mean of power at each of the frequency was computed in excel (for both the baseline and the post injection) and then graphed with their standard error of means. The same data was imported into SPSS for further analysis. The analysis between the different frequency bands was conducted in SPSS using repeated measures factorial ANOVA followed by LSD post hoc tests. This helped determine if there were any differences between the baseline and after

formalin injection and then after injecting it with morphine. A between-subjects factorial ANOVA was also conducted to back up the results obtained from the within-subjects analysis. All data was shown in mean ± standard error of mean. Significant difference was determined at $p < 0.05$.

Chapter 5

Results

5.1 tVTA (tail region of the ventral tegmental area)

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the its baseline and post formalin injection for the formalin group (administered at the 10-minute mark), for the **Delta wave** (Fig.10A), F (11,121) = 2.2.16, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .89, SE = .31), $p < .05$ and the time points at 15 minutes (M = 1.91, SE = .97), $p < .05$, 20 minutes (M = 1.89, SE = 1.19), $p < .05$, 25 minutes (M =1.84, SE = 1.08), $p < .05$, 30 minutes (M =2.03, SE = .997), $p < .05$, 35 minutes (M = 2.10, SE = 1.03), $p < .05$, 40 minutes (M = 1.78, SE = .96), $p < .05$, 45 minutes (M = 1.82, SE = 1.01), $p < .05$, 50 minutes (M = 1.68, SE = .95), $p < .05$, 55 minutes (M = 1.79, SE = 1.07), $p < .05$, and at the 60 minutes mark as well (M = 1.53, SE = .75), $p < .05$. A within subjects repeated measures ANOVA using SPSS also revealed a significant difference between the baseline and post formalin + morphine injection for the formalin + morphine group (both administered at the 10-minute mark), for the **Delta wave** (Fig.10A), F (11,121) = 1.876, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline and time points at 15 minutes (M = 1.33, SE = .30), $p < .05$, 30 minutes (M =1.32, SE = 4.02, $p < .05$, 35 minutes (M = 1.78, SE = .64), $p < .05$, 50 minutes (M = 1.63, SE = .94), $p < .05$, and 55 minutes (M = 1.67, SE = .84), $p < .05$. All the significant values corresponded to higher formalin response.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin group (administered at the 10-minute mark) at specific time points and between saline and formalin + morphine group at the 35 minute mark, for the **Delta wave** (Fig.10A), F (1,15) = 127.531, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 15 minutes between saline (M = .78, SE = .35), $p < .05$ and formalin (M = 1.91, SE = .96), $p < .05$, at 30 minutes between saline (M = .82, SE = .29), $p < .05$ and formalin (M = 2.03, SE = .99), 4$p < .05$, at 35 minutes between saline (M = .82, SE = .34), $p < .05$ and formalin (M = 2.10, SE = 1.03), $p < .05$ and between saline (M = .82, SE = .34) and formalin + morphine (M = 1.77, SE = .64), $p < .05$, , at 40 minutes between saline (M = .79, SE = .39), $p < .05$ and formalin (M = 1.77, SE = .966), $p < .05$, at 45 minutes between saline (M = .78, SE = .41), $p < .05$ and formalin (M = 1.81, SE = 1.01), $p < .05$. There was also significant difference observed between the pure morphine (M = .91, SE = .22), $p < .05$ and morphine + formalin group (M = 1.67, SE = .84), $p < .05$. Lastly There was also significant difference observed between the pure morphine (M = .98, SE = .17), $p < .05$ and formalin group (M = 2.03, SE = .99), $p < .05$. All the significant values corresponded to higher formalin response.

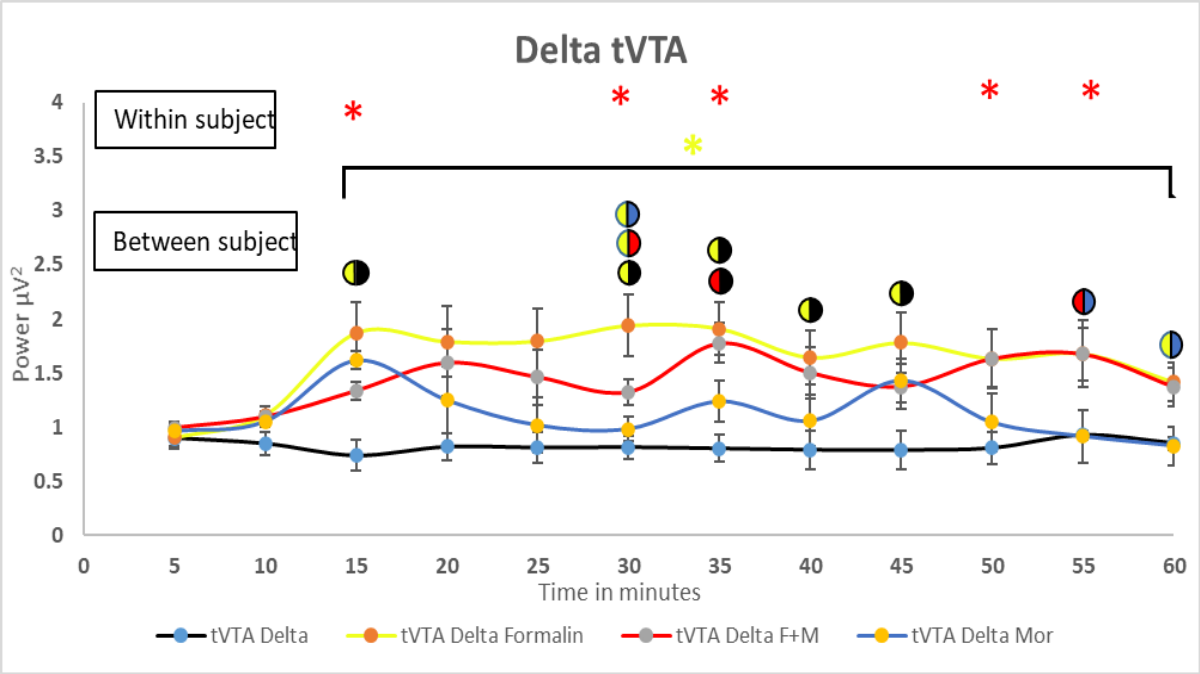


Figure 10A. The Delta wave within and between-subjects local field potential data for the tVTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Theta wave** (Fig.10B), F (11,121) = 1.810 $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .81, SE = .23), and the time points at 10 minutes (M = 1.09, SE = .29), $p < .05$, 15 minutes (M = 1.14, SE = .51), $p < .05$, 25 minutes (M =1.41, SE = .73), $p < .05$, 30 minutes (M =1.49, SE = .87), $p < .05$, 35 minutes (M = 1.65, SE = 1.04), $p < .05$, 55 minutes (M = 1.36, SE = .75), $p < .05$, and at the 60 minutes mark as well (M = 1.28, SE = .43), $p < .05$. All the significant values corresponded to higher formalin response.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin group (administered at the 10-minute mark) at specific time points for the **Theta wave** (Fig.10B), F (1,14) = 66.62, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 25 minutes between saline (M = .52, SE =

.36), $p < .05$ and formalin ($M = 1.41$, $SE = .74$), $p < .05$, at 30 minutes between saline ($M = .52$, $SE = .34$), $p < .05$ and formalin ($M = 1.65$, $SE = 1.04$), $p < .05$, at 60 minutes between saline ($M = .74$, $SE = .26$), $p < .05$ and formalin ($M = 1.28$, $SE = .43$), $p < .05$. A significant difference was also observed between formalin ($M = 1.41$, $SE = .74$), $p < .05$ and morphine ($M = .68$, $SE = .19$), $p < .05$ group at 25 minutes, F (1,16) = 125.70, $p < .05$ and at 60 minutes between formalin ($M = 1.28$, $SE = .43$), $p < .05$ and morphine ($M = .77$, $SE = .36$).

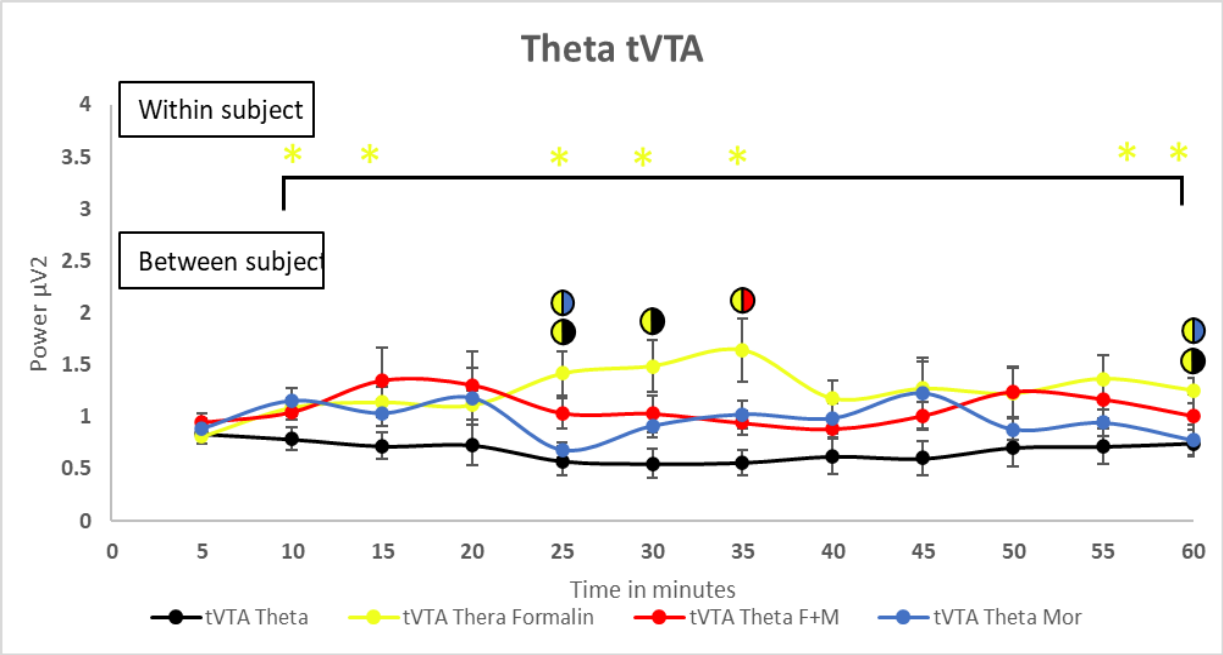


Figure 10B. The Theta wave within and between-subjects local field potential data for the tVTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Alpha wave** (Fig.10C), F (1, 11) = 139.63, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline ($M = .84$, $SE = .17$), $p < .05$ and the time points at 25 minutes ($M = 1.33$, $SE = .66$), $p < .05$, 30 minutes ($M = 1.30$, $SE = .59$), $p < .05$, and 35

minutes (*M* = 1.49, *SE* = .92), *p* < .05. All the significant values corresponded to higher formalin response.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin group (administered at the 10-minute mark) at specific time points for the **Alpha wave** (Fig. 10C), *F* (1,14) = 95.76, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 25 minutes between saline (*M* = .54, *SE* = .37), *p* < .05 and formalin (*M* = 1.34, *SE* = .66), *p* < .05, at 30 minutes between saline (*M* = .52, *SE* = .42), *p* < .05 and formalin (*M* = 1.30, *SE* = .59), *p* < .05, at 35 minutes between saline (*M* = .57, *SE* = .35), *p* < .05 and formalin (*M* = 1.49, *SE* = .92), *p* < .05. All the significant values corresponded to higher formalin response. A between subjects repeated measures ANOVA using SPSS also revealed a significant difference between the formalin + morphine and formalin group (administered at the 10-minute mark) at specific time points for the **Alpha wave** (Fig.10C), *F* (1,22) = 270.71, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 25 minutes between F+M (*M* = . 76, *SE* = .42), *p* < .05 and formalin (*M* = 1.34, *SE* = .66), *p* < .05, at 30 minutes between F+M (*M* = .81, *SE* = .56), *p* < .05 and formalin (*M* = 1.30, *SE* = .59), *p* < .05, at 35 minutes between F+M (*M* = .80, *SE* = .47), *p* < .05 and formalin (*M* = 1.49, *SE* = .92), *p* < .05 and at 40 minutes between F+M (*M* = .72, *SE* = .42), *p* < .05 and formalin (*M* = 1.49, *SE* = .92), *p* < .05.

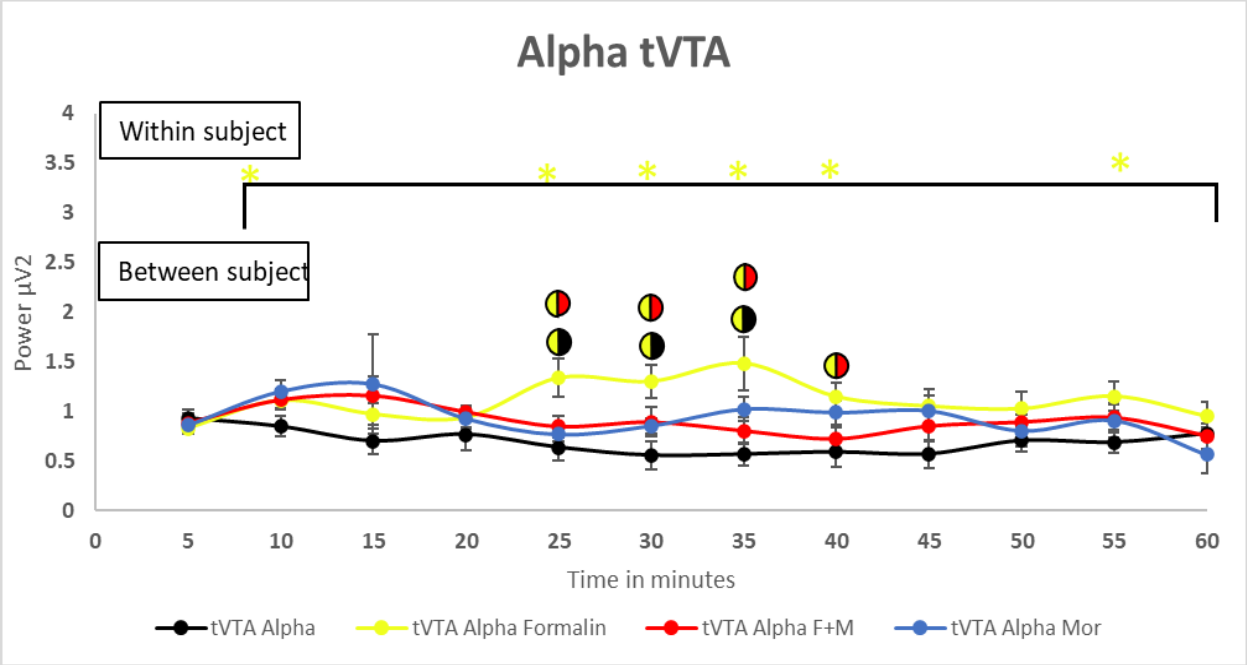


Figure 10C. The Alpha wave within and between-subjects local field potential data for the tVTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Beta wave** (Fig.10D), F (11, 121) = 2.661, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .93, SE = .11), $p < .05$ and the time points at 25 minutes (M = 1.46, SE = .79), $p < .05$, 30 minutes (M =1.71, SE = 1.10), $p < .05$, and 35 minutes (M = 1.58, SE = 1.18) $p < .05$. All the significant values corresponded to higher formalin response.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin group (administered at the 10-minute mark) at specific time points for the **Beta wave** (Fig.10D), F (1,14) = 75.427, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 25 minutes between saline (M = .59, SE =

.34), $p < .05$ and formalin ($M = 1.46$, $SE = .80$), $p < .05$, at 30 minutes between saline ($M = .59$, $SE = .38$), $p < .05$ and formalin ($M = 1.71$, $SE = 1.10$), $p < .05$, All the significant values corresponded to higher formalin response. A between subjects repeated measures ANOVA using SPSS also revealed a significant difference between the formalin + morphine and formalin group (administered at the 10-minute mark) at specific time points for the **Beta wave** (Fig.10D), $F(1,22) = 211.52$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 25 minutes between F+M ($M = .79$, $SE = .37$) $p < .05$ and formalin ($M = 1.46$, $SE = .80$), $p < .05$, at 30 minutes between F+M ($M = .84$, $SE = .57$), $p < .05$ and formalin ($M = 1.71$, $SE = 1.10$), $p < .05$, at 35 minutes between F+M ($M = .77$, $SE = .41$), $p < .05$ and formalin ($M = 1.57$, $SE = 1.18$), $p < .05$.

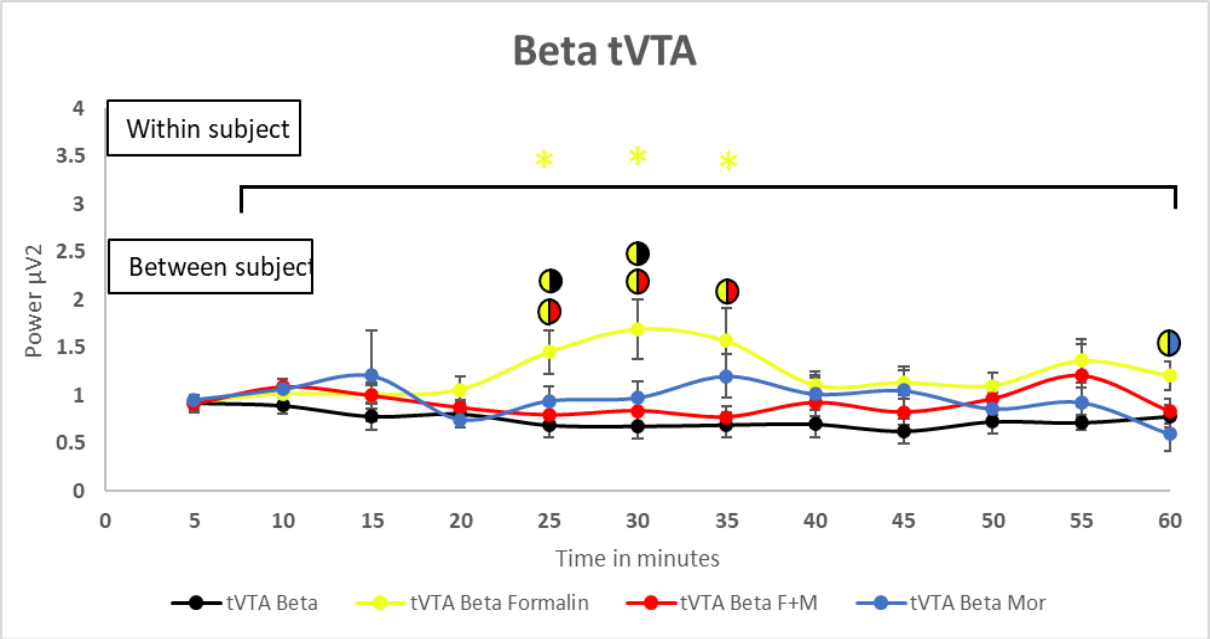


Figure 10D. The Beta wave within and between-subjects local field potential data for the tVTA region.

No significant differences were observed in a within subjects repeated measures ANOVA using SPSS. However, A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin group (administered at the 10-minute mark) at specific time points for the **Gamma wave** (Fig.10E), F (1,14) = 166.21, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline (M = .81, SE = .21), $p < .05$ and formalin (M = .99, SE = .12), $p < .05$, at 20 minutes between saline (M = .67, SE = .34), $p < .05$ and formalin (M = 1.03, SE = .27), $p < .05$.

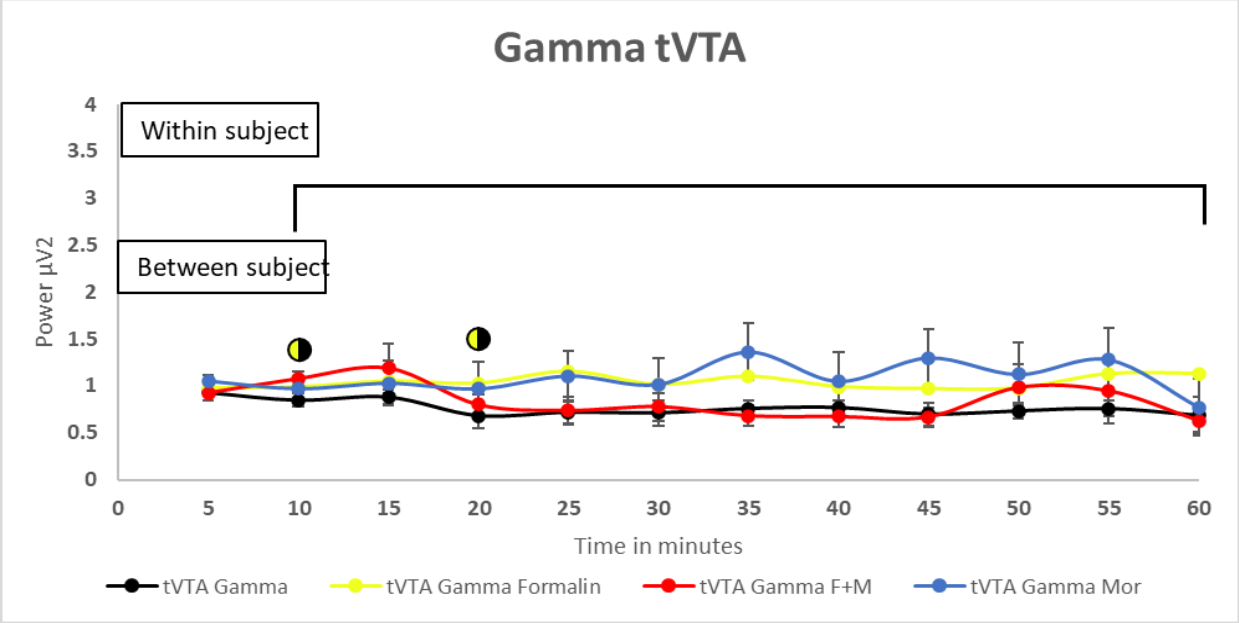


Figure 10E. The Gamma wave within and between-subjects local field potential data for the tVTA region

5.2 VTA (ventral tegmental area)

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Delta wave** (Fig.11A), F (11,121) = 3.768, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .91, SE = .34), $p < .05$ and the time points at 10 minutes (M = .66, SE = .28), $p < .05$, 15 minutes (M = 1.41, SE = .71), $p < .05$, 35 minutes (M = 1.44, SE = .45), $p < .05$, 50 minutes (M = 2.52, SE = 1.53), $p < .05$, 55 minutes (M = 1.79, SE = 1.80), $p < .05$. A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin + morphine group, for the **Delta wave** (Fig.11A), F (11,121) = 4.601, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 1.31, SE = .36), $p < .05$ and the time points at 15 minutes (M = .83, SE = .33), $p < .05$, 20 minutes (M = .84, SE = .48), $p < .05$, 30 minutes (M = .72, SE = .36), $p < .05$, 35 minutes (M = 2.01, SE = .90), $p < .05$. . A within subjects repeated measures ANOVA using SPSS also revealed a significant difference between the baseline and morphine group, for the **Delta wave** (Fig.11A), F (1, 11) = 302.87, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 1.47, SE = .85), $p < .05$ and the time points at 30 minutes (M = 1.33, SE = .24).

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time points for the **Delta wave** (Fig.11A), F (1,14) = 67.805, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 20 minutes between saline (M = .74, SE =

.24), $p < .05$ and formalin ($M = 1.49$, $SE = .66$), $p < .05$, and at 40 minutes between saline ($M = .59$, $SE = .25$), $p < .05$ and formalin ($M = 1.47$, $SE = .89$). A between subjects repeated measures ANOVA using SPSS also revealed a significant difference between the saline and morphine group at specific time point for the **Delta wave** (Fig.11A), $F(1,8) = 271.87$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 45 minutes between saline ($M = .65$, $SE = .15$), $p < .05$ and formalin ($M = .92$, $SE = .11$), $p < .05$.

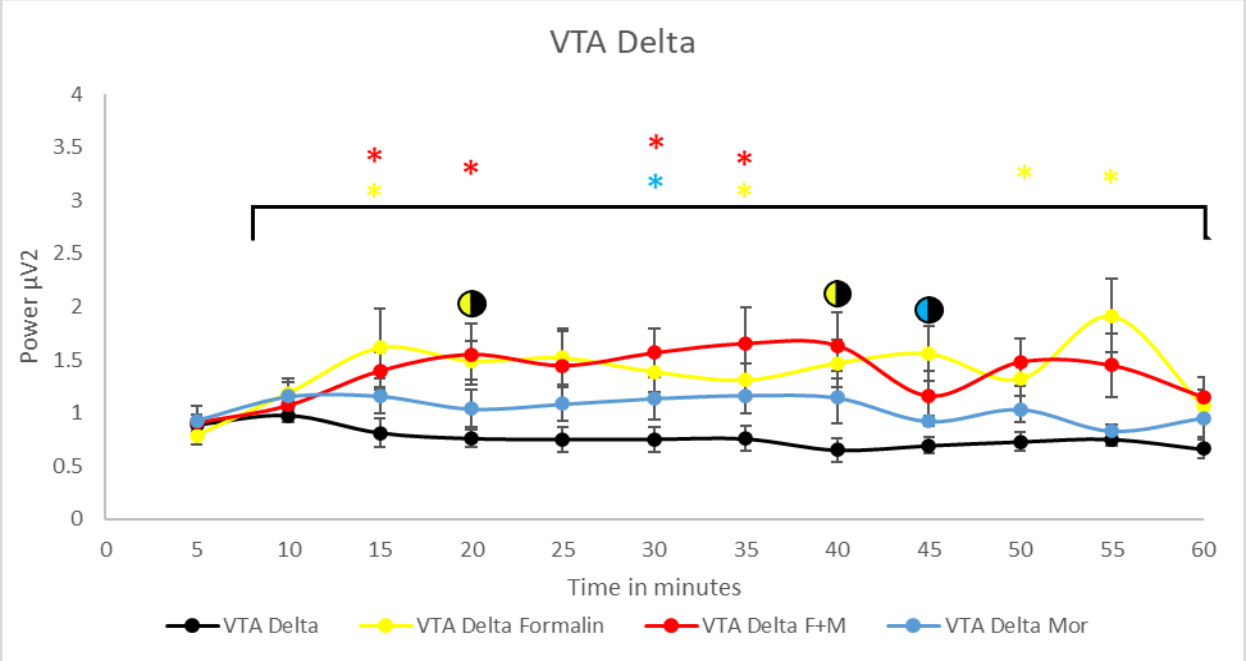


Figure 11A. The Delta wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Theta wave** (Fig. 11B), $F(11,121) = 5.888$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline ($M = 1.08$, $SE = .61$), $p < .05$ and the time points at 10 minutes ($M = 1.77$, $SE = .90$), $p < .05$, 45 minutes ($M = 1.69$, $SE = .38$), $p < .05$, 50

minutes (*M* = 3.40, *SE* = 1.77), *p* < .05, 55 minutes (*M* = 2.66, *SE* = 2.61), *p* < .05, 60 minutes (*M* = 1.79, *SE* = 1.80), *p* < .05. A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin + morphine group, for the **Theta wave** (Fig.11B), *F* (11,121) = 3.986, *p* < .05, Post hoc tests using the LSD correction revealed significant difference between baseline (*M* = .62, *SE* = .31), *p* < .05 and the time points at 10 minutes (*M* = 1.38, *SE* = .38), *p* < .05, 30 minutes (*M* = .95, *SE* = .03), *p* < .05, 45 minutes (*M* = 1.65, *SE* = 1.04), *p* < .05, 50 minutes (*M* = 1.33, *SE* = .53), *p* < .05, 55 minutes (*M* = 1.01, *SE* = .17), *p* < .05, and 60 minutes (*M* = 2.01, *SE* = .90), *p* < .05 A within subjects repeated measures ANOVA using SPSS also revealed a significant difference between the baseline and morphine group, for the **Theta wave** (Fig.11B), *F* (1, 11) = 302.87, *p* < .05, Post hoc tests using the LSD correction revealed significant difference between baseline (*M* = 1.47, *SE* = .85), *p* < .05 and the time points at 30 minutes (*M* = 1.18, *SE* = .21).

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time points for the **Theta wave** (Fig.11B), *F* (1,14) = 36.777, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 35 minutes between saline (*M* = .55, *SE* = .26), *p* < .05 and formalin (*M* = 1.77, *SE* = 1.25), *p* < .05, and at 40 minutes between saline (*M* = .54, *SE* = .24), *p* < .05 and formalin (*M* = 1.63, *SE* = 1.12). A between subjects repeated measures ANOVA using SPSS also revealed a significant difference between the saline and morphine group at specific time point for the **Theta wave** (Fig.11B), *F* (1,8) = 271.87, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 45 minutes between saline (*M* = .65, *SE* = .15), *p* < .05 and formalin (*M* = .92, *SE* = .11), *p* < .05.

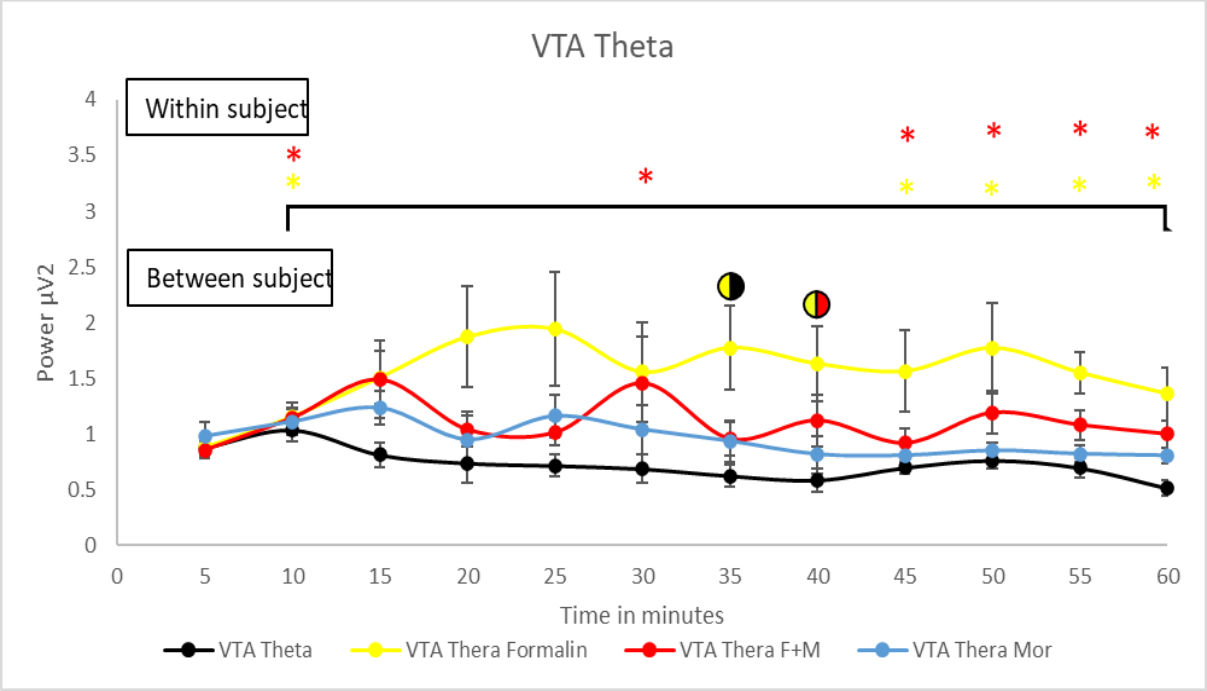


Figure 11B. The Theta wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Alpha wave** (Fig.11C), F (11,121) = 4.693, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 1.00, SE = .36), $p < .05$ and the time points at, 50 minutes (M = 2.22, SE = .95) $p < .05$, 55 minutes (M = 2.06, SE = 1.73), $p < .05$, and 60 minutes (M = 1.88, SE = .99), $p < .05$. A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin + morphine group, for the **Alpha wave** (Fig.11C), F (11,121) = 5.901, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 2.11, SE = .90), $p < .05$ and the time points at 10 minutes (M = 1.52, SE = .41), $p < .05$, 15 minutes (M = .40, SE = .45), $p < .05$, 30 minutes (M = .98, SE = .03), $p < .05$, 50 minutes (M = 1.04, SE = .40), $p < .05$, 55 minutes (M =

1.04, *SE* = .15), $p < .05$, and 60 minutes (*M* = .92, *SE* = .21), $p < .05$. A within subjects repeated measures ANOVA using SPSS also revealed a significant difference between the baseline and morphine group, for the **Alpha wave** (Fig.11C), $F(1, 11) = 204.67$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (*M* = 1.32, *SE* = .87), $p < .05$ and the time points at 30 minutes (*M* = 1.23, *SE* = .22).

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time points for the **Alpha wave** (Fig.11C), $F(1,14) = 91.821$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 40 minutes between saline (*M* = .62, *SE* = .21), $p < .05$ and formalin (*M* = 1.50, *SE* = .83), $p < .05$, and at 55 minutes between saline (*M* = .54, *SE* = .24), $p < .05$ and formalin (*M* = 1.63, *SE* = 1.12). A between subjects repeated measures ANOVA using SPSS also revealed a significant difference between the formalin and morphine group at specific time point for the **Alpha wave** (Fig.11C), $F(1,16) = 165.87$, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 55 minutes between saline (*M* = .81, *SE* = .09), $p < .05$ and formalin (*M* = 1.69, *SE* = .92), $p < .05$.

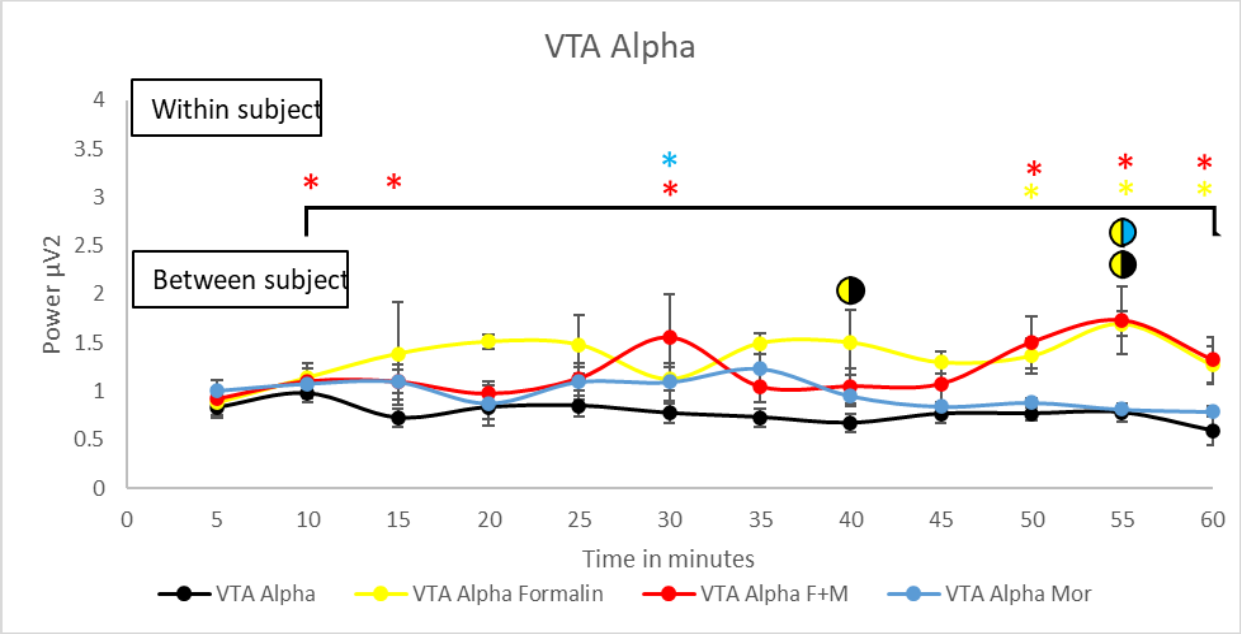


Figure 11C. The Alpha wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and post formalin injection (administered at the 10-minute mark), for the **Beta wave** (Fig.8D), F (11,121) = 4.717, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .86, SE = .14), $p < .05$ and the time points at 10 minutes (M = 1.96, SE = 1.19), $p < .05$, 15 minutes (M = 1.67, SE = .68), $p < .05$, 20 minutes (M = .50, SE = .41), $p < .05$, 30 minutes (M = 1.10, SE = .15), $p < .05$, 35 minutes (M = 1.42, SE = .27), $p < .05$. 50 minutes (M = 1.96, SE = 1.14), $p < .05$, 55 minutes (M = 1.51, SE = 1.32), $p < .05$, 60 minutes (M = 2.10, SE = 1.14), $p < .05$. A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin +morphine group, for the **Beta wave** (Fig.8D), F (11,121) = 7.913, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 2.18, SE = .84), $p < .05$ and the time points at 10 minutes (M = 1.23, SE = .42), $p < .05$, 15 minutes (M = .69, SE = .32), $p < .05$, 20 minutes (M =

1.01, *SE* = 1.03), *p* < .05, 30 minutes (*M* = .96, *SE* = .03), *p* < .05, 35 minutes (*M* = 1.09, *SE* = .20), *p* < .05. 50 minutes (*M* = .91, *SE* = .23), *p* < .05, 55 minutes (*M* = 1.06, *SE* = .16), *p* < .05, 60 minutes (*M* = 1.02, *SE* = .21), *p* < .05.

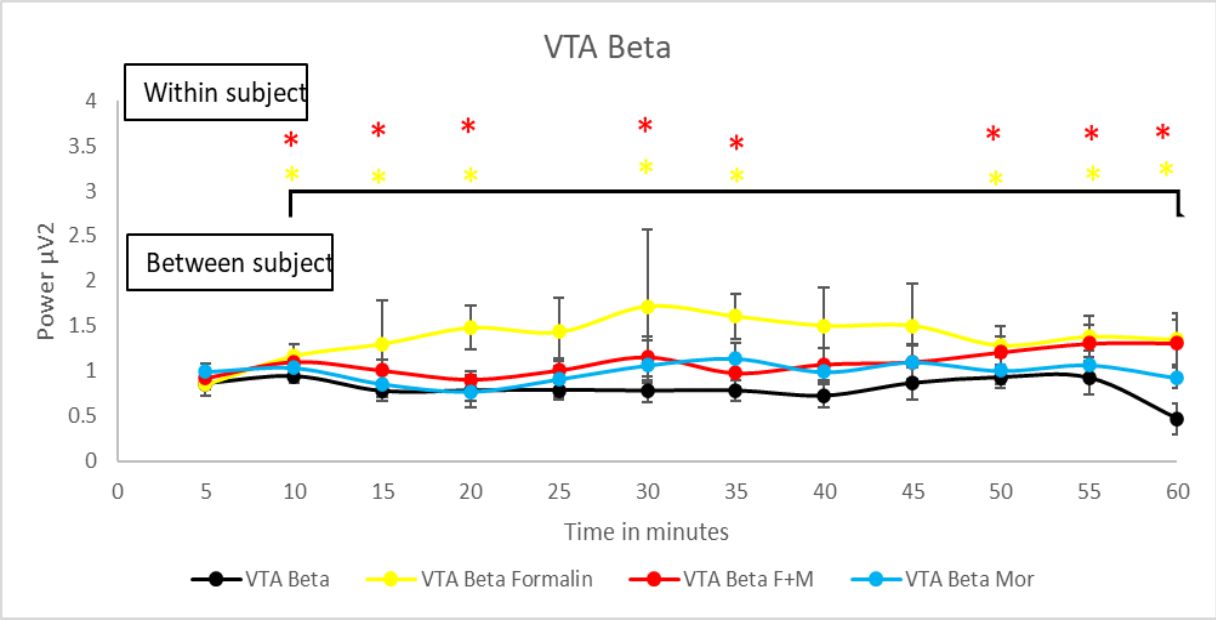


Figure 8D. The Beta wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin, for the **Gamma wave** (Fig.8E), *F* (1, 11) = 20.234, *p* < .05, Post hoc tests using the LSD correction revealed significant difference between baseline (*M* = 1.01, *SE* = .09), *p* < .05 and the time points at 10 minutes (*M* = 1.41, *SE* = .28), *p* < .05, 15 minutes (*M* = 1.87, *SE* = 1.19), *p* < .05, 20 minutes (*M* = .78, *SE* = .22), *p* < .05, 40 minutes (*M* = 1.49, *SE* = .37), *p* < .05, 45 minutes (*M* = 1.52, *SE* = .33), *p* < .05 and 60 minutes (*M* = 1.36, *SE* = .48), *p* < .05. A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and formalin + morphine, for the **Gamma wave** (Fig.8E), *F* (1,

11) = 24.712, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline ($M = .98$, $SE = .08$), $p < .05$ and the time points at 10 minutes ($M = 1.17$, $SE = .14$), $p < .05$, 15 minutes ($M = .36$, $SE = .35$), $p < .05$, 30 minutes ($M = 1.10$, $SE = .07$), $p < .05$, and 60 minutes ($M = .75$, $SE = .19$), $p < .05$.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the morphine and formalin group at specific time points for the **Gamma wave** (Fig.8E), F (1,16) = 141.655, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline ($M = .77$, $SE = .48$), $p < .05$ and formalin ($M = 1.13$, $SE = .19$), $p < .05$, at 20 minutes between saline ($M = .68$, $SE = .56$), $p < .05$ and formalin ($M = 1.21$, $SE = .45$) and at 55 minutes between saline ($M = .59$, $SE = .45$), $p < .05$ and formalin ($M = 1.55$, $SE = .81$).

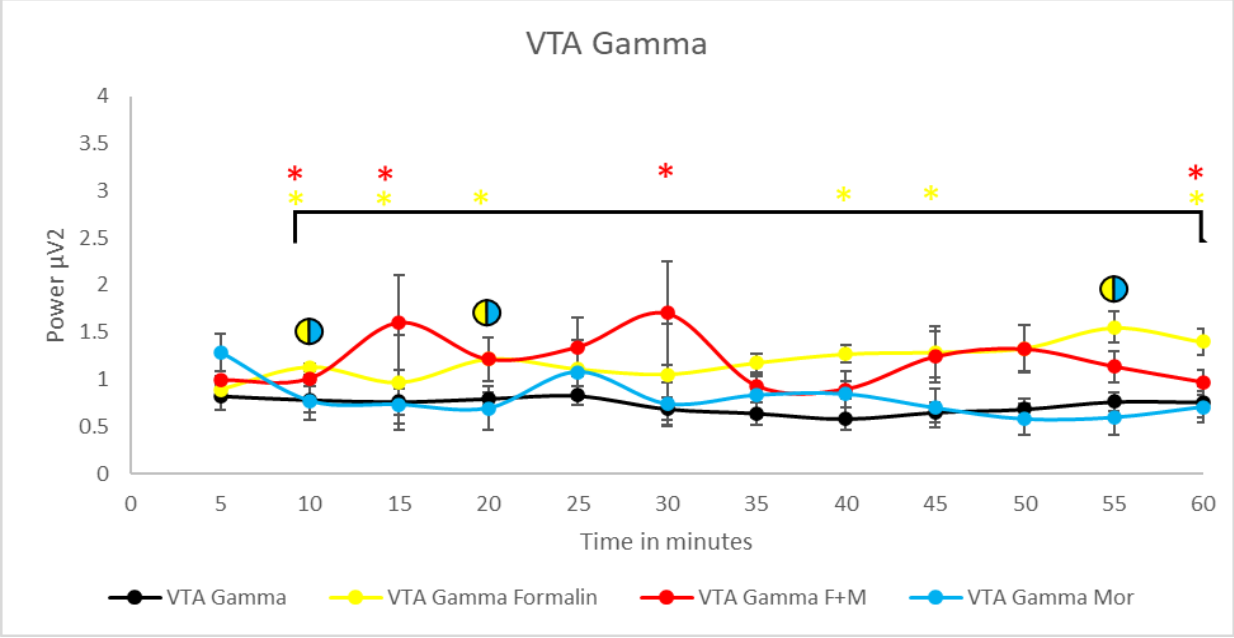


Figure 8E. The Beta wave within and between-subjects local field potential data for the VTA region.

5.3 NAc (Nucleus Accumbens)

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline and formalin +morphine group, for the **Delta wave** (Fig.11A), F (11,121) = 5.322, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 1.87, SE = .98), $p < .05$ and the time points at 15 minutes (M = 85, SE = .48), $p < .05$, 20 minutes (M = .61, SE = .40), $p < .05$, 30 minutes (M = .91, SE = .35), $p < .05$, and 45 minutes (M = 1.07, SE = .29), $p < .05$. A within subjects repeated measures ANOVA using SPSS also revealed a significant difference between the baseline and morphine group, for the **Delta wave** (Fig.11A), F (11,121) = 6.186, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = 1.46, SE = .84), $p < .05$ and the time points at 10 minutes (M = .63, SE = .22), $p < .05$, 35 minutes (M = 1.82, SE = .56), $p < .05$, 45 minutes (M = 2.59, SE = 1.11), $p < .05$ and 60 minutes (M = 2.13, SE = 1.28), $p < .05$.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and morphine group (administered at the 10-minute mark) at specific time points for the **Delta wave** (Fig.11A), F (1,8) = 86.044, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 35 minutes between saline (M = 1.61SE = .64), $p < .05$ and morphine (M = .69, SE = .35), $p < .05$. Another between subjects repeated measures ANOVA using SPSS reveled a significant difference between the formalin and morphine group (both administered at the 10-minute mark) at specific time points for the **Delta wave** (Fig.11A), F (1,16) = 88.752, $p < .05$, Post hoc tests using the LSD correction revealed

significant difference at 10 minutes between formalin (*M* = 1.16 *SE* = .33), *p* < .05 and morphine (*M* = .86, *SE* = .27), *p* < .05.

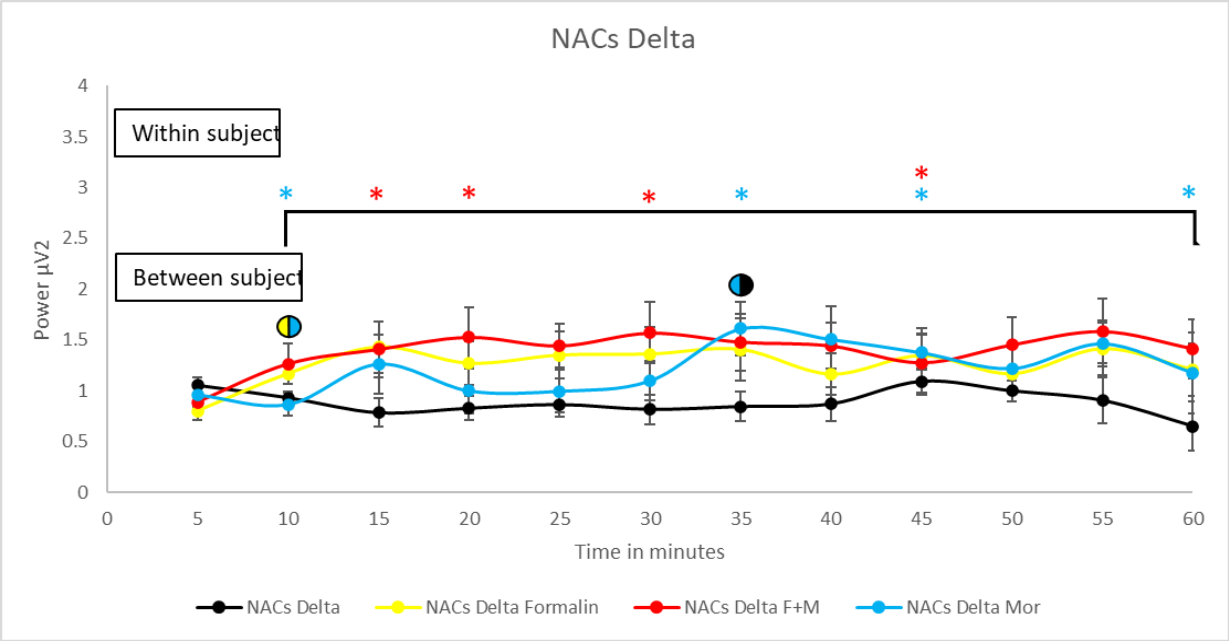


Figure 11A. The Delta wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline and morphine group, for the **Theta wave** (Fig.11B), *F* (11,121) = 8.323, *p* < .05, Post hoc tests using the LSD correction revealed significant difference between baseline (*M* = .70 *SE* = .30), *p* < .05 and the time points at 15 minutes (*M* = 65, *SE* = .25), *p* < .05, 30 minutes (*M* = 1.54, *SE* = .37), *p* < .05, 35 minutes (*M* = 2.21, *SE* = .63), *p* < .05, 40 minutes (*M* = 1.19, *SE* = .23), *p* < .05, 45 minutes (*M* = 2.95, *SE* = .14), *p* < .05, 55 minutes (*M* = 2.24, *SE* = 2.10), *p* < .05 and 60 minutes (*M* = 1.15, *SE* = .22), *p* < .05.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the formalin and morphine group (both administered at the 10-minute

mark) at specific time points for the **Theta wave** (Fig.11B), F (1,16) = 70.056, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 15 minutes between morphine (M = 1.43SE = .85), $p < .05$ and formalin (M = 1.33, SE = .75), $p < .05$, with a higher effect for morphine. Another between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and morphine group (both administered at the 10-minute mark) at specific time points for the **Theta wave** (Fig.11B), F (1,8) = 83.114, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 35 minutes between saline (M = 1.45 SE = .46), $p < .05$ and morphine (M = .84, SE = .26), $p < .05$, with a higher effect for morphine.

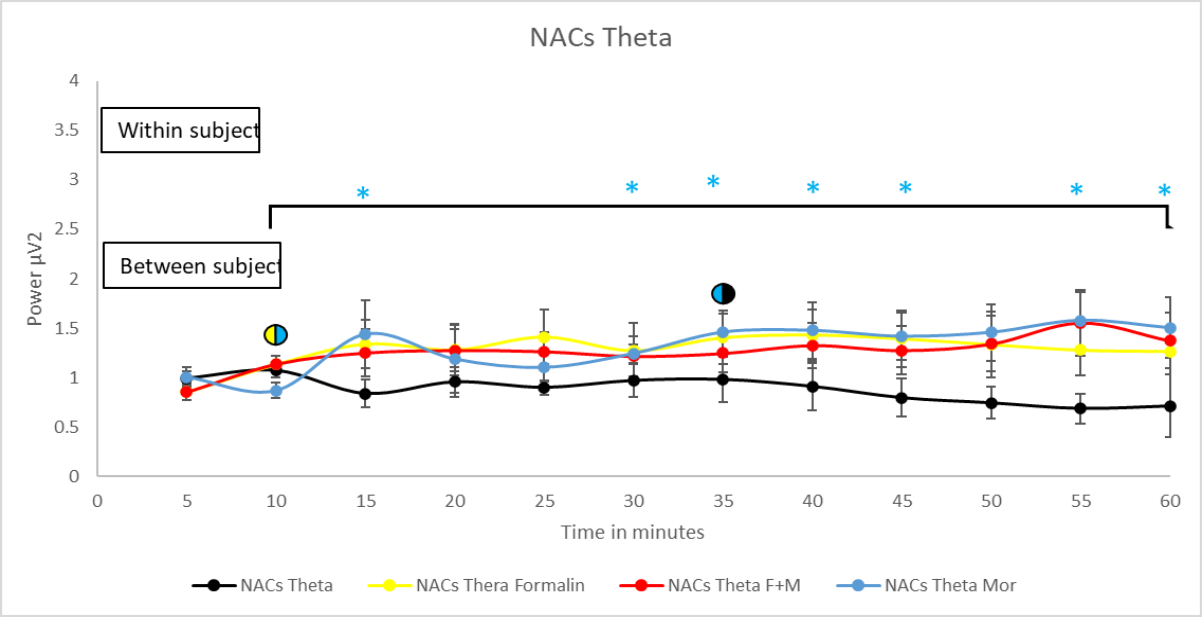


Figure 11B. The Theta wave within and between-subjects local field potential data for the VTA region

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline and morphine group, for the **Alpha wave** (Fig.11C), F (11,121) = 6.028, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between

baseline (*M* = .62 *SE* = .32), *p* < .05 and the time points at 15 minutes (*M* = 64, *SE* = .21), *p* < .05, 30 minutes (*M* = 1.39, *SE* = .31), *p* < .05, 35 minutes (*M* = 1.42, *SE* = .40), *p* < .05, 40 minutes (*M* = 1.30, *SE* = .27), *p* < .05, 45 minutes (*M* = 1.68, *SE* = .34), *p* < .05, 55 minutes (*M* = 2.42, *SE* = 2.29), *p* < .05 and 60 minutes (*M* = .91, *SE* = .21), *p* < .05.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the formalin and morphine group (both administered at the 10-minute mark) at specific time points for the **Alpha wave** (Fig.11C), *F* (1,16) = 72.055, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 15 minutes between morphine (*M* = 1.40 *SE* = .81), *p* < .05 and formalin (*M* = 1.23, *SE* = .45), *p* < .05, with a higher effect for morphine. Another between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and morphine group (both administered at the 10-minute mark) at specific time points for the **Alpha wave** (Fig.11C), *F* (1,8) = 63.121, *p* < .05, Post hoc tests using the LSD correction revealed significant difference at 30 minutes between saline (*M* = .25 *SE* = .36), *p* < .05 and morphine (*M* = .94, *SE* = .76), *p* < .05, with a higher effect for morphine.

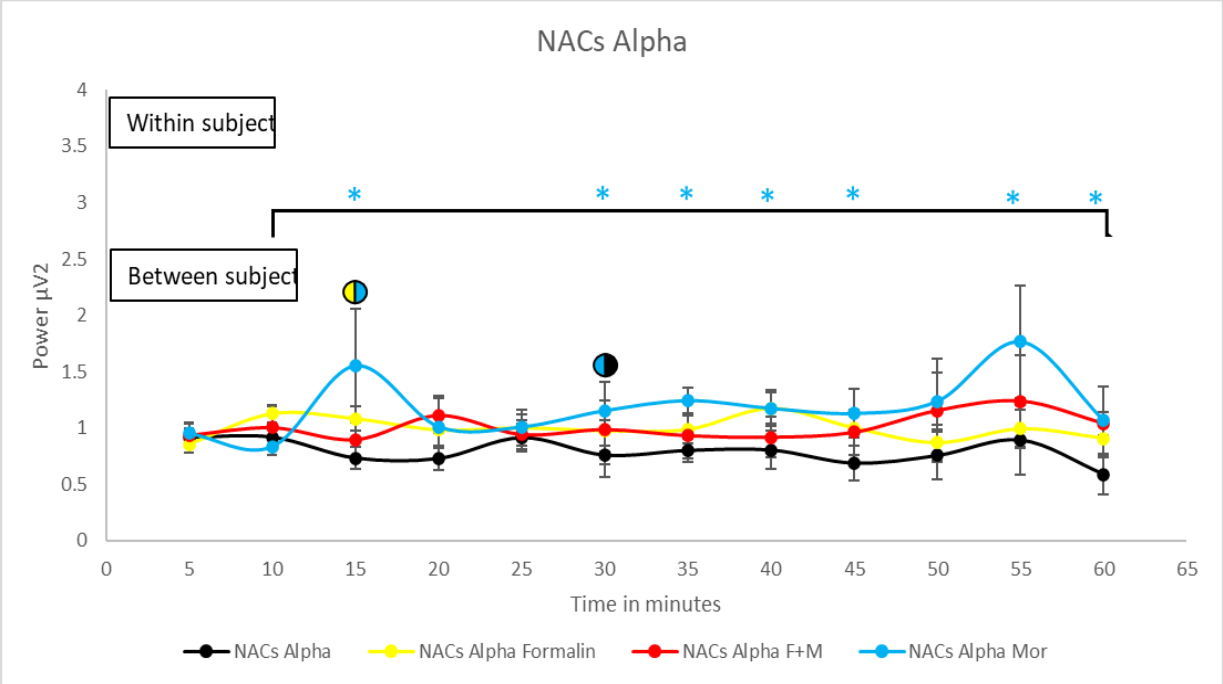


Figure 11C. The Alpha wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS reveled a significant difference between the baseline and morphine group, for the **Beta wave** (Fig.11D), F (11,121) = 5.193, p < .05, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .47, SE = .23), p < .05 and the time points at 15 minutes (M = 1.09, SE = .18), p < .05,, 30 minutes (M = 1.10, SE = .10), p < .05, 35 minutes (M = 1.45, SE = .42), p < .05, 40 minutes (M = 1.33, SE = .35), p < .05, 55 minutes (M = 2.06, SE = 1.77), p < .05, and 60 minutes (M = 1.03, SE = .21), p < .05. No significant results were observed for the between-subjects analysis. The Gamma wave also did not show any significant results.

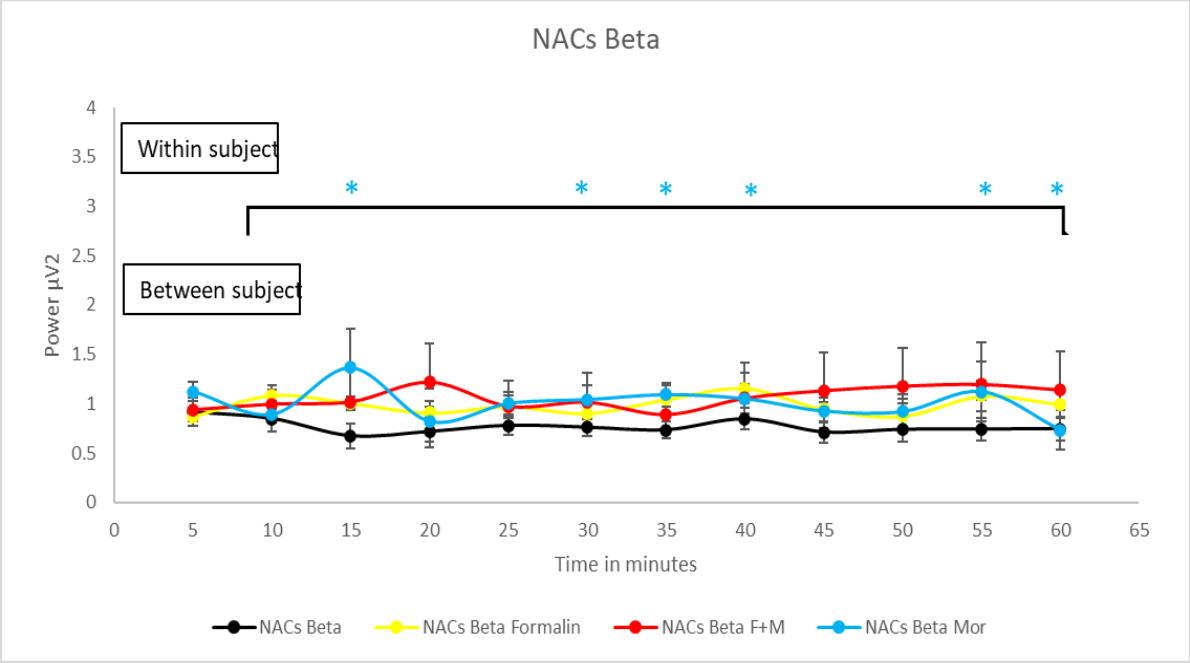


Figure 11D. The Beta wave within and between-subjects local field potential data for the VTA region.

5.4 ACC (Anterior Cingulate Cortex)

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline reading and when the formalin was administered, for the **Delta wave** (Fig.12A), F (11,121) = 1.845, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .811, SE = .31), $p < .05$ and the time points at 10 minutes (M = 1.19, SE = .31), $p < .05$, 15 minutes (M = 1.44, SE = .89), $p < .05$, 20 minutes (M = 1.72, SE = 1.00), $p < .05$, 45 minutes (M = 1.59, SE = 1.03), $p < .05$, 55 minutes (M = 1.72, SE = 1.06), $p < .05$. A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time points for the **Delta wave** (Fig.12A), F (1,15) = 88.740, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline (M = .81, SE = .17), $p < .05$ and formalin (M = 1.19, SE = .31), $p < .05$, and at 20 minutes between saline (M = .68, SE = .31), $p < .05$ and formalin (M = 1.44, SE = .89), with higher formalin effect as compared to the saline (control). A second between subjects repeated measures ANOVA using SPSS reveled a significant difference between the formalin and formalin + morphine group (both administered at the 10-minute mark) at specific time points for the **Delta wave** (Fig.12A), F (1,22) = 153.324, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 20 minutes between formalin + morphine (M = 1.01, SE = .59), $p < .05$ and formalin (M = 1.71, SE = 1.00), $p < .05$, and at 55 minutes between formalin + morphine (M = .93, SE = .60), $p < .05$ and formalin (M = 1.72, SE = 1.06), with higher formalin effect as compared to the formalin + morphine.

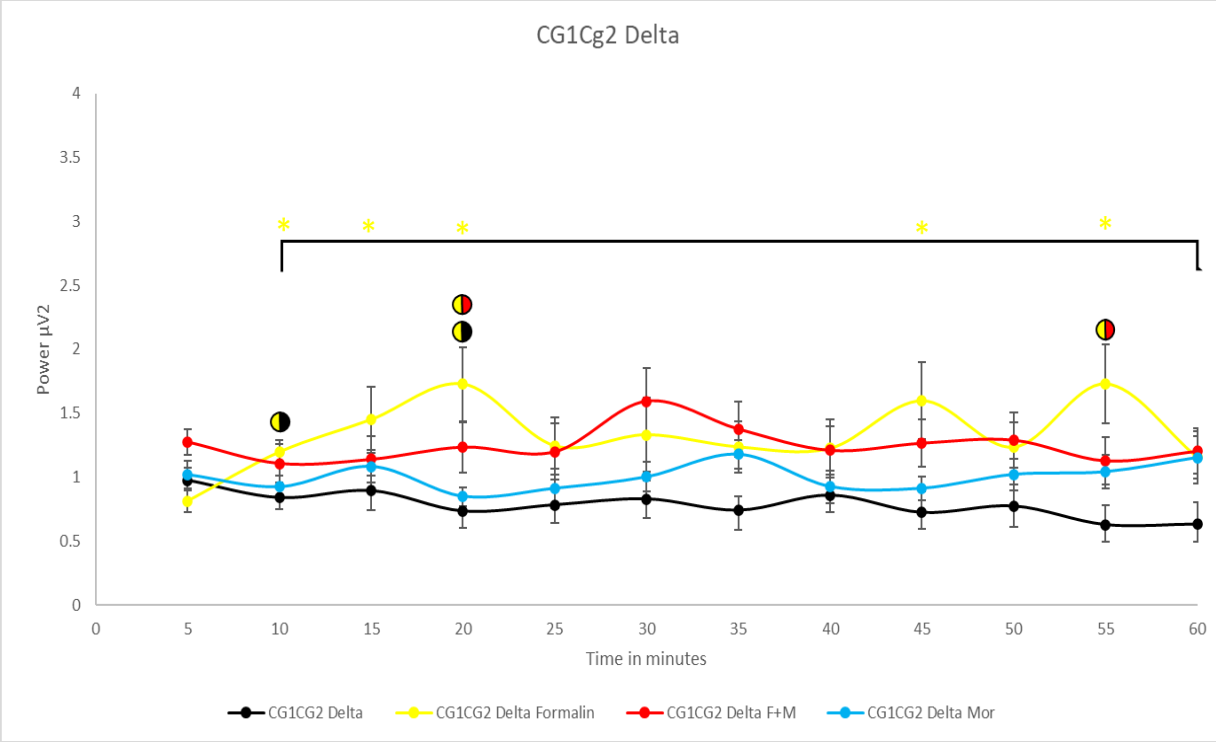


Figure 12A. The Delta wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline reading and when the formalin was administered, for the **Theta wave** (Fig.12B), F (11,121) = 4.419, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline ($M = .67$, $SE = .33$), $p < .05$ and the time points at 10 minutes ($M = 2.56$, $SE = 1.30$), $p < .05$, 20 minutes67 ($M = .96$, $SE = .36$), $p < .05$, 35 minutes ($M = 1.02$, $SE = .22$), $p < .05$, 45 minutes ($M = 1.53$, $SE = .30$), $p < .05$, 50 minutes ($M = 1.60$, $SE = .97$), $p < .05$, 60 minutes ($M = 1.34$, $SE = .26$), $p < .05$.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at

specific time points for the **Theta wave** (Fig.12B), F (1,15) = 103.108, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline (M = .87, SE = .16), $p < .05$ and formalin (M = 1.18, SE = .26), $p < .05$, at 40 minutes between saline (M = .69, SE = .28), $p < .05$ and formalin (M = 1.05, SE = .33), and at 55 minutes between saline (M = .66, SE = .26), $p < .05$ and formalin (M = 1.45, SE = .59), with higher formalin effect as compared to the saline (control). Another between subjects repeated measures ANOVA using SPSS reveled a significant difference between the morphine and formalin group (administered at the 10-minute mark) at specific time point for the **Theta wave** (Fig.12B), F (1,16) = 154.589, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 55 minutes between morphine (M = .75, SE = .33), $p < .05$ and formalin (M = 1.45, SE = .59), $p < .05$ with formalin having higher effect than morphine.

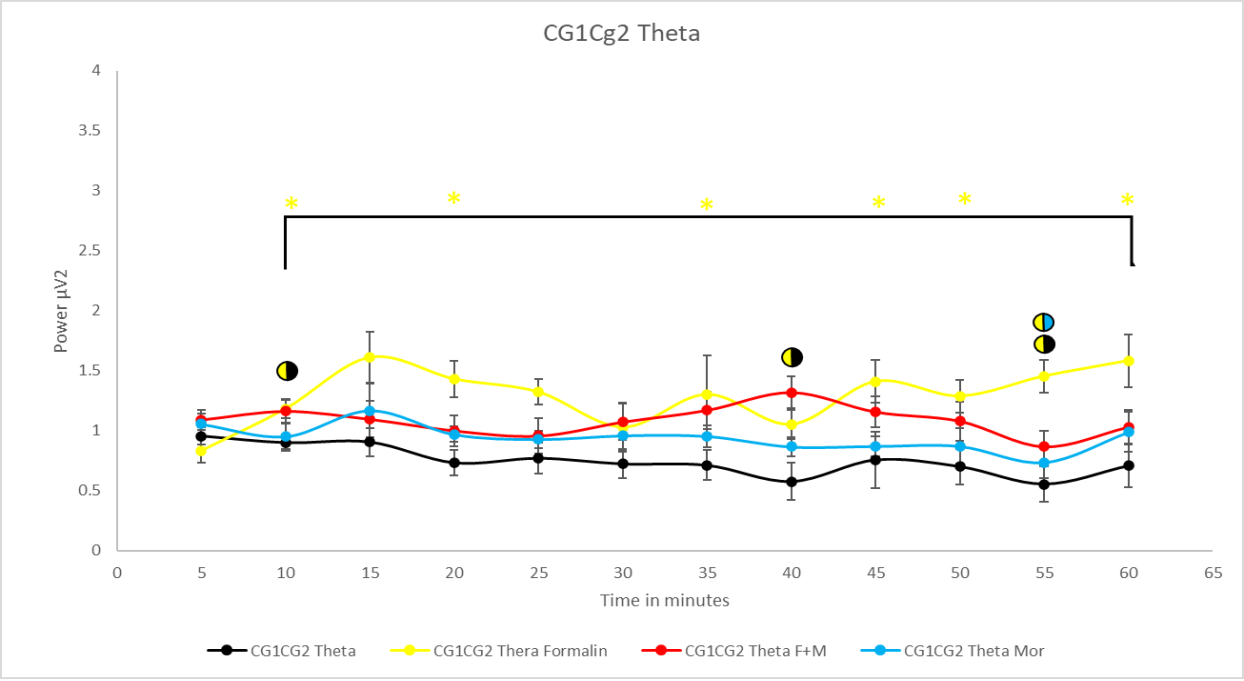


Figure 12B. The Theta wave within and between-subjects local field potential data for the VTA region.

I did not find any significant within subjects differences within the four groups when compared their baseline reading for the **Alpha wave,** however, a between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time point for the **Alpha wave** (Fig.12C), F (1,15) = 138.529, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline (M = .86, SE = .14), $p < .05$ and formalin (M = 1.15, SE = .23), $p < .05$, with clear higher formalin activity as compared to the control group. Another between subjects repeated measures ANOVA using SPSS reveled a significant difference between the morphine and formalin group (administered at the 10-minute mark) at specific time point for the **Alpha wave** (Fig.12C), F (1,16) = 240.650, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between morphine (M = .88, SE = .17), $p < .05$ and formalin (M = 1.15, SE = .23), $p < .05$ with formalin having higher effect than morphine.

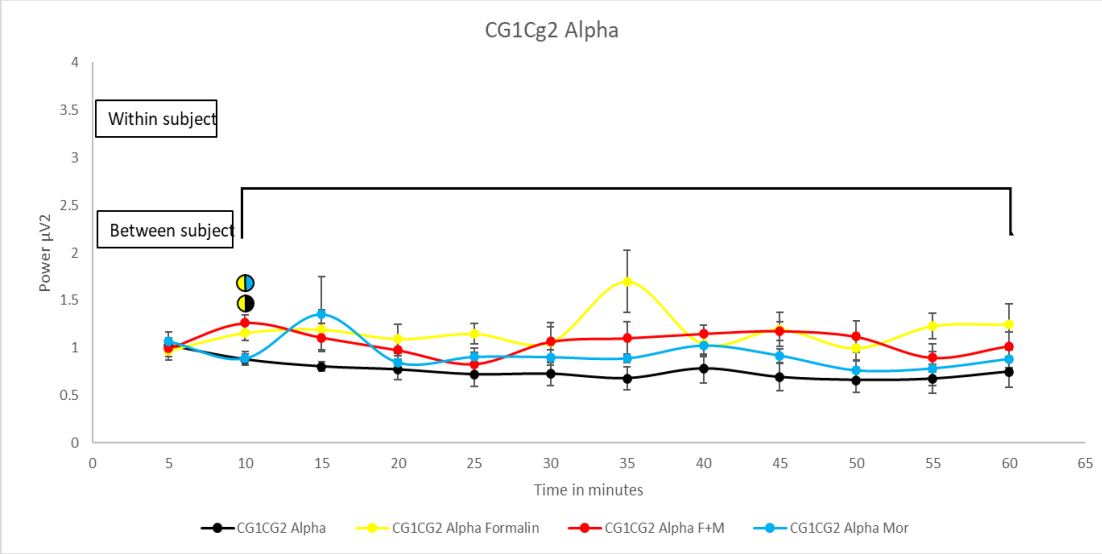


Figure 12C. The Alpha wave within and between-subjects local field potential data for the VTA region.

A within subjects repeated measures ANOVA using SPSS revealed a significant difference between the baseline reading and when the formalin was administered, for the **Beta wave** (Fig.12D), F (11,121) = 5.736, $p < .05$, Post hoc tests using the LSD correction revealed significant difference between baseline (M = .79, SE = .34), $p < .05$ and the time points at 10 minutes (M = 1.80, SE = .72), $p < .05$, 15 minutes (M = 1.12, SE = .10), $p < .05$, 35 minutes (M = 1.23, SE = .27), $p < .05$, 45 minutes (M = 1.07, SE = .14), $p < .05$, 50 minutes (M = 1.36, SE = .37), $p < .05$, 60 minutes (M = 1.19, SE = .30), $p < .05$.

A between subjects repeated measures ANOVA using SPSS reveled a significant difference between the saline and formalin group (administered at the 10-minute mark) at specific time points for the **Beta wave** (Fig.12D), F (1,15) = 201.253, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between saline (M = .83, SE = .22), $p < .05$ and formalin (M = 1.15, SE = .19), $p < .05$, at 20 minutes between saline (M = .73, SE = .26), $p < .05$ and formalin (M = 1.12, SE = .22), at 25 minutes between saline (M = .66, SE = .33), $p < .05$ and formalin (M = 1.23, SE = .41), at 45 minutes between saline (M = .80, SE = .34), $p < .05$ and formalin (M = 1.25, SE = .40), at 50 minutes between saline (M = .79, SE = .35), $p < .05$ and formalin (M = 1.15, SE = .30), at 55 minutes between saline (M = .70, SE = .35), $p < .05$ and formalin (M = 1.31, SE = .35), and at 60 minutes between saline (M = .56, SE = .39), $p < .05$ and formalin (M = 1.26, SE = .40) with higher formalin effect as compared to the saline (control).

A second between subjects repeated measures ANOVA using SPSS reveled a significant difference between the morphine and formalin group (administered at the 10-minute mark) at specific time points for the **Beta wave** (Fig.12D), F (1,16) = 375.169, $p < .05$, Post hoc tests using the LSD correction revealed significant difference at 10 minutes between morphine (M = .95, SE = .14), $p < .05$ and formalin (M = 1.15, SE = .19), $p < .05$, at 20 minutes between morphine (M = .89, SE = .11), $p < .05$ and formalin (M = 1.12, SE = .22), at 45 minutes between morphine (M = .83, SE = .04), $p < .05$ and formalin (M = 1.25, SE = .40), at 50 minutes between morphine (M = .78, SE = .10), $p < .05$ and formalin (M = 1.15, SE = .30), at 55 minutes between morphine (M = .87, SE = .20), $p < .05$ and formalin (M = 1.31, SE = .35), and at 60 minutes between morphine (M = .89, SE = .23), $p < .05$ and formalin (M = 1.26, SE = .40) with higher formalin effect as compared to the morphine (control). I did not find any significant differences for the Gamma wave.

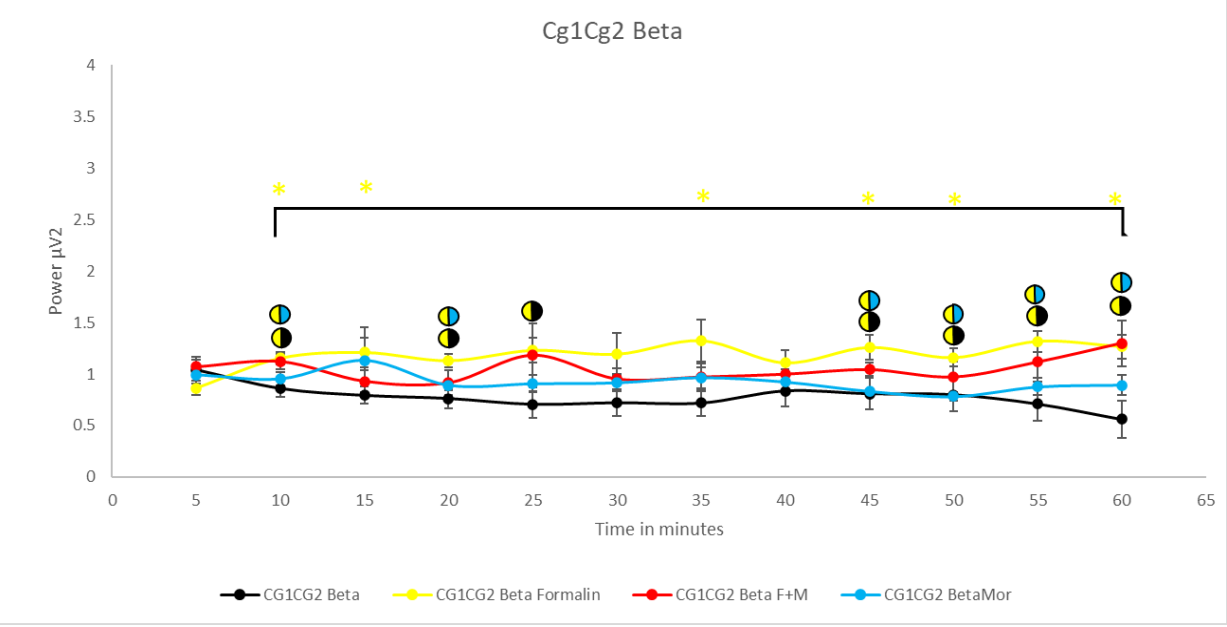
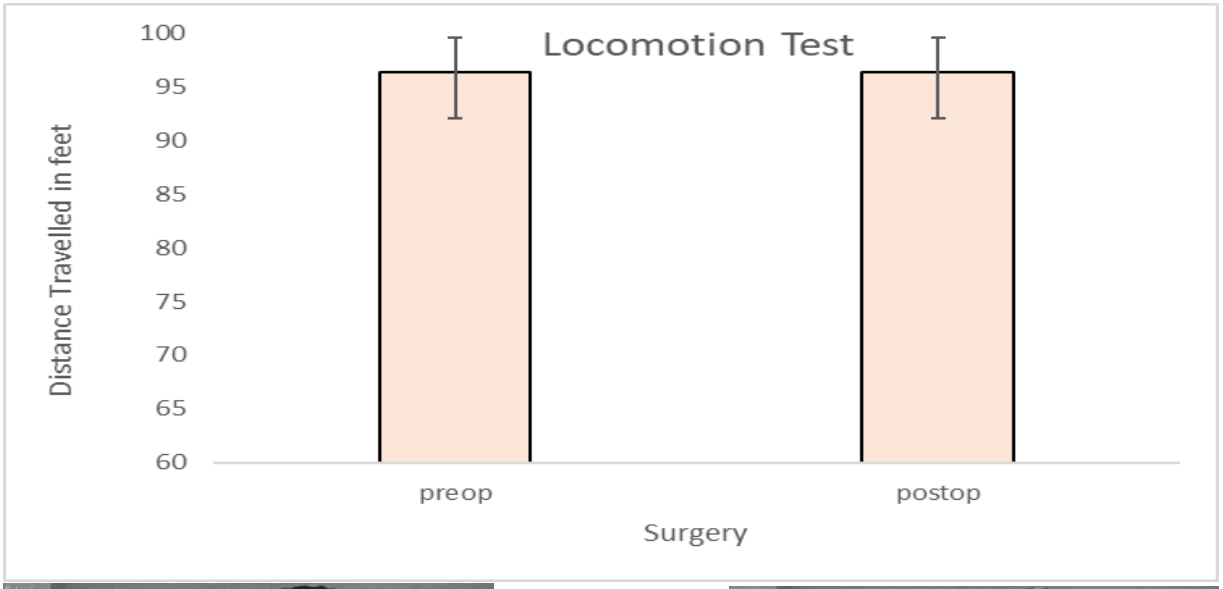


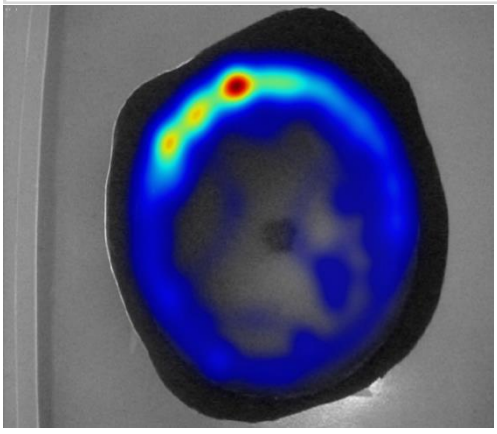
Figure 12D. The Beta wave within and between-subjects local field potential data for the VTA region.

5.5 Locomotive Test Result

Since all the results above were obtained from freely moving animals, it was important to confirm that the stereotaxic surgery did not affect the mobility of the animal. Hence a locomotive test was carried out, where the animals were placed in a chamber in a dark room with a video camera on top. The camera recorded their movements for 5 minutes. This test was carried out pre-surgery as well as post-surgery (after a week).

A





B

C

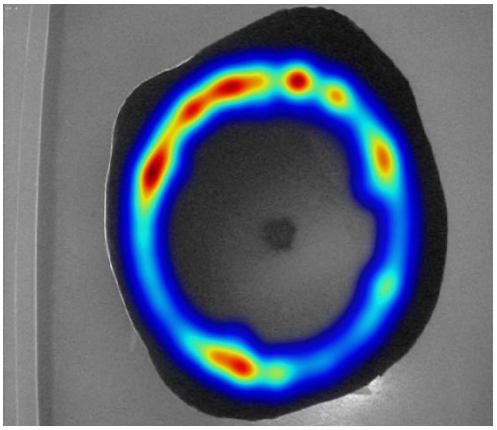


Fig. 13. The Locomotion Test. *A)* The average distance in feet travelled by the rats pre- and post-surgery. A t test was utilized to gauge the significant differences if any, but none were observed. B) A heat map of the rat's movements pre surgery and C) a heat map of the rats movements post-surgery.

A paired samples t-test was carried out to test any differences between the two values. t_{35} = 0.02, $p > 0.05$. No significant difference was observed between the pre- and post-op locomotion test, thus the surgery had no effect on the mobility on the animal or hampered it in any way.

5.6 Histology

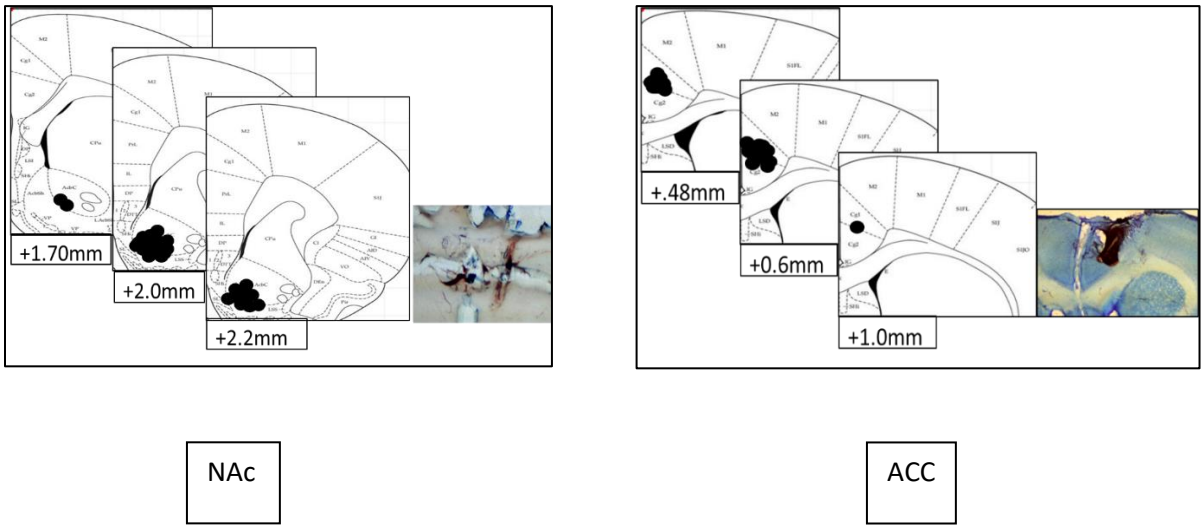


Figure 14 Histology NAc and ACC. Schematic representation of the localization of the electrodes' tips (black dots) on different coronal slices (modified from Paxinos & Waston, 1998) and an actual histology slide on the right.

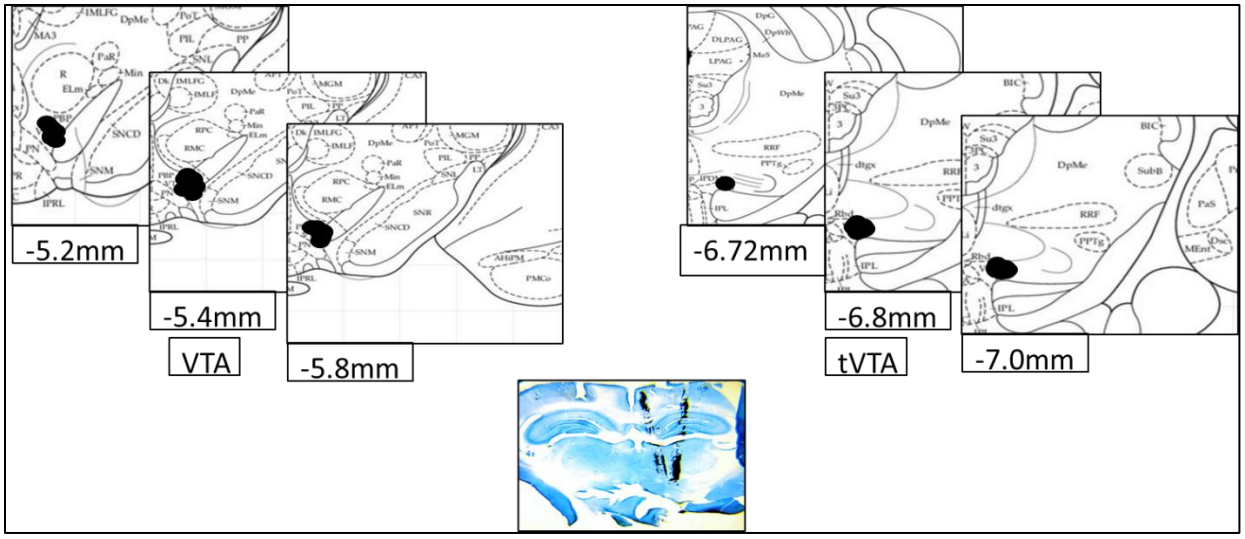


Figure 15 Histology tVTA and VTA. Schematic representation of the localization of the electrodes' tips (black dots) on different coronal slices (modified from Paxinos & Waston, 1998) and an actual histology slide on the bottom.

Chapter 6

Discussion

A number of studies have demonstrated neural circuitry of pain and opioid reward. However, until now there was no knowledge of opioid-indued LFPs in brain regions collectively known to contribute to reward and aversion (pain) responses. The main goal of this dissertation was observing opioid-induced alterations of local field potentials (LFPs), especially post noxious stimuli and the involvement of dopamine (DA) system in this process recorded simultaneously from the four brain regions. The major findings of this study are that LFP activity is differentially increased in each of the four brain regions studied post formalin and morphine administration. This unique pattern of activity adds to the body of knowledge about the involvement of tVTA-VTA-NAc-ACC pathway in nociception.

6.1 LFP activity increased in the tVTA after formalin administration

One of the ***aims*** of the experiment was to determine the changes in the local field potential activity after administering formalin in the four different brain areas. Administering formalin did produce significant changes in the LFP activity in the tVTA, with significant waves for Delta, Theta, Alpha and Beta. Gamma wave not significant. The significant waves were mainly observed in the low frequency waves. Thus, it can be postulated that tVTA might be involved in the nociceptive pathway since there was increase in the LFP activity after formalin injection as compared to the baseline. Comparison using a between-subjects analysis also proved that there were significant differences between the pure formalin group and the control

(saline) group at specific time points that coincide with the time associated with the biphasic formalin response. Thus, tVTA could play an important role in the nociception, or at least act as hub relaying the information forward. The reason for this increase could be attributed to the following reasons, based on previous research. The tVTA receives inputs from brain structures that are known for their role in aversive responses, namely, ACC (anterior cingulate cortex), septum, PAG (periaqueductal area), extended amygdala and the LHb (lateral habenula) (Matsumoto and Hikosaka, 2009) so there is a good possibility that tVTA might be involved in nociceptive stimuli pathways. Studies have shown that endomorphin-1 injections to the tVTA have reduced the nociceptive response in the second formalin phase in rats (Jhou et al., 2009). This could be due to dopamine dependent analgesic effect or due to the brainstem projects of the tVTA. Food restrictions as well as electric foot shocks have also been shown to increase the c-Fos expression in the tVTA (Jhou et al., 2012). It was also observed that these foot shocks also increased the overall activity of tVTA neurons. In mice, studies have observed that acute unpredictable electric shocks increased the excitatory signals form the LHb to the tVTA (Stamatakis and Stuber, 2012). Lesions to the tVTA have shown to produce reduced behavioral responses (freezing) to a conditional auditory tone (Jhou et al., 2012). In mice optogenetic stimulation of the lateral habenula terminals that are present within the tVTA induced active, passive as well as conditioned behavioral avoidance of the stimulation (Stamatakis and Stuber, 2012). Hence a significant increase in the local field potential activity was observed across all the waves expect the gamma wave.

6.1.1 No effect on LFP activity in the tVTA after morphine administration

Pertaining to other ***aim*** relating to morphine administration, no significant increase in LFP activity for any frequency band. The formalin + morphine group did show a significant increase for the Delta wave, but I think that result could very well be attributed to the formalin being the dominant force behind the effect. The main reason for no significant effect of morphine on tVTA could be explained based on the following research and analysis. Administering morphine has been shown to increase somatodendritic and axonal dopamine concentrations, however it was observed that there was no increase in the FosB/ΔFosB in the tVTA (Kaufling et al., 2010). The diffusion capacity of the dopamine induced my morphine is really low, and with the dopamine transporters still being active within the animal injected with morphine, diffusion of dopamine in turn is prevented. This might lead to the increased concentration of DA as aforementioned. tVTA neurons express μ-opioid receptors that get directly stimulated by the administered morphine, inhibiting the cAMP/PKA pathway further preventing the production of FosB/ΔFosB which is an important marker for neuronal activity (Kaufling et al., 2010). Hence no effect of morphine might have been observed within the tVTA, and a morphine + formalin only produced significant results at specific time points in the delta wave.

6. 2 LFP activity is increased in the VTA after formalin administration

One of the ***aims*** of the experiment was to determine the changes in the local field potential activity after administering formalin in the four different brain areas. Administering

formalin did produce significant changes in the LFP activity in the VTA, with significant waves for Delta, Theta, Alpha and Beta and Gamma. The significant waves were observed in the low and high frequency waves. Thus, it can be postulated that VTA might also be involved in the nociceptive pathway based on the increase in the LFP activity across the board after formalin injection as compared to the baseline. Comparison using a between-subjects analysis also proved that there were significant differences between the pure formalin group and the control (saline) group at specific time points that coincide with the time associated with the biphasic formalin response. This response was mainly observed in the low frequency waves. Thus, VTA could play an important role in the nociceptive pathway and relay the information. The reason for this increase could be attributed to the following reasons, based on previous research. The role of VTA in the reward system has been extensively studied but recent studies have also examined its role in nociception processing. Research has shown that acute noxious stimulations tend to produce phasic responses in the VTA DA neurons (Brischoux, Chakraborty, Brierley, & Ungless, 2009; Van'T Veer et al., 2012). Studies have examined the various excitatory as well has inhibitory afferents to the VTA (arising from the LHb, NAc, mPFC, tVTA), mainly arising from the cortical as well as the subcortical areas, many of whom are known to play an important role in nociceptive processing. A study by Hipólito et al., 2015, demonstrated that inflammatory pain led to the diminished function of the mu opioid receptors in the VTA. Damage to the VTA neurons due to lesions removed the morphine induced analgesia observed during any formalin testing (Morgan & Franklin, 1990). Another study also observed that damage to the VTA led to increased nociceptive behavior and sensitivity (Sotres-Bayón et al., 2001; Takeda et al., 2005). Administering dopamine agonist in the nucleus accumbens (NAc)

have been observed to inhibit analgesia produced by the VTA (Altier & Stewart, 1998). Elevated dopamine levels were observed in the NAc and the medial prefrontal cortex after receiving aversive stimuli which were confirmed using fast scan cyclic voltammetry (FSCV) and micro-dialysis (Bassareo, De Luca, & Di Chiara, 2002; Budygin et al., 2012). Experiments with direct electrical stimulation of the dopamine neurons within the VTA and also administration of selective agonist leading to the activation of D2 (dopamine D2) receptors in the nucleus accumbens, produced antinociceptive effects in rodent models of pain (Sotres-Bayón et al., 2001). On the other hand, administering D2 receptor antagonists in the nucleus accumbens caused increase in nociceptive sensitivity, suggesting the important role VTA plays in pain processing and also the dampening role of the dopaminergic signaling (Sotres-Bayón et al., 2001; Wood, 2008). Measuring D2 receptor binding levels is often tested as a direct response to nociception in humans (Hagelberg et al., 2002), and also patients suffering from Parkinson's disease (death of dopamine neurons) develop some form of chronic pain (Da Silva, Viana, & Barasnevicius Quagliato, 2008). It is also observed that there is activation of DA neurons and release of dopamine during nociception, and the enhanced activity likely exerts an inhibitory influence on the levels of nociception (Morales and Margolis, 2017). Hence form the above studies it could very well be postulated that VTA could play an important role in nociception processing. A similar outcome was also observed form the experiment, where administering formalin and a combination of formalin + morphine both produced significant differences post injection. A significant effect was observed in all the waves at different time intervals and a between subjects' analysis also showed a significant difference between the control (black) and

formalin (yellow) lines in all the waves except gamma, proving the involvement of VTA in nociception.

6.2.1 LFP activity increased in the VTA after morphine administration

Pertaining to other ***aim*** relating to morphine administration, there was a significant increase in LFP activity for any Delta and Theta band at the 30-35-minute timepoint. The formalin + morphine group did show a significant increase for the all the waves, but I think that result could very well be attributed to the formalin being the dominant force behind the effect. The main reason for no significant effect of morphine on tVTA could be explained based on the following research and analysis. The VTA disinhibitory mechanism is usually recruited upon opioid administration (Johnson & North, 1992). Studies have also observed that the VTA synaptic plasticity is heavily altered when subjected to drugs of abuse and stress in general (Niehaus, Murali, & Kauer, 2010). Such synaptic plasticity modifying factors have been postulated to be responsible for triggering addiction in individuals, while influencing long term changes in the excitatory and inhibitory synapses at the same time. One study has found that morphine could possibly disinhibit glutamatergic inputs present on the DA neurons in the VTA, which in turn could possibly trigger the pre-synaptic glutamate release (L. Yang et al., 2020). An increase in the LFP (local field potential) activity was observed after acute morphine administration in another electrophysiology-based analysis (Ahmadi Soleimani et al., 2018). However, another experiment did observe that VTA DA neuronal activity was higher (firing rate) in morphine dependent/addict rats when compared with naïve rats (Georges, Le Moine, & Aston-Jones, 2006). Another study observing the long-term potentiation of drug effects on VTA

found out that administering morphine (10 mg/kg i.p.) decreased the midbrain inhibitory synapse activity. Hence during my experiment, I did observe a statistically significant increase in morphine only animals in the delta and alpha wave. Between-subjects analysis did show difference between the blue (morphine) and the black (control) lines for the delta wave. Part of the reason as to why I may not have observed higher statistically significant data is that acute morphine administered in morphine naïve rats. Personal error could also be responsible for this.

6.3 No effect on the LFP activity in the NAc after formalin administration

One of the ***aims*** of the experiment was to determine the changes in the local field potential activity after administering formalin in the four different brain areas. Administering formalin did not produce any significant changes in the LFP activity in the NAc. The combination of formalin + morphine did produce significant effect in the low frequency Delta and Theta waves. Thus, it can be postulated that NAc might also be involved in the nociceptive pathway based on the increase in the LFP activity. Comparison using a between-subjects analysis also proved that there were significant differences between the pure formalin group and the control (saline) group at specific time points that coincide with the time associated with the biphasic formalin response. This response was mainly observed for the Delta, Theta, Alpha and Gamma waves. Thus, NAc could play an important role in the nociceptive pathway and relay the information. The reason for this increase could be attributed to the following reasons, based on previous research. Studies have shown that the NAc could very well be involved in nociceptive processing based on its connections to the other brain structures that are well known for

nociceptive modulations. There are dopaminergic afferent projecting from the VTA (ventral tegmental area) and substantia nigra, while it receives glutamatergic projections from the amygdala, thalamus, PFC (pre frontal cortex), hippocampus and the prelimbic cortex (Gupta & Young, 2018; Z. Li et al., 2018). The lateral habenulas has afferent connections from the NAc along with the medial frontal cortex (Bianco & Wilson, 2009). The LHb along with the prefrontal cortex has been known to involved in nociception, so it could very well be possible that NAc might be involved too along with being part of the pleasure pathway. NAc efferents also innervate amygdala and also to the ventral pallidum, two regions that have been studies to be heavily involved as nociceptive modulator (Kato, Ide, & Minami, 2016). Studies have also shown that both the core and shell region have feedback loops with regions of the brain that are directly involved with processing nociception, which could very well point towards the fact that NAc receives afferents and projects efferents to regions in the pain pathway (Harris & Peng, 2020; Seminowicz et al., 2019). However, there are studies that do counter and challenge this paradigm. According to a study by Becerra, Navratilova, Porreca, & Borsook, 2013, onset of nociceptive signal did not produce any activity change in the nucleus accumbens, however, offset of the pain or antinociception does produce an activity change in the NAc. They also observed that these changes were similar in both rats and human studies. Other studies have also reported similar result that signal valence within the nucleus accumbens was absent during the pain onset, while, the offset of the nociceptive signal produced a significant activity in the NAc (Baliki, Geha, Fields, & Apkarian, 2010; Lino Becerra & Borsook, 2008). These results fall in line with my experimental outcome, I did not see any significant results in the NAc after administering formalin however the combination of formalin and morphine which could

possibly have produce some antinociceptive effect did produce significant results in some of the waves.

6.3.1 LFP activity is increased in the NAc after morphine administration.

Pertaining to other ***aim*** relating to morphine administration, there was a significant increase in LFP activity for any Delta, Theta and Alpha band post morphine administration. The formalin + morphine group did show a significant increase for the all the waves, but I think that result could very well be attributed to the morphine being the dominant force behind the effect. The main reason for the robust significant effect of morphine on NAc could be explained based on the following research and analysis. Opiates have been long known to reduce nociceptive sensations by mainly mimicking the anti-nociceptive effect of endorphins. Research has shown that opiates primarily target medulla, midbrain and the pons to produce these antinociceptive effects (Erfanparast, Tamaddonfard, & Seyedin, 2018). Earlier studies mainly identified PAG, locus coeruleus and nucleus raphe magnus as the primary brain regions affected by morphine however recent studies have also identified the same antinociceptive effect exhibited by NAc increasing the neuronal activity (S. I. Hong et al., 2017; Yoshida, Nonaka, Nakamura, Araki, & Yamamoto, 2019). Another study by Corkrum et al., 2020 also observed the involvement of “astrocytes-neuron signaling mechanism” using calcium imaging as well as whole-cell patch clamp electrophysiological analysis in brain slices. These could be the reasons behind the increased neuronal activity in the NAc. Studies have also observed that ethanol has no effect on the dopamine uptake however in chronic ethanol treatment there was enhanced dopamine uptake (Budygin et al. 2007). FSCV studies pertaining to nicotine, cocaine both

increased the amplitude as well as the frequency of the spontaneous phasic DA release within the NAc (Wu et al. 2001). This is what was observed in my experiment as well which showed increased activity in the NAc post pure morphine administration in all the waves except beta and gamma. Between-subjects analysis was revealed significant differences between the black (control) and the blue (morphine) line in all of the waves at specific time points.

6.4 LFP activity is increased in the ACC after formalin administration.

One of the ***aims*** of the experiment was to determine the changes in the local field potential activity after administering formalin in the four different brain areas. Administering formalin did produce significant changes in the LFP activity in the ACC for Delta, Theta, Alpha and Beta waves. The combination of formalin + morphine did not produce any significant effect in the frequency bands. ACC has been known to be involved in the nociceptive pathway and based on the increase in the LFP activity, this is reaffirmed. Comparison using a between-subjects analysis also proved that there were significant differences between the pure formalin group and the control (saline) group at specific time points that coincide with the time associated with the biphasic formalin response. This response was mainly observed for the Delta, Theta, Alpha and Beta waves. The reason for this increase could be attributed to the following reasons, based on previous research. Studies have observed three major connections that are responsible for transmitting information from the visceral and somatic areas towards the ACC. First projection is from the thalamus specifically the ACC received this nociceptive afferent from the medial thalamus, which is turn received those signals from the spinal projections (spinothalamic tract) (Shyu & Vogt, 2009). The function aspect of this connection

pathway was also analyzed using electrophysiological studies (J. W. Yang, Shih, & Shyu, 2006). A recent study it was observed that the neurons within the mediodorsal thalamus directly innervate and stimulate the parvalbumin +ve interneurons located in the dorsal ACC. Its postulated that these neurons then further inhibit the pyramidal neurons within the layers II and III (Delevich, Tucciarone, Huang, & Li, 2015). The second projection towards the ACC originates at the amygdala, which includes the central nucleus receiving this nociceptive information via the parabrachial area (Ma & Peschanski, 1988). The last source of nociceptive signal originates from cortical regions such as S1 and insular cortex, towards the ACC. There are efferents originating from the pyramidal cells present in the layer V of the ACC innervating hypothalamus and the PAG, the later of the two is known to be involved in descending spinal sensory transmission. Studies have also revealed a direct projection from the ACC pyramidal cells to the dorsal horn and the spinal cord (Chen et al., 2014). Research has also shown connections between ACC and the amygdala, which is known to play an important role in fear and anxiety (Tovote, Fadok, & Lüthi, 2015). In another mammalian study directly stimulating the ACC produced vocalization which could have been an indicator of nociception and fear. It was observed that the connections between ACC and motor cortex were possibly involved (Devinsky, Morrell, & Vogt, 1995). ACC also projects to the locus coeruleus, a region that is known for its involvement in thermal nociceptive thresholds in mice (Aston-Jones & Cohen, 2005). Another reason why ACC might be involved in nociceptive information transmission is due to the cholinergic, dopaminergic, adrenergic as well as serotonergic connections its receives from the subcortical regions (Chandler, Lamperski, & Waterhouse, 2013). The results from my experiment did fall in line with the previous studies as significant increase in activity

was observed after formalin injection. Between -subjects analysis also revealed a significant difference between the black (Control) and yellow (formalin) line at various time points in almost all the waves.

6.4.1 No effect on the LFP activity in the ACC after morphine administration.

Pertaining to other ***aim*** relating to morphine administration, there was no significant increase in LFP activity for frequency bands. The formalin + morphine group also did not show a significant increase for any of the waves. The main reason for no significant effect of morphine on ACC could be explained based on the following research and analysis. Previous research has proven the importance of ACC in motivations, cognitive and evaluative functions (Navratilova et al., 2015) along with nociception (B. A. Vogt, 2005). In another study, ACC activated using glutamatergic inputs have shown to elicit pain-like responses in naïve rats (Johansen & Fields, 2004). Another study investigated the effect of opioid on the three sub regions of ACC and the RVM (rostral ventromedial medulla). Morphine was cranially microinjected to produce analgesic responses in RVM to thermal and mechanical noxious stimuli in injured rats, however similar morphine microinjections into the ACC had no significant effect on the thermal or mechanical responses and was responsible for reducing nociception only in one of the three ACC sites. Thus, it was postulated that systemically administered opioids acted preferentially within the ACC, helping to alleviate the emotional quality of pain without affecting the nociceptive threshold (Bingel et al., 2011; Navratilova et al., 2015). A study by Wang et al., 2020 also observed the importance of MORs in the ACC for opioid analgesia and the presence of inflammatory pain downregulated the number of MORs in the ACC thus diminishing the

morphine effect. My results also fall in line with these observations as I did not notice any significant changes in the LFP activity post morphine administration.

Chapter 7

Conclusion

Research over the past few decades has been successful in yielding tons of information regarding the brain and how it behaves during aversive and rewarding stimuli, however, till date the exact process of how these two mechanisms are interconnected is poorly understood. Recently the tVTA came out to be as an important GABAergic structure that has been postulated to be involved in aversion and rewarding stimuli. As seen from previous studies and form my own experiment tVTA could possibly play an important role. Other studies have also confirmed this paradigm but still more research is required to fully understand the complexity of this region. But this is a relatively newly identified structure and while the latest information regarding its involvement in reward, aversion and psychiatric disorders is available, more research is required to fully apply that knowledge in the clinical setting. There is still a major gap in research with regards to the pre-or-post synaptic receptors that are manipulated in the tVTA, (including the ones involved in glutamate and opioid transmission) that could influence the tVTA neurons to encode for aversive stimuli and inhibit the midbrain DA neurons. This could very well be the next project in the Peng/Perrotti lab. There should also be research involved with investigating the potential role played by the tVTA in substance abuse and other dysfunctions involving the dopamine transmissions, for e.g. Parkinson's disease as well as Schizophrenia. The findings from these could very well lead to a development of a therapeutic

intervention in neurological as well as psychiatric disorders. It could very well be postulated from previous research as well as my experiment that not all aversive stimuli pass through the tVTA and some avoidance behavior could very well develop in the absence of tVTA. Since this region further connects with VTA, NAc and ACC, I wanted to get a big picture of what is happening to this pathway in the event on an aversive response and during a antinociceptive effect. While I did observe data that could imply its involvement in both these processes, future research should involve other regions such as the habenula, thalamus, the dorsal horn, to fully understand this pathway. Acquiring results from a freely moving animal has its own challenges, the results could be better in the future if the rat's heads are fixed in place and the animal is kept awake. Personal error could not be discounted as with any scientific experiment. It would also be interesting to observe sex differences in the tVTA if any exists. Finally, Oversimplification, while trying to characterize simple functions to the tVTA, mainly for clarity, may mislead future research, as the complexity of the tVTA mirrors the complexity exhibited by the DA system itself.

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