# AN INVESTIGATION OF THE ROLE OF GALANIN IN BEHAVIORS ASSOCIATED WITH CHRONIC INFLAMMATORY PAIN AND STRESS

by

JESSICA M. SMITH

(Under the Direction of Philip V. Holmes)

## ABSTRACT

The neuropeptide galanin has wide distribution in the nervous system and has a role in diverse functions, such as pain modulation and behavioral responses to stress. A major mechanism of galanin's actions in the brain is modulation of catecholamine signaling. This has implications for conditions characterized by aberrant dopamine and norepinephrine activity, such as anxiety and depression. Galanin is also involved in pain modulation and emerging evidence suggests that it partially mediates the relationship between conditions of chronic inflammatory pain and depression. Dysregulation of the mesolimbic dopamine pathway is also implicated in the comorbidity of chronic inflammatory pain and depression. The following chapters provide evidence supporting the hypothesis that mesolimbic dopamine dysregulation underlies motivational deficits associated with chronic inflammatory pain. Furthermore, we provide further support for the role of galanin in modulating behavioral changes associated with stress and chronic inflammatory pain.

INDEX WORDS: Galanin, Depression, Anxiety, Arthritis, Complete Freund's adjuvant, Meoslimbic pathway, Locus coeruleus, Dopamine, Norepinephrine, Ventral tegmental area, Nucleus accumbens, Microdialysis, Contextual fear conditioning, Inflammatory pain

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Comorbidity of depression and inflammatory pain

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease characterized by synovial joint swelling, tenderness and pain, and destruction (Aletaha et al., 2010). A recent study determined that the overall prevalence of RA in the United States in 2014 was 0.53% and estimated that approximately 1.3 million adults were affected by RA in 2014 (Hunter et al., 2017). Hunter and colleagues (2017) also determined that the age-adjusted prevalence of RA was much higher in females (0.73% – 0.78%) than in males (0.29% – 0.31%) and that prevalence increased with age for both males and females. It is well accepted that RA has a high prevalence of comorbidity with depression (Anderson, Bradley, Young, McDaniel, & Wise, 1985; Dougados et al., 2014; Katz & Yelin, 1995). A recent meta-analysis estimated the annual prevalence rate of depression in RA patients to be 16.8%, compared to 4.1% for the general population (Matcham, Rayner, Steer, & Hotopf, 2013; Waraich, Goldner, Somers, & Hsu, 2004). Matcham and colleagues (2013) also found that 15% - 48% of RA patients report depressive symptoms, as measured by self-report screening questionnaires. RA patients with comorbid depression tend to experience increased pain, fatigue, reduced quality of life, increased health care costs, more comorbidities, and increased mortality (Roubille et al., 2015). Therefore, it is critical that RA patients are screened for comorbid depression and that the two conditions are treated concurrently. A better understanding of the neurobiological dysregulation linking the two disorders will lead to better treatment options and outcomes.

Several lines of pre-clinical research also support a relationship between inflammation, inflammatory pain, and depression. Manipulations that are frequently employed to induce a depression-like phenotype in rodents, such as olfactory bulbectomy, chronic mild stress, social defeat, and learned helplessness, also increase central and peripheral levels of pro-inflammatory cytokines (Audet, Jacobson-Pick, Wann, & Anisman, 2011; Chourbaji et al., 2006; Gomez-Lazaro et al., 2011; Goshen et al., 2008; Grippo, Francis, Beltz, Felder, & Johnson, 2005; Grippo, Sullivan, et al., 2005; Kubera et al., 1998; W. Liu et al., 2013; Patki, Solanki, Atrooz, Allam, & Salim, 2013; Rinwa & Kumar, 2013; Rinwa, Kumar, & Garg, 2013; Song, Zhang, & Manku, 2009; Steptoe, Willemsen, Owen, Flower, & Mohamed-Ali, 2001; Sukoff Rizzo et al., 2012; You et al., 2011). Genetically modified mice that lack genes for pro-inflammatory cytokines or their receptors are resilient to manipulations that induce a depression-related phenotype (Chourbaji et al., 2006; Goshen et al., 2008; Kaster, Gadotti, Calixto, Santos, & Rodrigues, 2012; Ulloa et al., 2010). Administration of pro-inflammatory cytokines or chemicals that induce inflammation, such as lipopolysaccharide (LPS), complete Freund's adjuvant (CFA), or Bacillus Calmette-Guerin (BCG) vaccine, lead to a depression-related phenotype (Bonaccorso et al., 2002; Kaster et al., 2012; Moreau et al., 2008). Finally, treatment with known anti-depressants or anti-inflammatory drugs normalizes both pro-inflammatory cytokine levels and depression-like behaviors in animal models of depression and decreases pro-inflammatory cytokine secretion from immune cells (Beurel, Harrington, & Jope, 2013; Brustolim, Ribeiro-dos-Santos, Kast, Altschuler, & Soares, 2006; Kubera, Holan, Mathison, & Maes, 2000; Kubera, Simbirtsev, Mathison, & Maes, 2000; Leitl, Onvani, et al., 2014; Maes et al., 1999; Song et al., 2009; Xia, DePierre, & Nassberger, 1996). Therefore, evidence from the animal literature is in

agreement with human literature and supports the claim that inflammatory pain is linked with depression.

**Comparison of animal models of inflammatory pain used for studying comorbid depression**

Intraplantar and intra-articular injection of CFA are two well-accepted rodent models of monoarthritis and can be used to study the neurobiology of RA. However, many rodent models are employed to study the neurobiology of inflammation- or inflammatory pain-induced sickness behavior and depression. Examples of other commonly used models of inflammation and inflammatory pain are provided below. The brief overviews provided focus on behavioral changes and neurobiological changes relevant to sickness behavior and depression. For information on immune activation and other pathophysiology induced by these and other animal models of arthritis see Bessis, Decker, Assier, Semerano, and Boissier (2017) and Fischer, Adeyemo, O'Leary, and Bottaro (2017). For reviews on acute pain models and nociceptive tests see Le Bars et al (2001) and on neuropathic pain models see Kumar, Kaur, and Singh (2018).

**CFA**

Intraplantar and intra-articular injection of CFA are useful models for the study of the neurobiology of the comorbidity of chronic inflammatory pain and depression. CFA administration rapidly induces local inflammation and allodynia that is sustained for at least six weeks (Butler, Godefroy, Besson, & Weil-Fugazza, 1992; Ren & Dubner, 1999; Stein, Millan, & Herz, 1988). Neuroimmune, neuroendocrine, and HPA-axis functions are disrupted by CFA. For example, CFA administration induces an acute increase in serum ACTH and prolactin and an acute decrease in serum growth hormone and thyroid stimulating hormone (Selgas, Arce, Esquifino, & Cardinali, 1997). CFA also induces prolonged increases in serum TNF-α, IL-1β, and VEGF (Santora, Rasa, Visco, Steinetz, & Bagnell, 2007; Zaringhalam et al., 2016) and

decreases melatonin in the pineal gland (Esquifino & Cardinali, 1999). Subcutaneous injection of CFA also increases whole brain IL-1β expression, but not in the cortex or hippocampus, and cortical COX-2 expression 2 weeks after injection (Maciel, Silva, Morrone, Calixto, & Campos, 2013). Furthermore, CFA injection alters expression of genes that encode dopamine receptors, corticotropin releasing hormone (CRH), and CRH receptors in pain- and stress-responsive brain regions (Monteiro et al., 2016).

In addition to physiological and neurochemical effects, intraplantar and intra-articular CFA induces a transient sickness response including decreased food consumption and weight loss (Iadarola, Brady, Draisci, & Dubner, 1988). Sickness behavior is followed by a sustained depression-related phenotype. Prolonged effects of CFA include alterations in sleep structure (Pankova, Popkova, Vetrile, Basharova, & Krupina, 2001), circadian rhythms (Cardinali & Esquifino, 2003) and HPA-axis activity (Grinevich et al., 2002), reduced sucrose consumption (Refsgaard, Hoffmann-Petersen, Sahlholt, Pickering, & Andreasen, 2016), increased immobility time during forced swim stress (G. Borges, Neto, Mico, & Berrocoso, 2014), and decreased social interaction time (Gregoire, Wattiez, Etienne, Marchand, & Ardid, 2014). Exploratory locomotor activity is also reduced for at least four weeks (Butler et al., 1992; Larsen & Arnt, 1985; Stein et al., 1988); however, it is difficult to disentangle the effects of pain from volition. Because the ensuing depression-like phenotype is sustained for several weeks after the initial sickness response has subsided, this model is suitable for the study of the comorbidity of inflammatory pain and depression.

**LPS**

Intraperitoneal injection of the bacterial endotoxin LPS is frequently employed to study effects of inflammation on the central nervous system. Through interactions with toll-like

receptor 4, LPS injection induces rapid systemic inflammation, fever, hyperalgesia, weight loss, and increased sleeping (Dunn & Swiergiel, 2005; Dunn, Swiergiel, & de Beaurepaire, 2005; Dunn, Wang, & Ando, 1999; Krueger, Majde, & Obal, 2003). LPS also induces acute increases in plasma ACTH, corticosterone, IL-6, TNF-α, and IL-10 (Gibb, Hayley, Poulter, & Anisman, 2011). Furthermore, LPS administration leads to altered monoamine signaling in several brain regions including the parietal cortex, hypothalamus, brainstem, and hippocampus. It also increases FOS expression in the hypothalamus, brainstem, and dorsal vagal complex, nucleus accumbens, amygdala, hippocampus, periaqueductal gray (PAG), and locus coeruleus (LC) (Dunn et al., 1999; Frenois et al., 2007; Wieczorek, Swiergiel, Pournajafi-Nazarloo, & Dunn, 2005). Brain levels of TNF-α, IL-1β, IL-6, and IFN-γ are also increased after LPS administration, in regions such as the medial prefrontal cortex and hippocampus (Gibb et al., 2011; O'Connor, Lawson, Andre, Moreau, et al., 2009; Fulenwider et al., 2018).

LPS administration induces a number of behavioral responses such as decreased consumption of regular chow and highly palatable food, increased immobility time in the forced swim and tail suspension tests, decreased open field exploration, decreased sucrose preference, decreased social interaction, and increased anxiety-like behaviors in the open field and light/dark box tests (Castanon, Bluthe, & Dantzer, 2001; Dunn & Swiergiel, 2005; Frenois et al., 2007; Salazar, Gonzalez-Rivera, Redus, Parrott, & O'Connor, 2012; Wieczorek et al., 2005). Importantly, the effects of LPS on physiology, behavior, and neurochemistry are transient (Dunn, 1992; Frenois et al., 2007; O'Connor, Lawson, Andre, Moreau, et al., 2009). Thus, although LPS administration is an excellent model for studying sickness behavior, it’s utility for studying inflammatory pain-induced neural changes that lead to a lasting depression-like phenotype is limited.

**Polyinosinic-polycytidylic acid I:C (Poly I:C)**

Poly I:C is a synthetic double stranded RNA that acts as a toll-like receptor-3 agonist. Intraperitoneal injection initiates a similar inflammatory response as viral infection (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Administration of poly I:C induces a transient sickness response that recovers within 72 hours, including decreased food intake, weight loss, fever, and decreased locomotor activity, rearing, burrowing, and sucrose consumption (Fortier et al., 2004; Gibney, McGuinness, Prendergast, Harkin, & Connor, 2013; Katafuchi et al., 2003; Murray et al., 2015). Treatment with poly I:C also suppresses voluntary wheel running for around 5-10 days depending on dose (Katafuchi, Kondo, Take, & Yoshimura, 2005; Katafuchi et al., 2003) Sickness behavior is accompanied by increased plasma IL-6, TNF-α, IL-1beta, IFN-beta, corticosterone, and kynurenine (Cunningham, Campion, Teeling, Felton, & Perry, 2007; Gandhi, Hayley, Gibb, Merali, & Anisman, 2007; Gibb et al., 2011; Murray et al., 2015). Poly I:C administration also increases IL-1β,  I, IL-6, and TNF-α in the frontal cortex, hippocampus, hypothalamus, brainstem, and cerebellum between 3- and 24-hours post-injection (Cunningham et al., 2007; Gibney et al., 2013; Konat, Borysiewicz, Fil, & James, 2009; Murray et al., 2015). Cortical, hippocampal, and hypothalamic IFN-α, but not IL-1beta, is increased 7-8 days post-injection (Katafuchi et al., 2005; Katafuchi et al., 2003). Hypothalamic IFN-β, microglial activation in the frontal cortex and hippocampus, and altered serotonin metabolism are also observed between 24- and 48-hours post injection (Gibney et al., 2013; Katafuchi et al., 2005). Expression of the serotonin transporter is also increased in the medial prefrontal cortex, dorsal raphe nucleus, parietal cortex, hippocampus, and hypothalamus within the first 8 days after poly I:C injection (Katafuchi et al., 2005). Poly I:C is an excellent model for studying the effects of acute inflammation on the neurobiology of sickness behavior. However, the poly I:C model is

more appropriate for the study of the immune and sickness responses associated with acute phase of viral infection, rather than those associated with injury or conditions of chronic inflammatory pain, such as RA.

**BCG**

BCG is an attenuated strain of *Mycobacterium bovis* and is commonly used to vaccinate against tuberculosis. Recently, this compound has begun to be employed to study inflammation-induced sickness behavior and depression (Moreau et al., 2008). Intraperitoneal BCG administration in rodents induces a transient loss in body weight, decrease of locomotor behavior, and decrease in food intake. BCG administration increases serum interferon-γ, TNF-α, IL-6, and kynurenine, for several weeks (Kwon et al., 2012; Moreau et al., 2008; Moreau et al., 2005; O'Connor, Lawson, Andre, Briley, et al., 2009). After seven days, BCG infection increases central and systemic indoleamine 2,3 dioxygenase (IDO), an enzyme that metabolizes tryptophan (O'Connor, Lawson, Andre, Briley, et al., 2009). Increased IDO activity in the brain lasts for approximately 3 weeks and mediates the effects of BCG on depression-related behavior (Moreau et al., 2005; O'Connor, Lawson, Andre, Briley, et al., 2009). Effects of BCG on depression-related behavior are also mediated by IFNγ and TNF-α, presumably through induction of IDO (O'Connor, Andre, et al., 2009).Mice injected with BCG show increased whole-brain norepinephrine levels, but no differences in whole brain serotonin or dopamine levels, 13 days later (Barneoud, Rivet, Vitiello, Le Moal, & Neveu, 1988). Furthermore, BCG administration causes are reduction of dopamine in the hippocampus 16 days later (Kwon et al., 2012)..

BCG administration increases immobility time in the tail suspension and forced swim tests and decreases sucrose consumption for two to three weeks (Kwon et al., 2012; Moreau et al., 2008). Administration of BCG also decreases voluntary wheel running for 1 week (Moreau et

al., 2008). Depression-like behaviors are attenuated by treatment with anti-depressants (Saleh et al., 2014; Vijaya Kumar et al., 2014). In sum, BCG administration induces transient sickness behavior that subsides within one week and is followed by a depression-related phenotype that lasts for at least three weeks. Therefore, BCG is an excellent model for studying the comorbidity of chronic inflammation and depression in mice. However, its utility as a model of chronic inflammatory pain that is more akin to RA is limited because BCG rapidly disseminates into all organs and induces wide-spread systemic inflammation (Tsenova, Bergtold, Freedman, Young, & Kaplan, 1999).

**Collagen-Induced Arthritis (CIA)**

CIA is one of the most frequently employed experimental models of autoimmune arthritis; however, few studies have investigated the behavioral effects of CIA. Arthritis is induced in genetically susceptible rodents by immunization with type II bovine collagen. Antibodies raised in the animal target the collagen in joint cartilage, resulting in a RA-like disease. Polyarthritis develops over several weeks before symptoms are observed and is sustained for at least 60 days (Asquith, Miller, McInnes, & Liew, 2009; Brown et al., 2018; del Rey et al., 2008). During the asymptomatic period, CIA rodents show increased plasma corticosterone and epinephrine (del Rey et al., 2008). They also show increased TNF-α, IL-1β, and IL-10 in lymphoid cells during the asymptomatic period. Corticosterone levels become decreased in CIA rodents relative to controls after onset of symptoms. While TNF-α levels remain elevated throughout disease progression, IL-1β and IL-10 levels return to normal within the first two weeks (del Rey et al., 2008). CIA mice show normal weight gain for at least two weeks post-treatment until symptoms manifest, at which point they begin to weigh less than controls, show swelling of joints and joint damage, and display hyperalgesia/allodynia (del Rey

et al., 2008; Inglis et al., 2007; Sasakawa, Sasakawa, Ohkubo, & Mutoh, 2005). After symptom onset, IFN-gamma and IL-10 levels in lymphoid cells increase (del Rey et al., 2008). Around this timepoint, CIA mice also show reduced sucrose preference and female urine sniffing, suggesting that CIA induces a major hallmark of depression, anhedonia. They also show increased reuptake of serotonin in the hippocampus suggesting enhanced activity of the serotonin transporter because serotonin transporter expression is unchanged. The effects of CIA on anhedonia and serotonin transporter function are dependent on increased TNF-α activity (Brown et al., 2018). CIA animals also show increased IL-1β and IL-6 mRNA in the hypothalamus for 2-3 weeks post-injection, but no differences in TNF-α mRNA, followed by altered hypothalamic monoamine levels (del Rey et al., 2008). Brain TNF-α receptors are upregulated, however (Brown et al., 2018). The long progression of this disease makes it a unique model for studying the neuropathology associated with arthritis. Specifically, the prolonged elevation of peripheral proinflammatory cytokines without concomitant increases in pain during the asymptomatic period allow experimenters to disentangle effects of chronic inflammation from chronic inflammatory pain on sickness- and depression-related behaviors.

## Mesolimbic dopamine dysregulation mediates the comorbidity of chronic inflammatory pain and depression

Although**,** the relationship between conditions of chronic inflammatory pain and depression is multifactorial and bidirectional (Margaretten, Julian, Katz, & Yelin, 2011; Remus & Dantzer, 2016), our laboratory is interested in how chronic inflammatory pain leads to dysregulation of neurobiological circuits that, in turn, induce a depression-like phenotype. A large body of evidence implicates dysregulation of the mesolimbic dopamine system in the comorbidity of conditions of chronic inflammatory pain and depression. However, chronic

inflammatory pain also disrupts other neurological processes that are implicated in the neurobiology of depression. A major emerging hypothesis of how inflammation induces depression centers on the role of tryptophan metabolism by the kynurenine pathway in mediating the effects of chronic inflammation on sickness behavior and depression (Remus & Dantzer, 2016). Trypotophan is converted to serotonin by 5-hydroxytryptophan or kynurenine by IDO or tryptophan 2,3 dioxygenase. Dantzer and colleagues have uncovered a role for kynurenine metabolites, quinolinic acid and kynurenic acid, in mediating effects of inflammation on depression-like behaviors via interactions with the NMDA receptor (Remus & Dantzer, 2016). Other groups have proposed that depression results from dysregulation of neurotrophic factors, neuroplasticity, and neurogenesis induced by inflammation (Doan, Manders, & Wang, 2015; Stepanichev, Dygalo, Grigoryan, Shishkina, & Gulyaeva, 2014). Dysregulation of glutamate and GABA signaling is also implicated in the comorbidity of pain and depression (Benson et al., 2015; Doan et al., 2015; Miladinovic, Nashed, & Singh, 2015; Remus & Dantzer, 2016). There is also extensive support for inflammatory pain-induced neuroendocrine dysfunction in depression (Blackburn-Munro, 2004; Mifflin et al., 2015). The following is a review of the known effects of chronic inflammatory pain on the mesolimbic dopamine system. Broader reviews of the mesolimbic dopamine system in acute and chronic pain processing can be found elsewhere (Mitsi & Zachariou, 2016; Navratilova & Porreca, 2014).

The ventral tegmental area (VTA) is a major dopamine nucleus in the brain. VTA projections to the nucleus accumbens and other limbic structures are commonly referred to as the mesolimbic dopamine pathway. The mesolimbic dopamine pathway is perhaps best known for its role in addiction (Volkow & Morales, 2015); however, impairments in motivated and reward-sensitive behaviors in depression are also attributed to mesolimbic dysregulation (Nestler &

Carlezon, 2006). It is now well-accepted that mesolimbic dopamine dysregulation in-part mediates the relationship between chronic inflammatory pain and depression.

Like other models of pain and inflammation, CFA administration has been shown to alter mesolimbic neurobiology and behaviors controlled by mesolimbic function. Operant responding for food and drug reward are decreased after CFA administration (Hipolito et al., 2015; Schwartz et al., 2014), as is intracranial self-stimulation (Leitl, Potter, et al., 2014). Intraplantar administration of CFA disrupts circadian rhythms of dopamine turnover in the striatum (Cano et al., 2001). CFA administration also blunts disinhibition of VTA neurons and dopamine release in the nucleus accumbens after heroin or DAMGO administration, suggesting that CFA alters the sensitivity of the mesolimbic dopamine system to drugs of abuse (Hipolito et al., 2015). In addition to affecting dopamine release, CFA administration also alters the excitability of medium spiny neurons in the nucleus accumbens (Schwartz et al., 2014).

How chronic inflammatory pain disrupts mesolimbic dopamine signaling remains unclear, as few studies have investigated the effects of intraplantar CFA on the VTA or brain regions upstream of the VTA. Hipolito and colleagues showed that altered mesolimbic dopamine signaling is in part accounted for by desensitization of opioid receptors in the VTA (Hipolito et al., 2015). Other research suggests that increased VTA dopamine neuron activity is mediated by upregulation of NF-κB in VTA astrocytes after reduced expression of microRNA miR-219-5p. Although reduced miR-219-5p causes an increase in proinflammatory cytokines in the VTA and mediates nociception induced by CFA, it does not seem to mediate the effects of CFA on affective behaviors (S. Zhang et al., 2017). Research on the effects of LPS suggest that changes to D3 receptor and BDNF expression in the VTA might also mediate the effects of inflammatory pain on affective behavior and mesolimbic dopamine signaling (J. Wang et al., 2018).

The LC is a major noradrenergic input to the VTA and impaired LC function after chronic pain is associated with a depression-like phenotype (Alba-Delgado et al., 2013). This suggests that LC-VTA connections mediate some effects of inflammatory pain on affective behavior. However, most research on the pain-modulatory effects of the LC has focused on its role in antinociception via descending pain modulation (Bassi et al., 2018; Brightwell & Taylor, 2009; Llorca-Torralba, Borges, Neto, Mico, & Berrocoso, 2016; Nonaka et al., 2017; Tsuruoka & Willis, 1996). In essence, research shows that the LC is activated by various structures, including the dorsal horn of the spinal cord and paragigantocelluar nucleus in the rostro-ventral medulla, in response to noxious stimuli (Berridge & Waterhouse, 2003; Ennis, Aston-Jones, & Shiekhattar, 1992; Hirata & Aston-Jones, 1994; Pertovaara, 2006). LC activation does not have a strong antinociceptive effect in response to acute noxious stimuli under basal conditions; however, when noxious stimulation is persistent in chronic pain conditions, the LC and other noradrenergic nuclei engage descending pain modulatory pathways that inhibit transmission of ascending pain signals from the spinal cord, primarily via α2 receptor stimulation (Pertovaara, 2006). Failure of α2 blockade to effect pain responses during acute noxious stimuli, suggest that LC activation likely serves to promote attention and arousal and possibly modulate cognitive and affective-motivational responses to the stimulus (Dennis, Melzack, Gutman, & Boucher, 1980; Pertovaara, 2006). How the LC modulates cognitive and affective-motivational properties of pain through ascending projections to limbic and cortical regions and supraspinal effects of the LC during chronic pain, however, are less-well understood (Llorca-Torralba et al., 2016).

Furthermore, the specific effects of intraplantar/intraarticular CFA on LC activity have not been well characterized. The LC is not strongly affected by CFA on the day of administration (Imbe et al., 2009). Rather, altered LC activation, measured by C-FOS and pERK expression,

manifests several weeks later (G. Borges et al., 2014), Likewise, Neto and colleagues (1999) showed that CFA-treated animals have increased metabolism in the LC and two major inputs to the LC, the lateral reticular nucleus and lateral paragigantocellular nucleus, two weeks after injection, but not 2 or 4 days later (Neto et al., 1999). Effects of CFA on LC activity might be mediated by downregulation of opioid receptor expression and increased CRF release from paraventricular nucleus of the hypothalamus (PVN) afferents (G. P. Borges, Mico, Neto, & Berrocoso, 2015; Jongeling, Johns, Murphy, & Hammond, 2009). Notably, CRF release in the LC seems to mediate the effects of CFA on ERK activation and affective behaviors, but not nociceptive behaviors (G. P. Borges et al., 2015), while electrical stimulation of the PVN reduces pain sensitivity, inflammation, and neutrophilic infiltration in arthritic rats (Bassi et al., 2018). Other research suggests that neural connections between the LC and the prefrontal cortex, anterior cingulate cortex, and amygdala also play a role in modulation of the affective and cognitive components of pain (Alba-Delgado et al., 2013; Barthas et al., 2015; Llorca-Torralba et al., 2016; Suto, Eisenach, & Hayashida, 2014); however, to our knowledge no one has investigated the effects of CFA on LC-VTA projections. Examining the effects of inflammatory pain on VTA specific projections of the LC will be an important step in understanding mesolimbic dysregulation in conditions of chronic inflammatory pain.

## The role of the neuropeptide galanin in stress- and pain-related disorders

### Galanin overview

Galanin is a well-conserved 29-amino acid neuropeptide that was isolated from porcine gut in 1983 (Kask, Langel, & Bartfai, 1995; Tatemoto, Rokaeus, Jornvall, McDonald, & Mutt, 1983). Over three decades of research have shown that galanin and galanin receptors have wide

expression throughout the body and are involved in a diverse array of physiological systems, including those which modulate pain and stress (Lang et al., 2015).

Several major mapping studies were performed shortly after discovery of galanin. Collectively it was revealed that galanin immunoreactivity and mRNA are widely expressed in the central nervous system. Notably, galanin is expressed in stress- and pain-responsive brain regions, such as the LC, nucleus of the solitary tract, amygdala, VTA, nucleus accumbens, and PVN, and the majority of forebrain galanin is colocalized with norepinephrine in LC-derived projections. Galanin is also expressed in the spinal cord and dorsal root ganglion (Ch'ng et al., 1985; Jacobowitz, Kresse, & Skofitsch, 2004; Melander, Hokfelt, & Rokaeus, 1986; Rokaeus et al., 1984; Skofitsch & Jacobowitz, 1985a, 1985b, 1986; Skofitsch, Sills, & Jacobowitz, 1986).

Galanin exerts its effects through three major G-protein couple receptor subtypes that are also widely expressed in the central nervous system (Jacobowitz et al., 2004; Skofitsch et al., 1986). The three receptor subtypes, GalR1, GalR2, and GalR3, show distinct, but overlapping, distribution in the central nervous system and couple to different signaling transduction pathways, allowing for diverse effects of galanin (Burgevin, Loquet, Quarteronet, & Habert-Ortoli, 1995; Gustafson, Smith, Durkin, Gerald, & Branchek, 1996; Jacobowitz et al., 2004; Lang et al., 2015; Melander et al., 1988; Mennicken, Hoffert, Pelletier, Ahmad, & O'Donnell, 2002; O'Donnell, Ahmad, Wahlestedt, & Walker, 1999). Stimulation of the GalR1 receptor inhibits production of cAMP, increases MAPK activity, and leads to the opening of G-protein coupled inward rectifying K+ (GIRK) channels. The effects on signal transduction are sensitive to pertussis toxin but independent of PKC, suggesting that GalR1 couples to $G_i$ proteins (Habert-Ortoli, Amiranoff, Loquet, Laburthe, & Mayaux, 1994; Parker et al., 1995; S. Wang, Hashemi, Fried, Clemmons, & Hawes, 1998). GalR2 simulation increases production of inositol phosphate,

an effect that is insensitive to pertussis-toxin. GalR2 stimulation also increases chloride conductance and calcium mobilization, suggesting that GalR2 couples to $G_{q/11}$ proteins (Fathi et al., 1997; Smith et al., 1997). One study showed that GalR2 stimulation induced a modest inhibition of cAMP production and increased MAPK activation in a pertussis toxin-sensitive manner. However, the effects of GalR2 stimulation on MAPK activation are also dependent on PKC. This suggests that GalR2 can also couple to $G_{i/o}$ proteins (S. Wang et al., 1998). Similar to GalR1, GalR3 inhibits the production of cAMP and stimulates GIRK channels, both in a pertussis toxin sensitive manner, suggesting coupling to $G_{i/o}$ proteins; however GalR3 has much lower expression than GalR1 and GalR2 in the rodent central nervous system (Mennicken et al., 2002; Smith et al., 1998).

**Galanin serves major neuromodulatory functions in the central nervous system**

Early studies also showed that galanin is frequently co-expressed with other neurotransmitters, including norepinephrine in the LC, dopamine in the hypothalamus, serotonin in the dorsal and medullary raphe nuclei, and acetylcholine in the cholinergic forebrain nuclei. Galanin is also colocalized with GABA, glutamate, and other neuropeptides (Hamill, Skofitsch, & Jacobowitz, 1986; Levin, Sawchenko, Howe, Bloom, & Polak, 1987; Melander, Hokfelt, Rokaeus, et al., 1986; Melander et al., 1985). Co-expression of galanin with other neurotransmitters suggests that galanin modulates neurotransmission. Indeed, a wealth of evidence indicates that galanin modulates neurotransmission of norepinephrine, acetylcholine, dopamine, serotonin and other neurotransmitters (Bartfai et al., 1991; Dutar, Lamour, & Nicoll, 1989; Picciotto, Brabant, Einstein, Kamens, & Neugebauer, 2010; Sundstrom & Melander, 1988; Z. Q. Xu, Zheng, & Hokfelt, 2005).

The interactions between galanin and catecholamine signaling are of particular interest to the current research because dysregulation of these transmitter systems is implicated in the neurobiology of stress-related disorders. Most noradrenergic cells in the LC co-express galanin and galanin is also co-expressed with norepinephrine in fibers and nerve terminals in brain regions that receive projections from the LC (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988; Melander, Hokfelt, Rokaeus, et al., 1986; Z. Q. Xu, Shi, & Hokfelt, 1998). Notably, the majority of galanin in the forebrain is in LC-derived projections that also express norepinephrine (Hokfelt, Xu, Shi, Holmberg, & Zhang, 1998; Z. Q. Xu, Shi, et al., 1998). Like other neuropeptides, galanin is preferentially released during burst firing (Bartfai, Iverfeldt, Fisone, & Serfozo, 1988; Consolo et al., 1994; Karhunen, Vilim, Alexeeva, Weiss, & Church, 2001; Lundberg & Hokfelt, 1983). Galanin inhibits the firing of LC noradrenergic neurons and norepinephrine release in an autocrine manner, primarily via GalR1 stimulation (Bartfai et al., 1991; Ma et al., 2001; Pieribone et al., 1995; Seutin, Verbanck, Massotte, & Dresse, 1989; Sevcik, Finta, & Illes, 1993; Tsuda, Yokoo, & Goldstein, 1989; Z. Q. Xu, Tong, & Hokfelt, 2001; Z. Q. Xu et al., 2005). Several lines of indirect evidence suggest that LC-derived galanin also inhibits neurotransmission in projection sites of the LC. That is, many LC targets express galanin receptors (Melander et al., 1988; Skofitsch et al., 1986), contain LC-derived galanin-positive fibers (Holets et al., 1988; Melander, Hokfelt, Rokaeus, et al., 1986; Z. Q. Xu, Shi, et al., 1998), and are inhibited by galanin, in terms of membrane hyperpolarization and cAMP production (Counts, Perez, Ginsberg, & Mufson, 2010; Hokfelt et al., 1998; Karelson, Laasik, & Sillard, 1995; Nishibori, Oishi, Itoh, & Saeki, 1988; Valkna et al., 1995; Z. Q. Xu, Zhang, Pieribone, Grillner, & Hokfelt, 1998). Therefore, modulation of noradrenergic neurons in the LC is a well-accepted major role of galanin.

Likewise, galanin modulates dopamine neurotransmission. Galanin receptors and galanin-positive fibers are expressed in the VTA, substantia nigra, and arcuate nucleus, which are three major dopaminergic nuclei. Galanin is also co-expressed with dopamine in the arcuate nucleus and has been shown to inhibit tuberoinfundibular dopamine neuron activity and dopamine release (Everitt et al., 1986; Gopalan, Tian, Moore, & Lookingland, 1993; Melander, Fuxe, Harfstrand, Eneroth, & Hokfelt, 1987; Melander, Hokfelt, Rokaeus, et al., 1986; Nordstrom, Melander, Hokfelt, Bartfai, & Goldstein, 1987). Galanin administration in the PVN increases dopamine release in the nucleus accumbens (Rada, Mark, & Hoebel, 1998). Galanin also inhibits dopamine secretion from pheochromocytoma cells (de Weille, Fosset, Schmid-Antomarchi, & Lazdunski, 1989) and galnon administration attenuates cocaine-induced dopamine release in the frontal cortex (Ogbonmwan et al., 2015). Similarly, chronic intracerebroventricular (ICV) galanin administration reduces footshock-induced release of dopamine in the frontal cortex (Sciolino et al., 2015). Furthermore, galanin has also been shown to inhibit mesolimbic dopamine synthesis and neurotransmission (Counts et al., 2002; Ericson & Ahlenius, 1999) and it may mediate the effects of LC hyperactivity on inhibition of VTA dopamine neurons (Grenhoff, Nisell, Ferre, Aston-Jones, & Svensson, 1993; Weiss, Bonsall, Demetrikopoulos, Emery, & West, 1998). Modulation of mesolimbic dopamine by galanin has important implications for stress-related disorders, particularly those which disrupt motivated and addiction-related behaviors (Picciotto et al., 2010).

**Galanin and depression**

It has long been accepted that disrupted norepinephrine signaling is involved in depression. Early hypotheses posited that depression-like behavioral changes were a result of "functional blockade" of auto-inhibitory α2 receptors on LC neurons that resulted in hyperactive

LC neurons (Weiss, Bailey, Pohorecky, Korzeniowski, & Grillione, 1980). However, it remained unclear how increased LC neuron activity, and therefore increased norepinephrine release, caused depression-like behaviors that are not strongly regulated by norepinephrine directly (Weiss et al., 1998). Dopamine, on the other hand, is strongly implicated in behaviors that are disrupted in depression. Therefore, it was posited that noradrenergic modulation of dopamine signaling could explain the link between hyperactive LC function and depression-like phenotype (Weiss et al., 1998). Indeed, the LC sends noradrenergic projections to the VTA, the VTA expresses noradrenergic receptors, and norepinephrine released from LC projections modulates VTA mesolimbic dopamine neuron activity (Grenhoff et al., 1993; Mejias-Aponte, Drouin, & Aston-Jones, 2009; Park, Bhimani, & Park, 2017). Low frequency stimulation of the LC excites VTA dopamine neurons through stimulation of α1 receptors, while high frequency stimulation (akin to burst firing) suppresses VTA dopamine neuron activity (Grenhoff et al., 1993). Thus, it was proposed that depression-related behavioral changes were a consequence of suppression of VTA dopamine neuron activity, and subsequent decreased dopamine release in the forebrain, due to increased burst firing of LC neurons (Weiss et al., 1998). Importantly though, Grenhoff and colleagues (1993) showed that the effect of LC burst firing on VTA dopamine neuron activity was not mediated by noradrenergic receptors. In the same paper it was proposed that the neuropeptide galanin, a known cotransmitter of norepinephrine in the LC, might mediate the effects of LC burst firing on suppression of mesolimbic dopamine neurons. A role for galanin in depression was subsequently proposed (Weiss et al., 1998). In essence, Weiss and colleagues proposed that galanin release in the VTA is increased during severe stress to compensate for functional blockade of α2 receptor-mediated autoinhibition of LC neurons, resulting in hyperpolarization of mesolimbic dopamine neurons and subsequent depression-like behaviors.

They then showed that galanin administration into the VTA induces, while blocking galanin receptors in the VTA suppresses, depression-like behavior in the forced swim test, lending support to their hypothesis (Weiss et al., 1998; Weiss et al., 2005).

Since the discoveries of Grenhoff and Weiss, as well as those of Fuxe and colleagues (Fuxe, Ogren, et al., 1988; Fuxe, von Euler, Agnati, & Ogren, 1988), substantial evidence has accumulated to support a role for galanin modulation of catecholamine signaling in depression (see Millon et al., 2017 for review). Much of the research on galanin's role in depression has consisted of measuring effects of pharmacological and genetic manipulations on behavior in the forced swim (Porsolt, Le Pichon, & Jalfre, 1977) and tail suspension (Steru, Chermat, Thierry, & Simon, 1985) tests, two well-accepted models for screening putative antidepressants. Both antidepressant and pro-depressant roles for galanin have been demonstrated with these assays (Millon et al., 2017). Other evidence for galanin's role in depression comes from measuring galanin and galanin receptor mRNA or protein expression after various manipulations. For example, galanin is upregulated in the LC after olfactory bulbectomy, a well-established rodent model of depression (P. V. Holmes & Crawley, 1996), and in the VTA of susceptible mice that show a depressive-like phenotype after social defeat stress (Krishnan et al., 2007). Similarly, Flinders Sensitive Line rats, an animal model of depression, have lower galanin receptor expression in the dorsal raphe than Flinders Resistant Line rats (Bellido et al., 2002). In contrast, galanin is also upregulated in the LC after chronic treatment with the antidepressants, including tricyclic anti-depressants, selective serotonin reuptake inhibitors, or monoamine oxidase inhibitors (P. V. Holmes, Yoo, & Dishman, 2006; Lu et al., 2005). Chronic treatment with antidepressants also reduces GalR2 mRNA in the VTA and increases GalR2 in the dorsal raphe nucleus (Lu et al., 2005; Rovin et al., 2012). Furthermore, galanin activity is necessary for the

antidepressant effects of chronic fluoxetine treatment during forced swim stress (Lu et al., 2005). Differential expression and signaling cascades of GalR1 and GalR2 receptors may explain the dual role of galanin in modulation of depression. In general, stimulation of the GalR1 receptor tends to have pro-depressant effects, while stimulation of the GalR2 receptor mediates antidepressant effects of galanin (Bartfai et al., 2004; de Souza et al., 2018; Kuteeva, Hokfelt, Wardi, & Ogren, 2008; Kuteeva, Wardi, Hokfelt, & Ogren, 2007; Le Maitre et al., 2011; Millon et al., 2014; Saar, Lahe, et al., 2013; Saar, Runesson, Jarv, Kurrikoff, & Langel, 2013; P. Wang et al., 2016). Evidence suggests that the GalR3 receptor may also promote depression-related behavior (Barr et al., 2006; Swanson et al., 2005). In addition to differential roles for galanin receptor subtypes, various homomer and heteromer galanin receptor complexes may also account for discrepant findings in attempts to elucidate galanin's role in depression (Fuxe et al., 2012; Millon et al., 2017).

It is noteworthy that several recent studies have also shown a role for galanin in depression in humans. Postmortem analysis of brain samples collected from depressed humans who committed suicide showed increased galanin and GalR3 transcription and decreased methylation in the LC and dorsal raphe (Barde et al., 2016). Another study showed that intravenous administration of galanin improved HAMD depression scores in depressed patients, compared to placebo (Murck et al., 2004). Several studies have shown that multiple single nucleotide polymorphisms in the galanin gene, its receptor genes, and a remote enhancer region are associated with risk for depression, symptom severity, and anti-depressant treatment response (da Conceicao Machado, de Souza, Rangel, Jara, & do Carmo Franco, 2018; Davidson et al., 2011; Juhasz et al., 2014; Unschuld et al., 2010; Wray et al., 2012). Furthermore, recent studies showed that several depression-risk polymorphisms interact with stress exposure, such as

childhood adversity and negative life events (Gonda et al., 2018; Juhasz et al., 2014). Strikingly, the interaction between galanin and galanin receptor gene variants and stress has a stronger effect on risk for depression than interactions of stress with variants of the serotonin transporter gene and BDNF (Gonda et al., 2018; Juhasz et al., 2014).

**Galanin and anxiety disorders**

Galanin also has a well-established role in the modulation of stress and anxiety-related behavior. Galanin and galanin receptors are expressed in brain regions involved in stress and anxiety, such as the LC, VTA, and amygdala (Melander, Hokfelt, Nilsson, & Brodin, 1986; Melander, Hokfelt, & Rokaeus, 1986; Melander et al., 1988). Several studies have shown that multiple single nucleotide polymorphisms in the galanin gene, its receptor genes, and a remote enhancer region are associated with risk for anxiety and stress-related disorders, such as panic disorder, post traumatic-stress disorder, and alcoholism, and interact with stress exposure (Belfer et al., 2006; Juhasz et al., 2014; Unschuld et al., 2008; Unschuld et al., 2010). Preprogalanin mRNA expression is upregulated in the LC after chronic exposure to a variety of stressors including social stress (P. V. Holmes, Blanchard, Blanchard, Brady, & Crawley, 1995), treadmill exercise (O'Neal, Van Hoomissen, Holmes, & Dishman, 2001), and voluntary wheel running (P. V. Holmes et al., 2006; Sciolino, Dishman, & Holmes, 2012). Galanin is also increased in the dorsomedial hypothalamus and amygdala after repeated immobilization stress (Makino, Asaba, Nishiyama, & Hashimoto, 1999; Sweerts, Jarrott, & Lawrence, 1999). Chronic voluntary exercise also increases galanin protein in the LC (Sciolino et al., 2015).

Studies investigating the role of galanin in anxiety-related behavior have yielded mixed results. Galanin has been shown to be anxiolytic in anxiety-related behavioral tests including Vogel punished drinking (Bing, Moller, Engel, Soderpalm, & Heilig, 1993), contextual fear-

conditioning (Karlsson, Holmes, Heilig, & Crawley, 2005), trace cued fear-conditioning (Bailey, Pavlova, Rohde, Hohmann, & Crawley, 2007; Kinney et al., 2002; Wrenn et al., 2004), shock-probe defensive burying (Echevarria, Hernandez, Diogenes, & Morilak, 2005) elevated zero maze, and the four-plate test (Rajarao et al., 2007). In addition, the anxiolytic effects of voluntary wheel running are mediated by galanin (Sciolino et al., 2015) and chronic ICV administration of galanin protects against stress-induced dendritic spine loss in the medial prefrontal cortex (Sciolino et al., 2015). Furthermore, genetic GalR1 knockout mice show an anxiogenic phenotype on the elevated plus maze, but not in the light/dark exploration test, the open field, or the emergence test (A. Holmes et al., 2003). Other studies have shown galanin to have no effect on anxiety-related behavior in the open field, elevated plus maze, light-dark exploration, and standard cued fear conditioning tasks (A. Holmes, Yang, & Crawley, 2002; Karlsson et al., 2005; Kinney et al., 2002; Wrenn et al., 2002). However, it is possible that these discrepant effects are due to the well-supported hypothesis that the anxiolytic effects of galanin preferentially manifest after stress. For example, galanin release in the central nucleus of the amygdala was necessary for the anxiolytic effect of noradrenergic activation by yohimbine when administered before mild acute immobilization stress; however, galanin blockade had no effect on anxiety-related behavior after mild acute immobilization stress (Khoshbouei, Cecchi, Dove, Javors, & Morilak, 2002). The same experiment showed that galanin administration in the central nucleus of the amygdala was sufficient to reduce anxiety related behavior after mild acute immobilization stress (Khoshbouei, Cecchi, Dove, et al., 2002). In contrast, galanin has also been shown to have an anxiogenic effect. Galanin activity in the bed nucleus of the stria terminalis facilitates acute stress-induced behavioral and neuroendocrine responses (Khoshbouei, Cecchi, & Morilak, 2002) and galanin activity in the central amygdala decreased Vogel punished drinking (Moller,

Sommer, Thorsell, & Heilig, 1999). Although galanin clearly modulates anxiety-related behavior, the precise role of the endogenous peptide remains unclear and seems to differ depending on the neural circuit, stress-level, and sensitivity of the paradigm to inherent stress-resilience or susceptibility of the animals (Krishnan et al., 2007).

**Galanin and modulation of pain and inflammation**

Galanin modulates acute and chronic pain at the peripheral, spinal, and supraspinal levels. Galanin and its receptors are expressed on sensory nerve afferents, in the dorsal root ganglia, in the dorsal horn of the spinal cord, and in brain regions associated with pain modulation, such as the LC, hypothalamus, amygdala, and PAG (Brumovsky, Mennicken, O'Donnell, & Hokfelt, 2006; Ch'ng et al., 1985; Gustafson et al., 1996; Holets et al., 1988; Melander, Hokfelt, & Rokaeus, 1986; O'Donnell et al., 1999; Rokaeus et al., 1984; Skofitsch & Jacobowitz, 1985a, 1985b; Skofitsch et al., 1986; Waters & Krause, 2000). Galanin generally exerts an inhibitory effect on spinal nociception; however, low doses of exogenous galanin have been shown to facilitate nociceptive responses (Lang et al., 2015; X. J. Xu, Hokfelt, & Wiesenfeld-Hallin, 2008). A discussion of evidence supporting galanin's role in pain modulation in the peripheral nervous system and spinal cord are beyond the scope of this review and have been addressed elsewhere (F. E. Holmes, Mahoney, & Wynick, 2005; Lang et al., 2015; H. X. Liu & Hokfelt, 2002; X. J. Xu et al., 2008). A review of galanin's role in supraspinal pain modulation follows.

ICV administration of galanin or a GalR1 agonist reduces the nociceptive response to mechanical and thermal stimulation in a PKC-dependent manner (Fu, Wang, Jiao, Wu, & Yu, 2011; Shi, Fu, & Yu, 2011). Intra-PAG administration of galanin increased latency to paw withdrawal for thermal and mechanical stimulation in healthy animals and in a model of

mononeuropathy. Galanin effects in the PAG are mediated by GalR1, but not GalR2 (Kong & Yu, 2013). Evidence suggests that there is a synergistic interaction between galanin and endogenous opioids in the PAG on pain modulation; the interaction is likely mediated by the μ-receptor subtype (Kong & Yu, 2013; Sun & Yu, 2005; D. Wang, Lundeberg, & Yu, 2000; D. Wang, Ye, Yu, & Lundeberg, 1999). Administration of galanin into the arcuate nucleus, tuberomammillary nucleus, nucleus accumbens, lateral habenula, anterior cingulate cortex, and central nucleus of the amygdala has similar effects in healthy rats and rats with inflammation (Fu et al., 2016; Jin, Liu, Liu, & Yu, 2010; Sun, Gu, Lundeberg, & Yu, 2003; Sun, Li, Yang, & Yu, 2004; S. L. Xu, Li, Zhang, & Yu, 2012; Yang et al., 2015; Zhang, Fu, & Yu, 2017). Anti-nociceptive effects are also seen after administration of galanin into the anterior cingulate cortex in animals with neuropathic pain (Zhang, Wang, Fu, & Yu, 2017). Furthermore, iontophoretically applied galanin suppressed neuronal responses in the gracile nucleus during mechanical stimulation after sciatic nerve ligation (Jung et al., 2009). Like in the PAG, effects of galanin in the central nucleus of the amygdala appear to also be mediated in-part by the μ-receptor subtype, as well as, the GalR1 receptor subtype (Jin et al., 2010; Li, Zhang, Xu, & Yu, 2012) and the effects in the nucleus accumbens and lateral habenula are in-part mediated by the GalR1 receptor subtype (Duan et al., 2015). Effects of galanin in the anterior cingulate cortex are mediated by GalR2 (M. L. Zhang, F. H. Fu, et al., 2017). One possible mechanism of supraspinal pain modulation by galanin is through stimulation of beta-endorphin expressing neurons in the arcuate nucleus and subsequent release of β-endorphin onto μ-receptors in the PAG (Sun, Gu, & Yu, 2007).

Although the majority of studies support an anti-nociceptive effect of supraspinal galanin signaling, a pronociceptive role has also been reported. Administration of galanin or a GalR1

agonist in the dorsomedial hypothalamus has a pronociceptive effect in healthy and in arthritic animals (Amorim et al., 2014). Evidence suggests that the pronociceptive effects of galanin in the dorsomedial hypothalamus might be mediated by descending pain modulatory pathways relayed through the dorsal reticular nucleus and serotonergic neurons in the rostral ventromedial medulla cells (Amorim et al., 2014; Amorim et al., 2015).

Other evidence in support of a role for galanin in supraspinal pain modulation comes from studies investigating changes in galanin and galanin receptor expression in the brain after induction of pain. Spared nerve injury, a rodent model of neuropathic pain, increases galanin-immunoreactivity, but has no effect on galanin mRNA, in the arcuate nucleus one month later (Gu, Sun, & Yu, 2007; Imbe et al., 2004). Cyclophosphamide-induced cystitis, a model of visceral pain, induces upregulation of galanin mRNA in the PVN and arcuate nucleus of the hypothalamus (Nishii, Nomura, Aono, Fujimoto, & Matsumoto, 2007). Sciatic nerve injury, but not carrageenan-induced inflammation, increases GalR1-immunoreactivity in the tuberomammillary nucleus (Sun et al., 2004). Sciatic nerve ligation and carrageenan-induced paw inflammation also increase GalR1 expression in the nucleus accumbens (Duan et al., 2015; Yang et al., 2015), as well as, galanin and GalR2 mRNA in the anterior cingulate cortex (M. L. Zhang, F. H. Fu, et al., 2017; M. L. Zhang, H. B. Wang, et al., 2017). GalR2 expression is also increased in the nucleus accumbens after carrageenan (Yang et al., 2015).

The modulatory effects of galanin in the central nervous system are complex and tend to depend on the receptor subtype that is stimulated, the neural circuit activated by the stimulus, and the environmental context. However, galanin is clearly involved in modulation of pain and stress-related behaviors. Although many researchers have investigated galanin's role in each process individually, whether galanin mediates interactions between various systems is not well

explored. The current research investigated whether galanin signaling mediates the interaction between neural systems that process inflammatory pain and control depression-related behavior.

## Proposed role for galanin in modulating effects of inflammatory pain on mesolimbic dopamine

It is well-accepted that disrupted mesolimbic dopamine signaling contributes to depression-like behavior induced by chronic inflammatory pain. However, precisely how chronic inflammatory pain leads to mesolimbic dysregulation is unclear. We propose that transmission of the neuropeptide galanin in the LC-VTA circuit mediates the behavioral effects of inflammatory pain based on the following working model.

Acute pain increases noradrenergic LC activation and the activity of a subset of mesolimbic dopamine neurons that encode the salience of the noxious stimulus (Brischoux, Chakraborty, Brierley, & Ungless, 2009; Navratilova & Porreca, 2014; Pertovaara, 2006). This triggers dopamine release into the nucleus accumbens core (Budygin et al., 2012). Dopamine activity in the striatum likely encodes salience of the aversive stimulus and mediates the motivation required to respond appropriately (Taylor, Becker, Schweinhardt, & Cahill, 2016). However, some evidence suggests that mesolimbic dopamine activity may also have antinociceptive effects (Mitsi & Zachariou, 2016; Taylor et al., 2016). Nevertheless, over time chronic pain changes mesolimbic dopamine and noradrenergic circuits. An important change observed in the LC in a model of chronic neuropathic pain is heightened excitation of the LC in response to noxious stimulation followed by an increased inhibitory period between the early and late phase of the biphasic LC neuron response to noxious stimuli, without concomitant changes to α2 receptors (Alba-Delgado, Borges, et al., 2012; Alba-Delgado, Mico, Sanchez-Blazquez, & Berrocoso, 2012). We propose that changes to galanin expression or signaling in noradrenergic

neurons that occur during chronic pain mediate changes observed in the VTA and LC. Following these changes, burst firing of the LC after noxious stimulation might induce galanin release onto VTA dopamine neurons and/or LC norepinephrine neurons. Increased galanin activity could in turn contribute to chronic downregulation of mesolimbic dopamine activity and induce a depression-like phenotype in several non-mutually exclusive ways. (i) Increased galanin release in the LC or VTA could facilitate auto-inhibitory feedback onto noradrenergic LC neurons, resulting in decreased stimulatory norepinephrine release in the VTA. (ii) Increased galanin release in the VTA or upregulation of galanin receptors in the VTA could directly hyperpolarize mesolimbic dopamine neurons. (iii) Repeated, increased stimulation of GalR2 receptors in the VTA could induce changes in transcription and translation of proteins that alter VTA dopamine neuron activity. In support of our proposal, previous work implicates galanin signaling in the nucleus accumbens in the comorbidity of pain and depression, but its effects in the VTA and upstream brain regions during inflammatory pain have not been investigated (Mitsi et al., 2015; Schwartz et al., 2014)

The current research was an investigation of whether galanin activity in the LC-VTA pathway could mediate mesolimbic dopamine dysregulation in comorbid pain and depression. We first characterized the long-term effects of intraplantar CFA on depression-like behavior in our hands. We also evaluated whether CFA would interact with stress and whether galnon, a galanin agonist, would be sufficient to mimic or exacerbate the effects of CFA. However, galnon and GalR2 agonists have been shown to have anti-depressant effects, therefore, we also asked whether galnon could have a therapeutic effect on treating comorbid pain and depression. Next, we determined whether CFA administration would decrease dopamine release in the nucleus accumbens, as this has never been directly investigated before. We also measured the effects of

CFA on monoamine levels in a variety of brain regions associated with the mesolimbic dopamine system to better characterize the effects of CFA on neurotransmitter synthesis and turnover. Finally, we evaluated the necessity of galanin signaling for the CFA-induced changes we found. In addition to investigating galanin regulation of mesolimbic dopamine in a model of chronic inflammatory pain, we also investigated the effects of galanin in healthy rats in a model of post-traumatic stress disorder to better understand the diverse stress-sensitive functions of this peptide neurotransmitter.

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# CHAPTER 2

# GALANIN ADMINISTRATION INTO THE PRELIMBIC CORTEX IMPAIRS CONSOLIDATION AND EXPRESSION OF CONTEXTUAL FEAR CONDITIONING[1]

[1] Hooversmith, J. M., Bhatti, D., & Holmes, P. V. Submitted to Neurobiology of Learning and Memory, 11/28/18

## Abstract

The neuropeptide galanin is a potential therapeutic target for treating stress-related disorders, such as post-traumatic stress disorder (PTSD); however, it's effects on contextual fear conditioning (CFC), an accepted animal model of PTSD, are not well understood. Dysregulation of the medial prefrontal cortex (mPFC) is implicated in PTSD. We investigated the effects of galanin (1 ug) administrated bilaterally into the prelimbic cortex, a division of the mPFC, on the consolidation, expression, and extinction of CFC of male Sprague-Dawley rats. We also measured the effects of CFC and galanin on PSD95 expression in the mPFC. Galanin administration significantly reduced consolidation and expression of CFC, but had no effect on retention or retrieval of extinction learning. Furthermore, both galanin administration and CFC alone reduced PSD95 expression in the prelimbic cortex. Galanin somewhat attenuated the effects of CFC on PSD95, although the effect was not significant. These data further implicate galanin as a potential therapeutic target for treating stress-related disorders, particularly those characterized by aberrant emotional memory.

## Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder characterized by persistent intrusive and recurrent memories of the trauma, negative changes in mood, altered reactivity and arousal, and avoidance behaviors following traumatic life events. The lifetime prevalence of PTSD, as defined by DSM-V, is approximately 6.1% (Goldstein et al., 2016). A variety of drugs, including anti-depressants, anxiolytics, and antipsychotics, are commonly prescribed to treat symptoms of PTSD; however, recent metanalyses show that the effect sizes of such treatments compared to placebo are low (Cipriani et al., 2017; Hoskins et al., 2015). Therefore, novel pharmacological strategies for treating or preventing PTSD are needed.

The contextual fear conditioning (CFC) paradigm is commonly employed to investigate potential therapeutic strategies for targeting the traumatic memory features of PTSD in a rodent model (Bertaina-Anglade, O'Connor, & Andriambeloson, 2017). CFC is a form of aversively-motivated Pavlovian conditioning. During CFC training, an unconditioned aversive stimulus, typically foot-shock, is paired with a neutral context. If associative learning is intact, the organism displays a fear-related behavior, typically freezing for rodents, in response to the conditioned context upon re-exposure. In healthy organisms, repeated exposure to the conditioned context in the absence of the unconditioned stimulus results in extinction of the conditioned fear-response.

Acquisition, expression, and extinction of fear conditioning is largely controlled by bidirectional connectivity between the basolateral amygdala and the medial prefrontal cortex (mPFC; Likhtik & Paz, 2015). The mPFC can be further anatomically and functionally divided into the dorsal prelimbic cortex and the ventral infralimbic cortex. The prelimbic and infralimbic cortices have distinct roles in modulation of CFC; the prelimbic cortex is necessary for fear

expression, but not extinction memory, while the infralimbic cortex is necessary for extinction memory, but not fear expression (Milad, Vidal-Gonzalez, & Quirk, 2004; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). Moreover, activity in the prelimbic cortex is higher in rats that fail to recall extinction learning (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009). The prelimbic and infralimbic cortices in rodents are analogous to the ventromedial prefrontal cortex in primates, which is hypoactive in people with PTSD (Etkin & Wager, 2007). Therefore, the mPFC is a promising therapeutic target for PTSD because it regulates emotional behavior in rodents and primates, is implicated in mediating extinction of fear conditioning, and shows aberrant function in people with PTSD.

It is well established that noradrenergic neurons in the locus coeruleus are responsive to stress and modulate behavioral and physiological responses to threatening stimuli. Norepinephrine also plays a major role in memory formation and fear conditioning (T. O'Donnell, Hegadoren, & Coupland, 2004). Therefore, it is unsurprising that evidence supports a role for a hyperactive noradrenergic system in the neuropathology of PTSD (T. O'Donnell et al., 2004; Southwick et al., 1997). Pharmacological reduction of noradrenergic activity in the central nervous system, with noradrenergic antagonists such as prazosin and clonidine, is a current strategy for alleviating symptoms of PTSD. However, sustained improvement of symptoms requires chronic prescription of these drugs (Boehnlein & Kinzie, 2007; Hudson, Whiteside, Lorenz, & Wargo, 2012). As a result, a novel strategy to modulate noradrenergic activity to treat PTSD is needed.

Galanin, a 29-amino acid neuropeptide, is co-localized with norepinephrine in at least 80% of noradrenergic cells in the locus coeruleus of rats (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988; Melander et al., 1986), as well as, in mice (Perez, Wynick, Steiner, & Mufson,

2001) and humans (Le Maitre, Barde, Palkovits, Diaz-Heijtz, & Hokfelt, 2013). Galanin inhibits noradrenergic neurons in the locus coeruleus (Xu, Zheng, & Hokfelt, 2005), presumably through autocrine and paracrine signaling (Pieribone et al., 1995; Vila-Porcile et al., 2009). In addition to its neuromodulatory functions, galanin has also been shown to have neurotrophic effects (Elliott-Hunt et al., 2011; Hobson, Vanderplank, Pope, Kerr, & Wynick, 2013; Sciolino et al., 2015). Both neuromodulation of norepinephrine and neurotrophic effects of galanin are implicated in galanin's well-established role in reducing anxiety-like behavior and promoting stress-resilience (Weinshenker & Holmes, 2016). Notably, galanin administration was sufficient to prevent a stress-induced reduction in dendritic spine density in the medial prefrontal cortex, an effect which may underlie its role in stress-resilience (Sciolino et al., 2015). Furthermore, several studies have shown that several single nucleotide polymorphisms in the galanin gene and a remote enhancer region are associated with risk for depression and anxiety disorders (Belfer et al., 2006; Davidson et al., 2011; Juhasz et al., 2014; Unschuld et al., 2010; Wray et al., 2012). Thus, the galanin system is a potential therapeutic target for treating PTSD through modulation of noradrenergic hyperactivity, promotion of stress-resilient neuroplasticity in the mPFC, or both mechanisms. However, few studies have investigated the effects of the galanin system on CFC.

Previous studies demonstrate that galanin administration into the lateral ventricle of rats reduced expression of cued fear conditioning in a trace cued paradigm, but not cued or contextual fear expression in a standard paradigm (Kinney et al., 2002). A similar effect was shown in galanin-overexpressing mice (Kinney et al., 2002; Wrenn et al., 2002). Galanin administration into the lateral ventricle of mice reduced expression of contextual fear (Karlsson, Holmes, Heilig, & Crawley, 2005). The receptor subtype that mediates the known effects of galanin remain unclear; however, preliminary findings suggest the GalR1 receptor, but not the

GalR2 receptor may be important for trace cued fear conditioning (Bailey, Pavlova, Rohde, Hohmann, & Crawley, 2007; Wrenn et al., 2004). Although previous work implicates the galanin system in fear conditioning, the role of endogenous galanin in the consolidation, expression, and extinction of CFC remains unclear. Furthermore, the effects of exogenous galanin on CFC, and thereby the therapeutic potential of galanin for PTSD, also remain unclear.

We investigated the effects of galanin in the prelimbic cortex on CFC consolidation, expression, and extinction. We also measured the post-synaptic protein PSD95 as a marker for synaptic density to determine if the effects of galanin were mediated by a mechanism related to its effects on neuroplasticity. We hypothesized that galanin would serve a stress-protective role and decrease CFC consolidation and expression. Furthermore, we hypothesized that galanin would prevent CFC-induced changes in PSD95 expression.

## Experimental Methods

### Subjects

Male Sprague-Dawley rats (N = 43; Harlan, Prattville, AL) were obtained at 175 – 200 g and given ad libitum food and water. Rats were housed individually in clear polycarbonate cages (50 x 30 x 30 cm) at 23 ± 3 °C on a 12:12 reverse light:dark cycle. All behavioral testing occurred during the dark cycle. Procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 – 23, revised 2013). Experiments were approved by University of Georgia IACUC.

### General Experimental Methods

Rats were undisturbed for 1 week upon arrival to the facility. Following this habituation period, rats underwent stereotaxic surgeries for implantation of guide cannula into the prelimbic cortex for acute drug delivery. Following a 1-week recovery period rats underwent testing in a

CFC paradigm. Galanin or artificial cerebrospinal fluid (aCSF) was administered through the guide cannula immediately after the CFC conditioning trial (consolidation experiment) or 10 minutes before the CFC retrieval trial (expression experiment), which occurred 24-hour after conditioning (Figure 2.1), to assess the effects of galanin on the consolidation and expression of CFC, respectively. Final group sample sizes for the consolidation experiment were: No CFC/aCSF n = 6, No CFC/galanin n = 6, CFC/aCSF n = 6, and CFC/galanin n = 7. Final group sample sizes for the expression experiment were: aCSF n = 9 and galanin n = 9. After behavioral testing, rats were euthanized via rapid decapitation and brains were extracted for further processing.

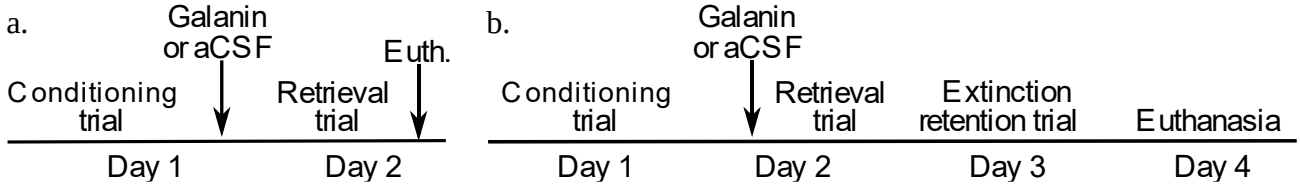


**Figure 2.1** Experimental timelines for the consolidation and expression experiments. (a) For the consolidation experiment, rats underwent a 7-min conditioning trial on Day 1 where they were either exposed to three foot-shocks (n = 13) or remained shock-naïve (n = 12). All rats received an intra-prelimbic injection of aCSF (n = 12) or galanin (1 ug; n = 13) immediately after the conditioning trial. All rats were placed back in the conditioning chamber 24-hr later for the retrieval trial and were euthanized afterwards. (b) For the expression experiment, rats underwent a 7-min conditioning trial on Day 1 where they were exposed to three foot-shocks. 24-hr later, all rats received an intra-prelimbic injection of aCSF (n = 9) or galanin (1 ug; n = 9) 10 min before the retrieval trial. Rats were placed in the conditioning chamber again 24-hr later for the extinction retention trial and were euthanized the following day.

**Stereotaxic Surgery and Drug Administration**

Rats were anesthetized with isoflurane (1-5%) and bilateral guide cannula (22G C232G-1.0; Plastics1) were stereotaxically implanted into the prelimbic cortex (3 mm AP, +/- 0.5 mm ML, and 4 mm DV) relative to Bregma using Paxinos and Watson (1998). Cannula were secured

using surgical screws and epoxy cement. Rats were administered Meloxicam (1.0 mg/kg) or Banamine (2.0 mg/kg) immediately and 24 hr after surgery.

Galanin (Tocris; 1 ug/side) or aCSF (Harvard Apparatus) was administered through the guide cannula using 30G needles attached to a Hamilton syringe extending 1 mm beyond the guide cannulae at a rate of 1 ul/min. Syringe needles were left in place for an additional 30 s after the infusion to allow for drug diffusion away from the needle tip. Injection volumes were 1 ul/side or 0.5 ul/side for the consolidation and expression experiments, respectively.

**Contextual Fear Conditioning**

On Day 1 of behavioral testing (fear conditioning trial), rats were placed into the center of a transparent plexiglass open field chamber (43.3 cm long X 43.3 cm wide X 30.5 cm high) and allowed to habituate to the environment for 4 min. Foot shock was delivered to a subset of rats through a grid floor on minutes 4, 5, and 6 (0.8 mA, 0.5 s, 60 s ITI; ENV-4145, Med Associates, St. Albans, VT). Rats were returned to their home cages 1 min after the final shock. Rats were placed back in the open field without foot shock for 10 min 24-hr after the conditioning trial for the retrieval trial. Freezing within the context in the absence of foot-shock was measured to determine the strength of the context-shock association formed during the conditioning trial. To determine if galanin administered before the retrieval trial affected extinction learning, rats were returned to the open field without foot shock for an additional 10 min trial (extinction retention trial) 48 hr after conditioning; these rats were euthanized 24 hr later. Freezing behavior, defined as behavioral immobility except for the movement needed for respiration (Blanchard & Blanchard, 1969), was recorded from video by an experimenter using JWatcher (http://www.jwatcher.ucla.edu). Other behaviors were recorded by infrared beam

breaks that track the coordinate position and movement of the rat (ENV-520, SOF-810; Med Associates) for CFC rats, as well as, a subset of rats that did not receive foot shocks.

**Tissue processing and Enzyme-linked immunosorbent assay (ELISA)**

After rapid decapitation, brains were extracted and the mPFC was dissected for ELISA. The mPFC was divided into infralimbic and prelimbic regions for rats that received galanin immediately after the consolidation trial. All tissue was frozen on dry ice and stored at -80°C until use. Cannula placement was visually verified during the dissection or brains were sectioned coronally (12 μm) using a cryostat microtome and nissl stained using thionin for verification.

For protein extraction, the tissue was weighed and put in test tubes containing PBS (500 μL of 0.01 mol/L, pH=7.2), homogenized (15s; PowerGen 125, Fisher), put through two freeze-thaw cycles, and centrifuged (5 min at 4°C, 3000 rpm at 5000× g; Beckman Model TJ-6). Supernatant was removed, placed into separate tubes, and diluted (1:1000) with PBS. The dilute samples were then processed for the post-synaptic protein PSD95 according to the manufacturer's instructions for the 96-well plate PSD95 (DLG4) Rat ELISA kits **(**MBS2019180, MyBioSource, San Diego, CA**)**. Wells were read at 450nm. A curve of best fit was used to calibrate readings to the standards, and data for each subject were averaged across duplicates.

**Statistical Analysis**

Statistics were performed using GraphPad Prism 7.03 software. Outliers were removed using the ROUT method. Independent t-tests were performed on freezing behavior for all rats and open field behavior and PSD95 protein expression for the rats that received galanin or aCSF immediately before the retrieval trial. Two-way ANOVAs (Drug x CFC) were performed on open field behavior and PSD95 protein expression for rats that received galanin or aCSF

immediately after the fear conditioning trial. Fisher's LSD post hoc tests were performed following the ANOVAs.

## Results

### Effects of Galanin on Consolidation of CFC

To verify that the two groups of rats were no different prior to drug administration, an independent t-test was performed on freezing behavior during the fear conditioning trial (data not shown). There were no significant differences in freezing behavior between the two groups during the fear conditioning trial, $t(11) = 0.49$, $p = 0.63$. An independent t-test was performed to determine the effects of galanin administration immediately after the conditioning trial on freezing behavior during the retrieval trial (Figure 2.2). Galanin administration immediately after the conditioning trial significantly reduced freezing 24 hr later during the retrieval trial, compared to aCSF administration, $t(10) = 2.25$, $p = 0.05$.

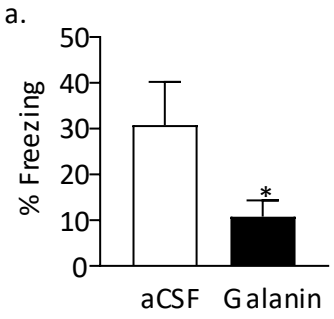


**Figure 2.2.** Effect of galanin on consolidation of contextual fear conditioning. Administration of galanin into the prelimbic cortex immediately after the fear conditioning trial reduced time spent freezing during the retrieval trial, compared to aCSF administration. *$p < 0.05$. Error bars represent SEM.

To determine the effects of foot-shock on behavior in the open field during the conditioning trial and to verify that the groups of rats were no different prior to drug administration, 2X2 ANOVAs were performed on ambulatory time, rearing time, and entries into the center zone (data not shown). As expected, prior to drug treatment, the two groups of rats were no different on ambulatory time, $F(1, 21) = 1.1$, $p = 0.31$, rearing time, $F(1, 21) = 2.4$, $p =$

0.14, or entries into the center zone, F(1, 21) = 0.19, $p = 0.54$, in the open field during the conditioning trial. Rats that underwent foot-shock in the open field during the conditioning trial spent significantly less time in ambulation, F(1, 21) = 5.6, $p = 0.03$, and less time rearing, F(1, 21) = 13, $p = 0.002$, compared to rats that did not receive foot-shock. Foot-shock exposure did not affect entries into the center zone, F(1, 21) = 2.9, $p = 0.10$, during the conditioning trial.

To determine the effects of foot-shock and drug treatment on behavior in the open field during the retrieval trial, 2X2 ANOVAs were performed on ambulatory time, rearing time, and entries into the center zone (Figure 2.3). Galanin-treated rats were no different from aCSF-treated rats on ambulatory time, F(1, 21) = 0.26, $p = 0.62$, rearing time, F(1, 21) = 0.003, $p = 0.96$, or entries into the center zone, F(1, 20) = 0.48, $p = 0.50$, in the open field during the retrieval trial. Rats that underwent foot-shock in the open field during the conditioning trial spent significantly less time in ambulation, F(1, 21) = 33, $p < .0001$, less time rearing, F(1, 21) = 39, $p < 0.001$, and entered the center zone significantly less, F(1, 20) = 29, $p < 0.001$, in the open field during the retrieval trial than rats that did not receive foot-shock.

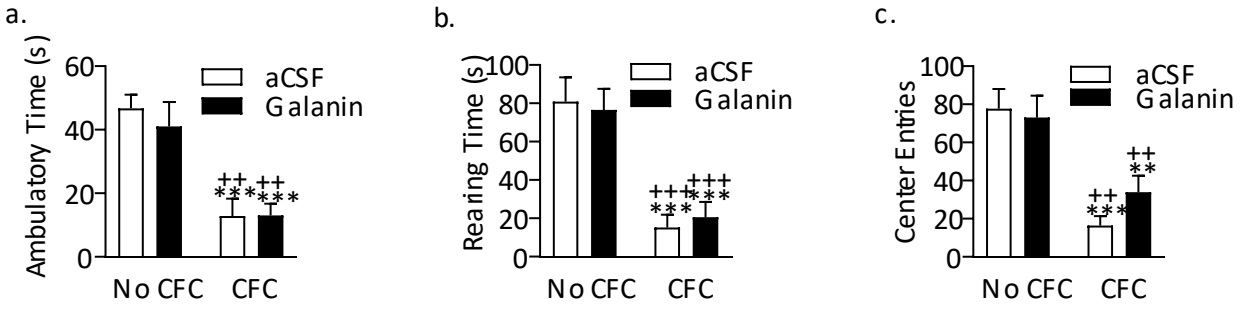


**Figure 2.3.** Effects of contextual fear conditioning (CFC) and galanin administration into the prelimbic cortex immediately after the conditioning trial on ambulatory time (a), rearing time (b), and center entries (c) in the open field during the retrieval trial for rats that received foot-shock (CFC) compared to rats that did not receive foot-shock (No CFC) during the conditioning trial. *p < 0.05, **p < 0.01, ***p < 0.0001 compared to No CFC-aCSF; +p < 0.05, ++p < 0.01, +++p < 0.0001 compared to No CFC-Galanin. Error bars represent SEM.

**Effect of Galanin on Expression of CFC**

To verify that the two groups of rats were no different prior to drug administration, an independent t-test was performed on freezing behavior during the fear conditioning trial (data not shown). Prior to drug treatment, the two groups of rats were no different on freezing behavior during the fear conditioning trial, t(16) = 0.12, $p$ = 0.90. Independent t-tests were also performed to determine the effects of galanin on freezing behavior during the retrieval and extinction retention trials (Figure 2.4). Galanin administration 10 minutes before the retrieval trial reduced freezing during the retrieval trial, t(15) = 2.32, $p$ = 0.04, but not the extinction retention trial, t(16) = 0.61, $p$ = 0.55, compared to aCSF administration.

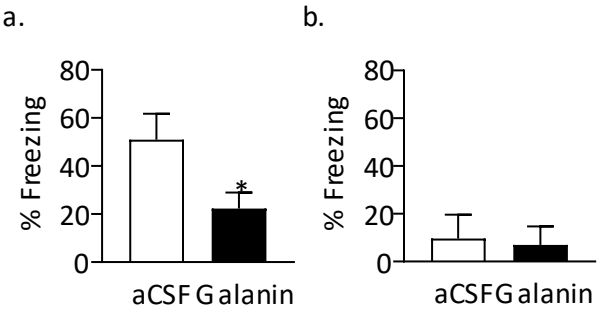


**Figure 2.4.** Administration of galanin into the prelimbic cortex 10 minutes before the retrieval trial reduced time spent freezing during the retrieval trial (a), but not the extinction retention trial (b), compared to aCSF administration. *p < 0.05. Error bars represent SEM.

To verify that the two groups of rats were no different in exploratory behavior prior to drug administration, independent t-tests were performed on ambulatory time and rearing time in the open field during the conditioning trial (data not shown). As expected, prior to drug treatment, the two groups of rats were no different on ambulatory time, t(16) = 0.41, $p$ = 0.69 or rearing time, t(16) = 0.39, $p$ = 0.70 in the open field during the fear conditioning trial. Independent t-tests were also performed to determine the effects of galanin on ambulatory and rearing behavior during the retrieval and extinction retention trials (Figure 2.5). After drug treatment, galanin-treated rats were no different from aCSF-treated rats on ambulatory time,

t(16) = 0.43, $p$ = 0.67 or rearing time, t(16) = 0.84, $p$ = 0.41 in the open field during the retrieval trial. Nor were galanin-treated rats different from aCSF-treated rats on ambulatory time, t(16) = 1.06, $p$ = 0.30 or rearing time, t(16) = 0.07, $p$ = 0.95 in the open field during the extinction retention trial.

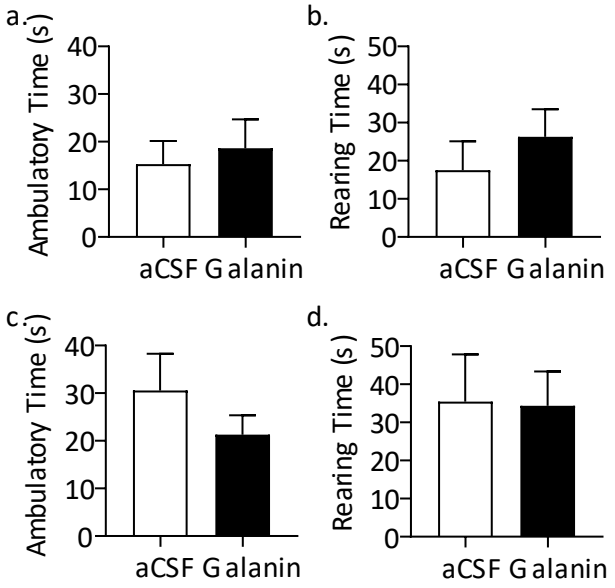


**Figure 2.5.** Neither galanin nor aCSF administration in the prelimbic cortex 10 minutes before the retrieval trial affected ambulatory time (a, c) or rearing time (b, d) during the retrieval trial (a, b) or extinction retention trial (c, d).

To verify that the two groups of rats were no different on anxiety-like behavior prior to drug administration, independent t-tests were performed on center time and entries in the open field during the conditioning trial (data not shown). As expected, prior to drug administration, the two groups of rats were no different on center entries, t(16) = 0.76, $p$ = 0.46, or center time, t(15) = 0.53, p= 0.60, in the open field during the conditioning trial. Independent t-tests were also performed to determine the effects of galanin on anxiety-like behaviors during the retrieval and extinction retention trials (Figure 2.6). Following drug administration, galanin-treated rats were no different from aCSF-treated rats on center entries during the retrieval trial, t(15) = 0.53, $p$ = 0.60, or extinction retention trial, t(16) = 0.80, $p$ = 0.44. However, galanin-treated rats spent

significantly less time in the center zone of the open field than aCSF-treated rats during the retrieval trial, t(12) = 2.25, $p = 0.04$, although they were no different during the extinction retention trial, t(12) = 0.41, $p = 0.69$.

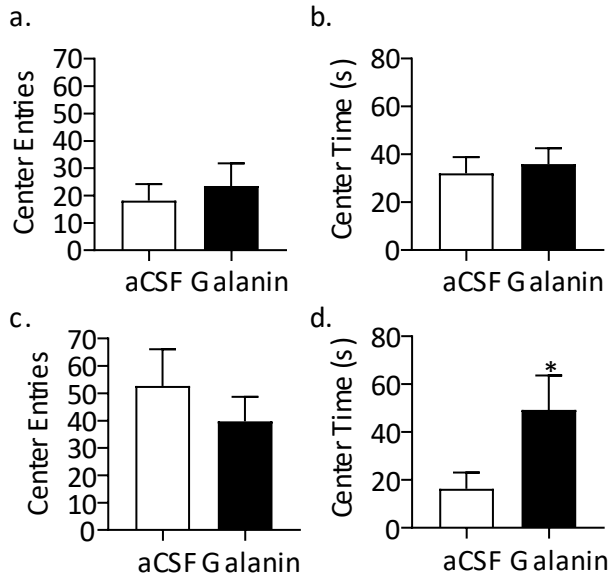


**Figure 2.6**. Effects of galanin and aCSF administration in the prelimbic cortex 10 minutes before the retrieval trial on center entries (a, c) and center time (b, d) during the retrieval trial (a, b) and the extinction retention trial (c, d). *p < 0.05

**Effects of Galanin on PSD95 Expression**

2X2 ANOVAs were performed to determine the effects of foot-shock and galanin administration immediately after the conditioning trial on PSD95 expression in the prelimbic and infralimbic cortices (Figure 2.7). There was a significant interaction between drug and foot-shock exposure for PSD95 expression in the prelimbic cortex when galanin or aCSF was administered immediately after the conditioning trial, F(1,16) = 24, $p = 0.0002$. Galanin administration significantly decreased PSD95 expression in rats that did not undergo CFC conditioning, $p < 0.001$. Exposure to foot-shock during CFC conditioning significantly reduced PSD95 expression in rats exposed to aCSF, $p < 0.001$, or galanin, $p < 0.01$. Galanin

administration attenuated the effects of foot-shock on PSD95 reduction; however, the effect was not significant, $p = 0.09$. Neither galanin administration immediately after the conditioning trial, $F(1, 13) = 0.29$, $p = 0.60$, nor exposure to foot shock during CFC conditioning, $F(1, 13) = 0.12$, $p = 0.73$, effected PSD95 expression in the infralimbic cortex.

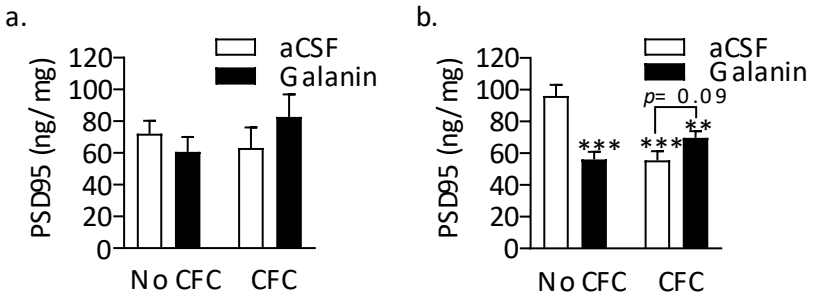


**Figure 2.7.** Effects of contextual fear conditioning (CFC) and galanin administration into the ventromedial prefrontal cortex immediately after the fear conditioning trial on PSD95 protein expression in the prelimbic (a) and infralimbic (b) cortices. **p < 0.01, ***p < 0.001 compared to No CFC-aCSF.

## Discussion

We measured the effects of galanin administered into the prelimbic cortex on the consolidation, retrieval, and extinction of CFC. We also investigated whether the effects of galanin were mediated by a mechanism related to its effects on neuroplasticity. We showed that galanin administration into the prelimbic cortex immediately after the fear conditioning trial impaired consolidation of the emotional memory, as evidenced by decreased freezing in response to the shock-paired context 24-hr later. Furthermore, galanin administration into the prelimbic cortex immediately before the retrieval trial impaired expression of the fear response to the shock-paired context, as shown by reduced freezing in galanin-treated rats, compared to aCSF rats. The same manipulation did not affect retention or expression of extinction learning, as freezing behavior in rats previously treated with galanin was no different from that of aCSF-

treated rats during the extinction retention trial. However, whether galanin facilitates acquisition of extinction learning is not clear from our data. Our results suggest that stimulation of galanin receptors in the prelimbic cortex may promote stress-resilience after trauma by reducing consolidation or retrieval of emotional memories, thereby reducing expression of the emotional memory during test trials. Our results are consistent with a role for the prelimbic cortex in expression of fear after CFC (Knapska et al., 2012; Sierra-Mercado et al., 2011; Vidal-Gonzalez et al., 2006).

The mechanism by which galanin suppresses fear expression in the prelimbic cortex is unclear; however, we postulate that three non-mutually exclusive mechanisms are possible: (i) neuromodulation of norepinephrine release in the mPFC, (ii) direct inhibition of prelimbic neurons by galanin, and (iii) stress-protective neuroplasticity. Galanin is co-expressed with norepinephrine in nerve terminals in the frontal cortex (Holets et al., 1988) and galanin receptors are present in the mPFC (Jungnickel & Gundlach, 2005). Furthermore, evidence suggests that galanin suppresses noradrenergic activity in projection sites, in addition to at the level of the locus coeruleus (Xu et al., 2005). Therefore, galanin administration into the prelimbic cortex could have reduced fear consolidation and expression by suppressing norepinephrine release in the prefrontal cortex after conditioning or during retrieval of the emotional memory. This would be consistent with other studies showing that norepinephrine is involved in formation of emotional memories (McGaugh, 2004). However, although the effects of norepinephrine in the amygdala on consolidation and expression of fear memories and a role for norepinephrine in extinction learning are well-studied (McGaugh, 2004; Mueller & Cahill, 2010), the specific effects of norepinephrine release in the prelimbic cortex on the consolidation and expression emotional memory are unclear. Research suggests that that norepinephrine release during fear

conditioning alters prelimbic neuron activity through beta-adrenergic receptors and is involved in consolidation and expression of the emotional memory (Chalermpalanupap et al., 2018; Davies et al., 2004; Do-Monte et al., 2010; Fitzgerald, Giustino, Seemann, & Maren, 2015; Gazarini, Stern, Carobrez, & Bertoglio, 2013; Murchison, Schutsky, Jin, & Thomas, 2011; Murchison et al., 2004; Rodriguez-Romaguera, Sotres-Bayon, Mueller, & Quirk, 2009). Therefore, suppression of norepinephrine activity in the prelimbic cortex by galanin could explain our findings.

Galanin administration could also affect prelimbic activity independent of norepinephrine. Galanin receptors are expressed in the prelimbic cortex (Jungnickel & Gundlach, 2005) and exogenous galanin administration is known to suppress neural activity (Habert-Ortoli, Amiranoff, Loquet, Laburthe, & Mayaux, 1994; Smith et al., 1998; Wang, Hashemi, Fried, Clemmons, & Hawes, 1998). GalR1 receptors are coupled to $G_{i/o}$ signaling pathways, and thus are predominantly inhibitory to cell function (Lang et al., 2015). Therefore, galanin could suppress prelimbic activity via neural inhibition through GalR1 signaling. However, expression of GalR1 receptors is extremely low in the prelimbic cortex (D. O'Donnell, Ahmad, Wahlestedt, & Walker, 1999). Alternatively, the effects of galanin could be mediated by GalR2 receptor signaling. GalR2 receptor expression is higher in the prelimbic cortex than GalR1 expression (Burazin, Larm, Ryan, & Gundlach, 2000). The GalR2 receptor subtype has more complex signaling cascades through $G_{i/o}$ and $G_{q/11}$ (Lang et al., 2015) and is implicated in the neurotrophic and neuroprotective effects of galanin (Counts, Perez, Ginsberg, & Mufson, 2010; Elliott-Hunt et al., 2011; Elliott-Hunt et al., 2004; Elliott-Hunt, Pope, Vanderplank, & Wynick, 2007; Hobson et al., 2008; Hobson, Holmes, Kerr, Pope, & Wynick, 2006; Hobson et al., 2013; Pirondi et al., 2005). Our laboratory has previously shown that daily administration of galanin is sufficient to

protect against stress-induced reductions in mPFC dendritic spine density (Sciolino et al., 2015). Therefore, galanin may reduce consolidation or expression of conditioned fear by promoting stress-resilient neuroplasticity. Indeed, when galanin was administered immediately after conditioning, it attenuated the CFC-induced reduction of PSD95 in the prelimbic cortex and reduced fear expression 24 hours later. These persistent changes suggest that galanin may have altered neuroplasticity that normally underlies classical conditioning through GalR2 signaling; however, this hypothesis requires further testing.

The effects of galanin on CFC are also consistent with the inhibitory effects of galanin on memory (McDonald, Gleason, Robinson, & Crawley, 1998). It is important to note; however, that while galanin administration into the prelimbic cortex decreased freezing behavior, it did not alter the effects of foot-shock during the CFC conditioning trial on other behaviors measured during the retention trial. Specifically, galanin treated animals were no different from saline treated animals on exploratory behavior (ambulatory and rearing time) or anxiety-like behavior (entries into the center zone) during the retrieval trial. Our results suggest that the effects of galanin on memory may be dose-dependent, region-dependent, or limited to stressful or emotional circumstances. Alternatively, galanin may selectively modulate the behavioral response to memory (e.g., freezing), rather than impose a general inhibitory effect on memory.

We showed that exogenous galanin administration into the prelimbic cortex is sufficient to reduce the expression of freezing behavior in response to a learned association between a previously neutral context and foot-shock. These effects are consistent with the role of the prelimbic cortex in fear expression. The effects of galanin administered before the expression trial are consistent with the anxiolytic and neuromodulatory effects of galanin. The effects of galanin administered after the conditioning trial may be more in line with its role in

neuroplasticity; however, the precise neural mechanisms that explain the effect of galanin on consolidation are still unknown. Furthermore, it remains unclear whether endogenous galanin is involved in prelimbic modulation of CFC expression, consolidation, and extinction. Further investigation into the role of galanin in CFC is needed to determine the therapeutic potential of targeting the galanin system for treating PTSD and other anxiety disorders.

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# CHAPTER 3

# EFFECTS OF GALNON ON DEPRESSION-LIKE BEHAVIOR AND BRAIN MONOAMINE CONTENT IN A MODEL OF CHRONIC INFLAMMATORY PAIN[2]

[2]Hooversmith, J. M., Williams III, K. C., Moore, E., Ali, A., Harbert, A. L., MohanKumar, S. M. J., MohanKumar, P. S., Miller, L. L., & Holmes, P. V. To be submitted to Progress in Neuro-Psychopharmacology and Biological Psychiatry

## Abstract

Novel strategies to treat comorbid pain and depression concurrently are needed. Dysregulation of the mesolimbic dopamine system is implicated in comorbid pain and depression. Projections from the locus coeruleus modulate mesolimbic dopamine activity via norepinephrine and galanin release, which are both involved in stress, pain, anxiety, and depression. Thus, the galanin system is a potential therapeutic target for treating comorbid pain and depression. We induced a model of chronic inflammatory pain in male Sprague-Dawley rats via intraplantar complete Freund's adjuvant (CFA; 50 ul) administration, or saline for control. The galanin agonist galnon (2 mg/kg, i.p.) or saline was then administered for 9 days, after which anxiety- and depression-related behaviors were measured. Whole tissue monoamine content was also measured in the ventral midbrain, ventral striatum, locus coeruleus, and medial prefrontal cortex using HPLC. CFA administration reduced sucrose consumption one and seven days after injections and increased immobility time during forced swim stress eight days after injections. Galnon did not alter the effects of CFA on sucrose consumption, but did attenuate its effects on forced swim stress. CFA administration did not affect anxiety-like behaviors measured on the elevated plus maze or in the open field. CFA administration reduced dopamine levels in the ventral midbrain, but this effect was not altered by galnon administration. These data are consistent with dysregulation of the mesolimbic dopamine system in comorbid pain and depression, but do not provide strong support for a therapeutic role for galnon.

## Introduction

Comorbid chronic pain and depression pose a major health concern. People with conditions characterized by chronic pain, such as rheumatoid arthritis and fibromyalgia, are more likely to experience symptoms of depression than the general population. Furthermore, people with depression report pain symptoms more frequently than non-depressed populations. The presence of comorbid pain is associated with increased severity of depression and poorer treatment outcomes. Likewise, the presence of comorbid depression is associated with poorer treatment outcomes for patients with conditions characterized by chronic pain (Bair, Robinson, Katon, & Kroenke, 2003). Anti-depressants are commonly prescribed to treat comorbid pain and depression; however, evidence of their efficacy in treating both conditions is limited (Bair et al., 2003; Richards, Whittle, & Buchbinder, 2011). Therefore, development of novel strategies to effectively treat both conditions concurrently is critical.

Intraplantar administration of complete Freund's adjuvant (CFA) is a well-established rodent model of chronic inflammatory pain (Ren & Dubner, 1999). CFA injection induces a rapid and robust increase in inflammation and hyperalgesia that lasts for several weeks without significantly altering locomotor behavior or causing tissue damage (Ren & Dubner, 1999). Intraplantar CFA can be employed to study the shared neurobiology of comorbid chronic pain and depression because administration of CFA also induces a depression-like behavioral phenotype in the rodent. For example, intraplantar CFA injection has been shown to alter sleep structure (Pankova, Popkova, Vetrile, Basharova, & Krupina, 2001) and reduce sucrose consumption, a putative measure of anhedonia-like behavior in rodents (Refsgaard, Hoffmann-Petersen, Sahlholt, Pickering, & Andreasen, 2016; Shi, Wang, & Luo, 2010). CFA administration has also been shown to increase immobility time during forced swim (Borges,

Neto, Mico, & Berrocoso, 2014; G. F. Zhang et al., 2016) and decrease social interaction time (Gregoire, Wattiez, Etienne, Marchand, & Ardid, 2014; Parent et al., 2012).

Other known behavioral effects of CFA administration implicate dysregulation of the mesolimbic dopamine system in the link between chronic pain and depression (Betourne et al., 2008; Hipolito et al., 2015; Schwartz et al., 2014). For example, intraplantar administration of CFA has been shown to transiently decrease the rate of intracranial self-stimulation of the medial forebrain bundle (Leitl et al., 2014). Evidence suggests that such behavioral changes might be mediated by altered function of dopaminergic projections from the ventral tegmental area to the nucleus accumbens (Cano et al., 2001; Hipolito et al., 2015; Pais-Vieira, Mendes-Pinto, Lima, & Galhardo, 2009; Schwartz et al., 2014). However, the effects of CFA administration on monoamine content in the mesolimbic dopamine pathway and upstream regions are not well-characterized.

One upstream circuit that is critical for mesolimbic dopamine activity is the noradrenergic locus coeruleus. Galanin is a 29-amino acid neuropeptide that is extensively colocalized in these noradrenergic neurons (Lang et al., 2015; Melander, Hokfelt, Nilsson, & Brodin, 1986; Melander, Hokfelt, & Rokaeus, 1986). (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988; Melander, Hokfelt, Rokaeus, et al., 1986; Xu & Hokfelt, 1997) and modulates their activity (Counts et al., 2002; Ericson & Ahlenius, 1999; Tsuda, Tsuda, Nishio, Masuyama, & Goldstein, 1998; Xu, Zhang, Pieribone, Grillner, & Hokfelt, 1998; Xu, Zheng, & Hokfelt, 2005). Galanin has well-established roles in modulating anxiety-like behavior (Weinshenker & Holmes, 2016), depression-like behavior (Millon et al., 2017), and pain at spinal and supraspinal levels (Lang et al., 2015). Notably, galanin has been shown to exert antinociceptive effects in models of inflammatory pain (Hua et al., 2004; Hua et al., 2005; Sun, Gu, Lundeberg, & Yu,

2003; Xiong, Gao, Sapra, & Yu, 2005; Yang et al., 2015; M. L. Zhang, Fu, & Yu, 2017). Therefore, the galanin system is a potential therapeutic target for treating comorbid pain and depression.

We investigated the effects of chronic inflammatory pain induced by intraplantar CFA injection on anxiety- and depression-related behaviors and monoamine levels within the mesolimbic dopamine system. Because stress is well-known to increase vulnerability to depression, we also evaluated whether the effects of CFA would be exacerbated by stress. Furthermore, we investigated the therapeutic potential for galnon, a non-specific, non-peptide agonist of the galanin receptors (Saar et al., 2002), with anxiolytic (Kozlovsky, Matar, Kaplan, Zohar, & Cohen, 2009; Rajarao et al., 2007), anti-depressant (Lu et al., 2005; Rajarao et al., 2007), and analgesic properties (W. P. Wu et al., 2003). We hypothesized that galnon would attenuate the neurobiological and behavioral effects of chronic inflammatory pain.

## Experimental Methods

### Subjects

Male Sprague-Dawley rats (N = 48; Envigo, Prattville, AL) were obtained at 175 – 200 g and given ad libitum food and water. Rats were housed individually in clear polycarbonate cages (50 x 30 x 30 cm) at 23 ± 3 °C on a 12:12 reverse light:dark cycle. All behavioral testing occurred during the dark cycle. Procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 – 23, revised 2013). Experiments were approved by University of Georgia IACUC.

### General Experimental Protocol

Rats were acclimated to the facility for 6-10 days upon arrival. Following acclimation, rats were injected with saline (n = 32) or CFA (n = 33), as described below, to induce chronic

inflammatory pain. Intraplantar injections were followed by 9 days of drug treatment with galnon (n = 16) or saline (n = 32). Rats in the stress group (n = 33) were exposed to forced swim stress 8 days after intraplantar injections; control rats (n = 32) were brought into the testing room but remained undisturbed in their home cages. All rats underwent a battery of behavioral tests 9 days after intraplantar injections. Rats were euthanized via rapid decapitation following behavioral testing on day 9. Non-stressed groups were: rats that received intraplantar injections of saline and daily intraperitoneal injections of vehicle (Sal/Veh; n= 8) or galnon (Sal/Gal; n = 8) and rats that received intraplantar injections of CFA and daily intraperitoneal injections of vehicle (CFA/Veh; n = 8) or galnon (CFA/Gal; n =8). Stressed groups were: rats that received intraplantar injections of saline (fssSal/Veh; n= 8) or CFA (fssCFA/Veh; n = 8) and daily intraperitoneal injections of vehicle.

**Inflammation Induction and Drug Administration**

Rats were briefly anesthetized with isoflurane (1-5% in O2) and the left hindpaw was swabbed with 70% ETOH. Rats were then injected with CFA (50 ul; Sigma Aldrich) or saline into the intraplantar region of the left hindpaw. Injections were delivered through a 26G needle over 30 sec. Needles were left in place for an additional 30 sec to minimize backflow. Calipers were used to measure the dorsal-ventral width (mm) of the paw at the injection site immediately before and every day after intraplantar injections for the duration of the experiment. Beginning immediately after intraplantar injections, rats received daily intraperitoneal injections of galnon sonicated with 1% DMSO in saline (2 mg/kg; Bachem Americas) or saline for 9 days in a volume of 2 ml/kg.

**Behavioral Manipulations and Testing**

*Forced swim stress*

Rats were placed in the center of a cylindrical glass tank (61 cm tall, 20 cm diameter) with 23-25 °C water filled to a depth of 30 cm for 15 min. Rats were able to swim or float without their hindlimbs touching the bottom. Time spent immobile and swimming was recorded. After the test, rats were towel dried and placed back in their home cages. Control rats were transported to the testing room for 15 min but did not undergo forced swim stress.

*Sucrose preference*

Training for the sucrose preference test began three days before intraplantar injections. For the first training trial, two sipper tubes were filled with tap water and placed in the home cage for 24 hours to allow rats to habituate to the presence of two bottles. The water in both bottles was replaced with 1% sucrose solution the following day for 24 hr to allow the rats to habituate to the sucrose solution. On test days the rats were presented with one bottle of tap water and one bottle of 1% sucrose solution for 24 hours. Bottles were weighed before each trial and 1 and 24 hours after each trial began to determine the amount of volume consumed from each bottle. Sucrose consumption was measured one day before CFA, one day after CFA, and seven days after CFA.

*Open field*

The apparatus was a transparent plexiglass open-field chamber (43.3 cm long x 43.4 cm wide x 30.5 cm high; Med Associates, St. Albans, VT). Rats were placed in the back-left corner of the arena and undisturbed for 10 min. Behavior was continuously recorded via infrared beam breaks (ENV-520, Med Associates) and monitored by an experimenter blind to experimental

conditions. Total distance traveled, rearing, and center time and entries were measured. Animals were returned to their home cages after testing.

*Elevated plus maze*

The wooden elevated plus maze was elevated 50cm above the floor and consisted of two opposite open arms (45 cm x 9 cm), two opposite closed arms (45 cm x 9 cm x 38 cm), and a central platform (9 cm x 9 cm). A 15 W bulb placed 1 m above maze illuminated the center platform (15 lx). Each rat was placed in the center of the maze facing an open arm of the maze opposite the experimenter. Behavior was recorded for 5 min. An experimenter blind to group assignment scored open and closed arm time and entries from a video monitor hidden behind a divider during testing.

**Tissue and Serum Processing**

*Blood collection and corticosterone quantification*

Rats were euthanized via rapid decapitation between 95 and 105 min after being tested on the elevated plus maze. Trunk blood was collected into serum tubes. After clot formation, samples were centrifuged at 1000 x g for 10 min at 4 °C. Supernatant was stored in 0.5 ml Eppendorf microcentrifuge tubes at -20 °C until further processing.

Corticosterone levels (ng/ml) were measured in serum using a corticosterone parameter assay kit (R&D Systems; KGE009) according to instructions provided with the kit.

*Tissue collection and high-performance liquid chromatography (HPLC)*

Brains were extracted, and the ventral midbrain, ventral striatum, medial prefrontal cortex, and dorsal pons were dissected on a cold metal plate, placed into 1.5 ml microcentrifuge tubes, frozen on dry ice, and stored at -80 °C until use.

Tissue was analyzed for norepinephrine, dopamine, DOPAC, serotonin, and 5-HIAA using HPLC with electrochemical detection (HPLC-EC). Tissue was homogenized in 400 ul of KRH buffer. 25 ul of the homogenate was added to 125 ul of 0.05 M perchloric acid. An aliquot of the sample was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). Samples were then centrifuged at 13,000 x g for 7 min at 25 C. The supernatant was injected with the internal standard, dihydroxybenzylamine (DHBA; 0.05 M) into the autoinjector (SIL-20AC) for HPLC-EC analysis. The rest of the HPLC-EC system consisted of a Luna 5 um C18 reverse phase column (250 x 4.6 mm; Phenomenex, Torrance, CA), a CTO-20AC column oven (Shimadzu, Columbia, MD; 37 C), and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase (153.10 mM chloroacetic acid, 1.55 mM octanesulfunic acid, 0.86 mM Ultrapure EDTA, 116.30 mM sodium hydroxide, 46.90 mM acetonitrile, 1.20 mM tetrahydrofuran) was set to 1.8 ml/min using an LC-20AD pump (Shimadzu, Columbia, MD). Neurotransmitter concentration in tissue samples were expressed as pg/ug of protein.

### Statistical Analysis

Statistics were performed using GraphPad Prism 7.03 software. Outliers were removed using the ROUT method. Unless otherwise stated, two-way (inflammation x drug treatment) ANOVAs were performed and were followed by Fisher's LSD post hoc tests.

## Results

### Weight and Paw Inflammation

A two-way (group x day) repeated measures ANOVA was performed to determine the effects of CFA and galnon on paw swelling over the 10-day experiment (Figure 3.1). Because stress occurred on Day 9 and had no effect on paw width on Day 10, stressed and non-stressed

rats were combined for analysis. There was a significant interaction between group and day, $F(27, 549) = 27.00$, $p<0.001$. Fisher's LSD post-hoc analyses revealed that there were no differences in baseline paw width between groups on Day 1, but that CFA/Veh rats and CFA/Gal rats had increased paw width on Days 2-10 compared to Sal/Veh and Sal/Gal rats.

A two-way (group x day) repeated measures ANOVA was performed to determine the effects of CFA and galnon on weight over the 10-day experiment (Figure 3.1). Because stress occurred on Day 9 and had no effect on weight on Day 10, stressed and non-stressed rats were combined for analysis. There was a significant interaction between group and day, $F(27, 549) = 6.20$, $p<0.001$. Fisher's LSD post-hoc analyses revealed that there were no differences in baseline weight between groups on Day 1, but that Sal/Veh rats weighed significantly more than all other groups on Days 2 through 10.

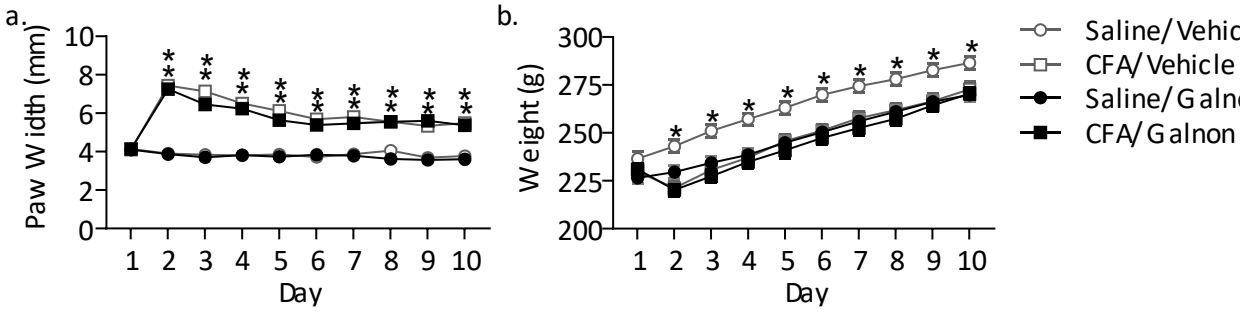


**Figure 3.1.** (a) Intraplantar administration of complete Freund's adjuvant (CFA) induced localized swelling of the left hind paw for at least 9 days post-injection in galnon and saline treated rats. *p ≤ 0.0001 compared to saline-saline same day. (b) CFA and galnon administration decreased weight for the duration of the experiment. *p ≤ 0.01 Saline/Vehicle compared to all other groups same day. Error bars represent SEM.

**Sucrose Preference**

A two-way (inflammation x drug) ANOVA was performed on baseline sucrose consumption one day before intraplantar injections (data not shown). Results showed that there

were no differences between rats on baseline measures of sucrose consumption, $F(1, 60) = 0.67$, $p = 0.42$ (inflammation), $F(1, 60) = 0.53$, $p = 0.47$ (drug).

A two-way (inflammation x drug) ANOVA was performed on sucrose consumption one day after intraplantar injections (Figure 3.2). Results showed that there was a significant interaction between inflammation and drug on sucrose consumption one-day post injections, $F(1, 59) = 7.40$, $p = 0.01$. Fisher's LSD post-hoc analyses showed that CFA reduced sucrose consumption in CFA/Veh rats, $p < 0.001$, and CFA/Gal rats, $p = 0.01$, compared to Sal/Veh rats. Furthermore, galnon reduced sucrose consumption in Sal/Gal rats, $p = 0.01$, compared to Sal/Veh rats.

A two-way (inflammation x drug) ANOVA was performed on sucrose consumption seven days after intraplantar injections (Figure 3.2). Results showed that there was a main effect of inflammation on sucrose consumption seven-days post injection, $F(1, 59) = 7.00$, $p = 0.01$; CFA/Sal and CFA/Gal rats consumed less sucrose than Sal/Veh rats. Galnon alone did not affect sucrose consumption, $F(1, 59) = 0.07$, $p = 0.79$.

**Forced Swim Stress**

A subset of animals was exposed to 15 min of forced swim stress eight days after intraplantar injections of saline or CFA. Two-way (inflammation x drug) ANOVAs were performed on immobility and swim time (Figure 3.3). Results showed that there were near-significant interactions between inflammation and drug on immobility time, $F(1, 26) = 3.80$, $p = 0.06$, and swim time, $F(1, 26) = 3.80$, $p$ 0.06. Because the trend was nearly significant, Fisher's LSD post-hoc analyses were performed. Post-hocs showed that fssCFA/Veh animals spent significantly more time immobile, $p = 0.03$, and less time swimming, $p = 0.03$, than fssSal/Veh

animals. Neither fssSal/Gal nor fssCFA/Gal rats were different from fssSal/Veh controls on immobility or swim time.

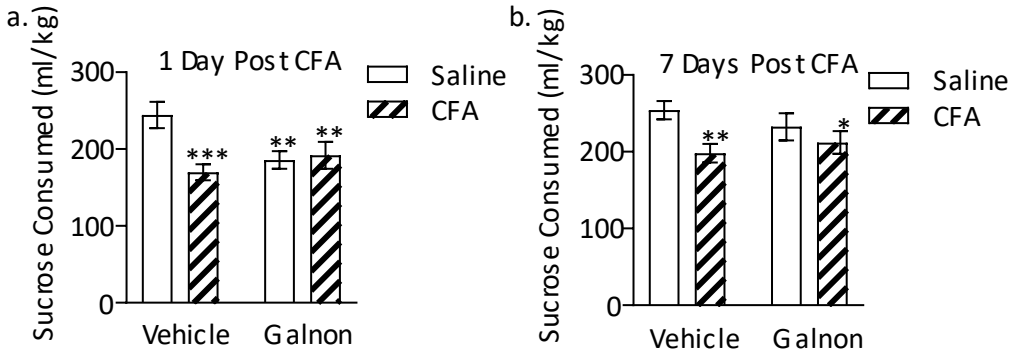


**Figure 3.2.** Administration of complete Freund's adjuvant (CFA) decreased sucrose consumption one (a) and seven (b) days later in vehicle- and galnon-treated rats. Daily administration of galnon decreased sucrose consumption in saline-treated rats one day (a), but not seven days (b), after intraplantar injections. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared to Saline/Vehicle. Error bars represent SEM.

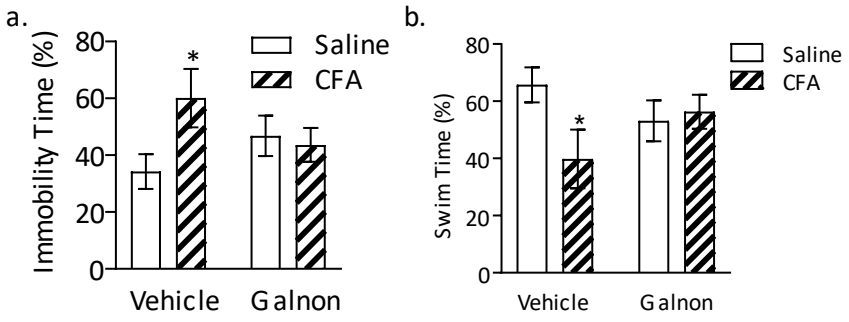


**Figure 3.3.** Intraplantar administration of complete Freund's adjuvant (CFA) increased the percent of time spent immobile during the 15-min forced swim stress procedure for vehicle-treated rats eight days later. Galnon blunted the effects of CFA. *p ≤ 0.05 compared to Saline/Vehicle. Error bars represent SEM.

**Effects of CFA and Galnon on Anxiety-Related Behaviors in Non-Stressed Animals**

Two-way (inflammation x drug) ANOVAs were performed on behaviors measured in the open field nine days after intraplantar injections in non-stressed rats (Figure 3.4). Results showed that there was no effect of inflammation, $F(1, 27) = 0.22$, $p = 0.64$, nor drug, $F(1, 27) = 0.29$, $p =$

0.59, on ambulatory time. There was a significant main effect of inflammation on rearing time, F(1, 27) = 4.40, $p = 0.05$, with CFA-treated rats rearing less than saline-treated rats. There was a significant interaction between inflammation and drug on center time, F(1, 26) = 6.6, $p = 0.02$. Post-hoc analyses revealed that Sal/Gal rats spent more time in the center than Sal/Veh rats, $p = 0.001$, CFA/Veh rats, $p = 0.01$, and CFA/Gal rats, $p = 0.01$. CFA/Gal rats were no different from Sal/Veh controls, $p = 0.36$. There was a significant main effect of drug on perimeter time, F(1, 28) = 5.00, $p = 0.03$, with galnon-treated rats spending less time in the perimeter than vehicle-treated rats.

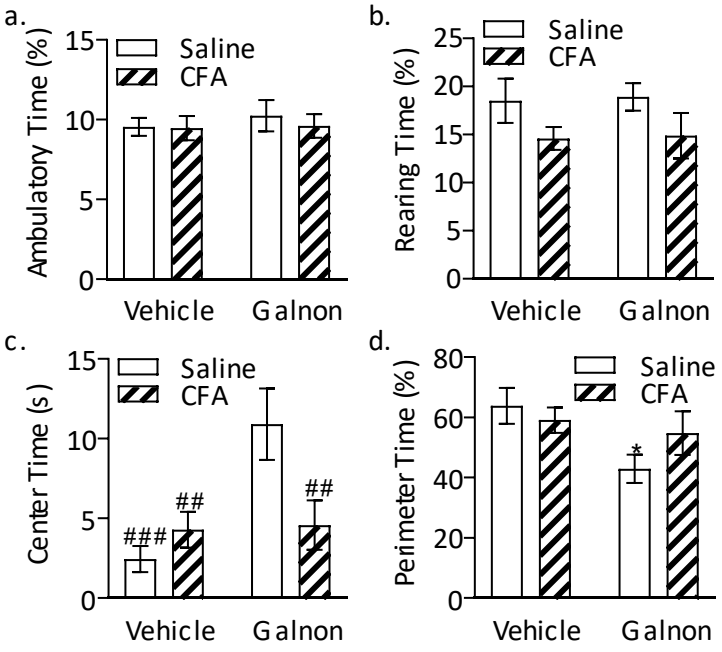


**Figure 3.4.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) or saline and chronic treatment with galnon or vehicle on behavior of non-stressed rats during 10-min exploration of a novel open field chamber 9 days later. *$p \leq 0.05$ compared to Saline/Vehicle. $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ compared to saline/galnon. Error bars represent SEM.

Two-way (inflammation x drug) ANOVAs were performed on behaviors measured on the elevated plus maze nine days after intraplantar injections in non-stressed rats (Figure 3.5). Results showed that there was no effect of drug, F(1, 25) = 0.86, $p = 0.36$, or inflammation, F(1, 25) = 2.60, $p = 0.12$, on open arm time. However, there was a significant interaction between

drug and inflammation on open arm entries, F(1, 26) = 4.40, $p = 0.05$. Post-hoc analyses showed that galnon increased open arm entries in Sal/Gal rats, $p = 0.08$, but not CFA/Gal rats, $p = 0.98$. There was no effect of drug, F(1, 27) = 0.016, $p = 0.69$, or inflammation, F(1, 27) = 0.02, $p = 0.88$, on closed arm time. Nor was there an effect of drug, F(1, 27) = 0.17, $p = 0.68$, nor inflammation, F(1, 27) = 0.22, $p = 0.64$, on closed arm entries. There was also no effect of drug, F(1, 27) = 0.72, $p = 0.40$, or inflammation, F(1, 27) = 0.22, $p = 0.65$, on total arm entries.

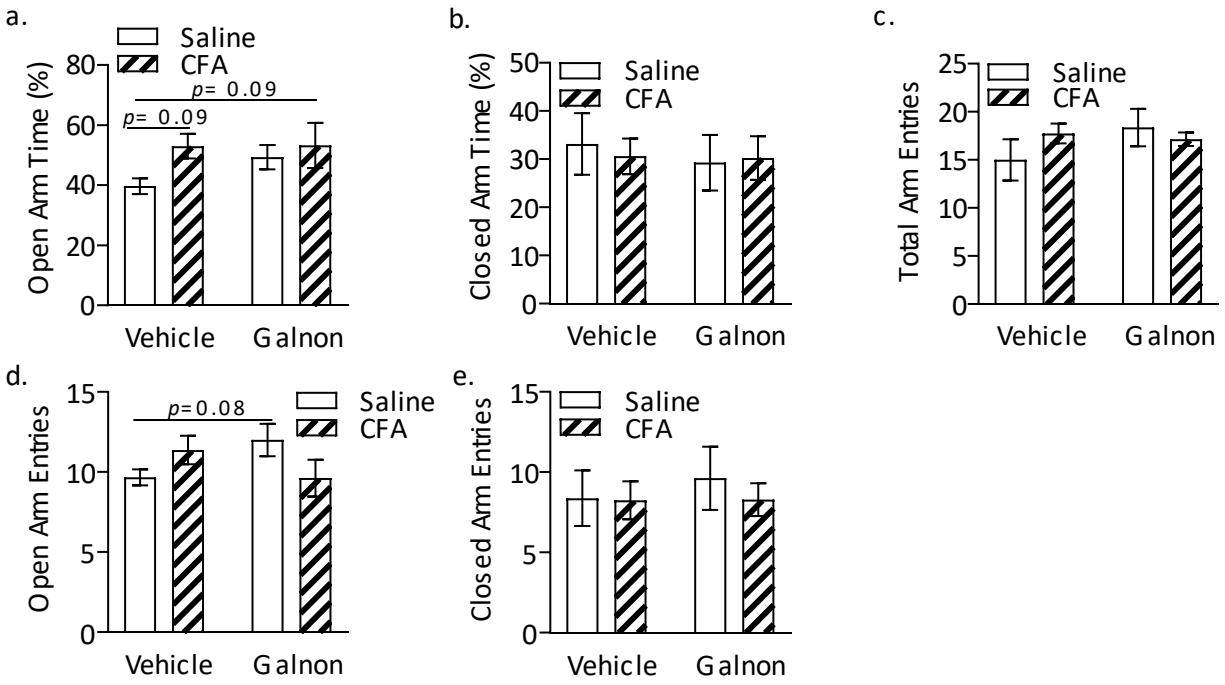


**Figure 3.5.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) or saline and chronic treatment with galnon or vehicle on behavior of non-stressed rats during 5-min exploration of an elevated plus maze 9 days later. Error bars represent SEM.

**Effects of CFA and Stress on Anxiety-Related Behaviors in Vehicle-Treated Animals**

Two-way (inflammation x stress) ANOVAs were performed on behaviors measured in the open field nine days after intraplantar injections in vehicle-treated rats (Figure 3.6). Results showed that there was no effect of stress, F(1, 28) = 0.55, $p = 0.46$, or inflammation, F(1, 28) =

0.82, $p = 0.37$, on ambulatory time in the open field for vehicle-treated animals. There was a main effect of inflammation on rearing time, $F(1, 26) = 6.90$, $p = 0.01$, with CFA-treated animals rearing less than saline-treated animals, but no effect of stress on rearing time, $F(1, 26) = 0.18$, $p = 0.68$. There was no effect of stress on center time, $F(1, 24) = 1.30$, $p = 0.26$, or perimeter time, $F(1, 28) = 0.94$, $p = 0.34$. Nor was there an effect of inflammation on center time, $F(1, 24) = 0.10$, $p = 0.75$, or perimeter time, $F(1, 28) = 0.45$, $p = 0.51$.

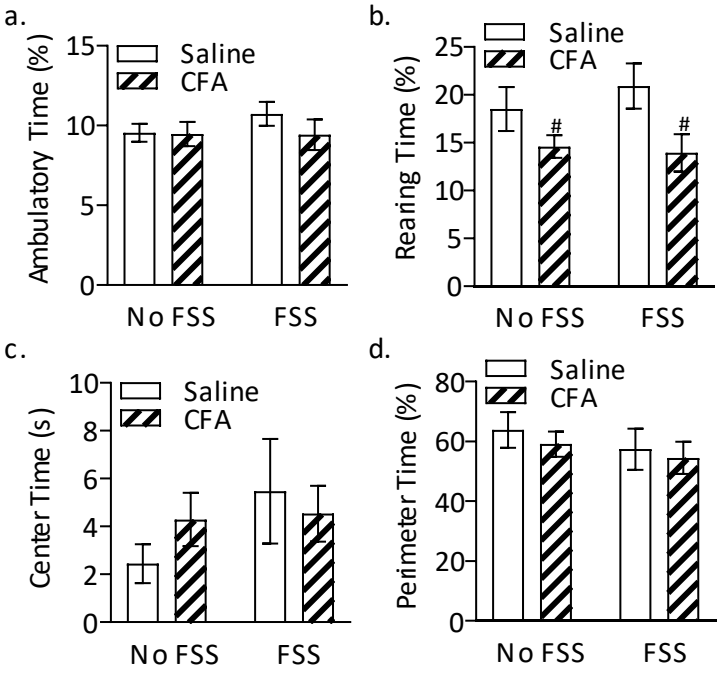


**Figure 3.6.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) or saline in non-stressed (No FSS) and stressed (FSS) vehicle-treated rats on behavior during 10-min exploration of a novel open field chamber 9 days later. $^{\#}p \leq 0.05$ compared to CFA/FSS. Error bars represent SEM.

Two-way (inflammation x stress) ANOVAs were performed on behaviors measured in the elevated plus maze nine days after intraplantar injections in vehicle-treated rats (Figure 3.7). There were significant main effects of stress, $F(1, 26) = 6.40$, $p = 0.02$, and inflammation, $F(1, 26) = 6.20$, $p = 0.02$, on open arm time. Stressed animals spent more time on the open arm than non-stressed animals and animals treated with CFA spent more time on the open arm than animals treated with saline. There was a significant main effect of inflammation on open arm

entries, F(1, 25) = 5.30, $p = 0.03$, with CFA-treated animals entering the open arm more than animals treated with saline, but there was no effect of stress on open arm entries, F(1, 25) = 1.20, $p = 0.28$. There was no effect of stress on closed arm time, F(1, 28) = 0.77, $p = 0.39$, or entries, F(1, 27) = 0.38, $p = 0.54$. Neither was there an effect of inflammation on closed arm time, F(1, 28) = 2.10, $p = 0.16$, or entries, F(1, 27) = 0.28, $p = 0.60$. There was also no effect of stress, F(1, 25) = 1.60, $p = 0.21$, or inflammation, F(1, 25) = 0.01, $p = 0.93$, on total arm entries.

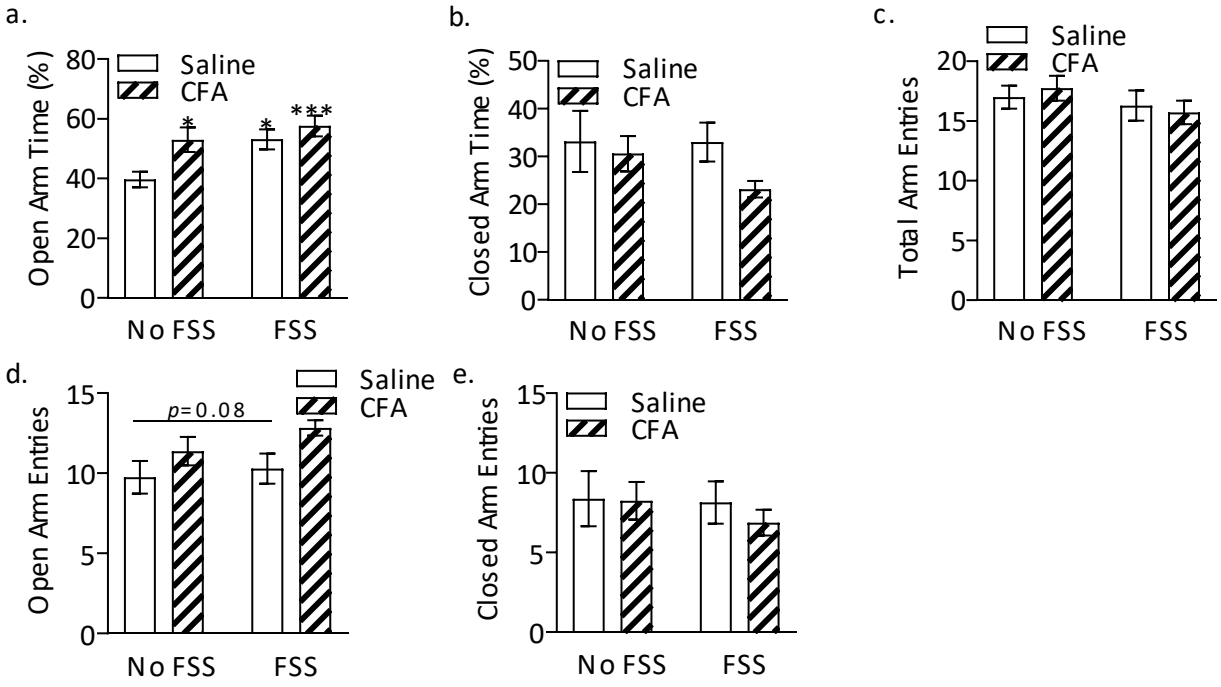


**Figure 3.7.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) or saline in non-stressed (No FSS) and stressed (FSS) vehicle-treated rats on behavior during 5-min exploration of an elevated plus maze 9 days later. *$p \leq 0.05$, ***$p \leq 0.001$ compared to Saline/No FSS. Error bars represent SEM.

**Effects of CFA and Galnon on Brain Monoamine Content in Non-Stressed Animals**

Two-way (inflammation x drug) ANOVAs were performed on monoamine levels in the ventral striatum nine days after intraplantar injections in non-stressed rats (Table 3.1). There was

a trend towards a main effect of drug, F(1, 26) = 3.50, $p = 0.07$, but no effect of inflammation, F(1, 26) = 0.003, $p = 0.95$, on norepinephrine in the ventral striatum; galnon decreased norepinephrine levels in the ventral striatum. There was no effect of drug, F(1, 24) = 0.02, $p = 0.90$, or inflammation, F(1, 24) = 0.38, $p = 0.54$, on DOPAC in the ventral striatum (Figure 3.8). There was a significant interaction between drug and inflammation on dopamine in the ventral striatum, F(1, 26) = 5.20, $p = 0.03$ (Figure 8). Galnon significantly increased dopamine levels in the ventral striatum of saline-treated rats ($p < 0.01$), but not CFA-treated rats ($p = 0.49$), compared to Sal/Veh rats. CFA alone had no effect on dopamine levels in the ventral striatum ($p = 0.41$). There was no effect of drug, F(1, 24) = 2.60, $p = 0.12$, or inflammation, F(1, 24) = 0.02, $p = 0.90$, on 5HIAA in the ventral striatum. There was no effect of drug, F(1, 26) = 1.20, $p = 0.28$, or inflammation, F(1, 26) = 1.10, $p = 0.30$, on serotonin in the ventral striatum.

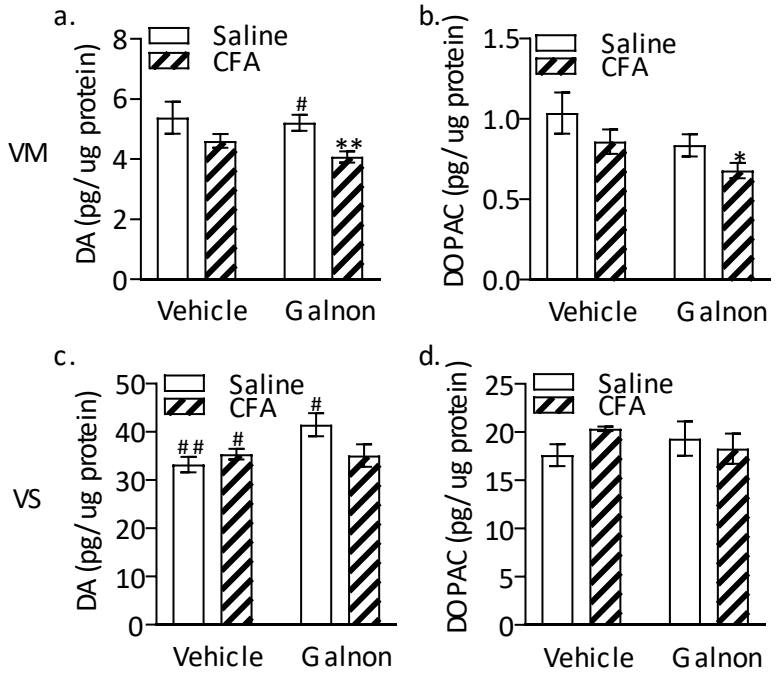


**Figure 3.8.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) and galnon on dopamine (DA; a, c) and DOPAC (b, d) in the ventral midbrain (VM; a, b) and ventral striatum (VS; c, d) of non-stressed animals. CFA significantly reduced dopamine ($p = 0.01$) and DOPAC ($p = 0.08$) levels in the VM. There was a significant interaction between drug and inflammation on dopamine in the VS (VS; $p = 0.03$). *$p \leq 0.05$, **$p \leq 0.01$ compared to Saline/Vehicle, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ compared to CFA/Galnon.

Two-way (inflammation x drug) ANOVAs were performed on monoamine levels in the ventral midbrain nine days after intraplantar injections in non-stressed rats (Table 3.1). There was a significant interaction between inflammation and drug on norepinephrine levels in the ventral midbrain, $F(1, 26) = 7.10$, $p = 0.01$. Neither CFA ($p = 0.58$) nor galnon ($p = 0.45$) alone affected norepinephrine levels in the ventral midbrain. However, ventral midbrain norepinephrine levels were lower in CFA/Gal animals, compared to Sal/Veh animals ($p = 0.02$). There was a significant main effect of drug, $F(1, 26) = 4.40$, $p = 0.05$, on DOPAC levels in the ventral midbrain and a trend towards a significant main effect of inflammation, $F(1, 26) = 3.40$, $p = 0.08$ (Figure 3.8); CFA reduced and galnon significantly reduced DOPAC in the ventral midbrain. There was no effect of drug, $F(1,28) = 1.10$, $p = 0.30$, on dopamine in the ventral midbrain, but there was a significant main effect of inflammation, $F(1, 28) = 8.20$, $p = 0.01$ (Figure 3.8); CFA significantly reduced dopamine in the ventral midbrain. There was no effect of drug, $F(1, 27) = 2.6$, $p = 0.12$, or inflammation, $F(1, 27) = 0.24$, $p = 0.63$, on 5HIAA in the ventral midbrain. There was a significant main effect of drug, $F(1, 27) = 4.60$, $p = 0.04$, and a trend towards a significant main effect of inflammation, $F(1, 27) = 3.30$, $p = 0.08$, on serotonin levels in the ventral midbrain; CFA decreased and galnon significantly decreased serotonin in the ventral midbrain.

Two-way (inflammation x drug) ANOVAs were performed on monoamine levels in the dorsal pons nine days after intraplantar injections in non-stressed rats (Table 3.1). There was no effect of drug, $F(1, 25) = 0.05$ , $p = 0.82$, or inflammation, $F(1, 25) = 0.10$, $p = 0.76$, on norepinephrine levels in the dorsal pons. There were significant interactions between drug and inflammation on DOPAC levels, $F(1, 25) = 4.50$, $p = 0.04$, and dopamine levels, $F(1, 25) = 5.7$, $p = 0.03$, in the dorsal pons. Post-hoc analyses showed that neither CFA ($p = 0.45$), nor galnon ($p =$

0.44) alone effected DOPAC levels in the dorsal pons. CFA/Gal rats had higher levels of DOPAC in the dorsal pons compared to CFA/Veh rats ($p = 0.04$) and CFA/Gal rats ($p = 0.04$), but not Sal/Veh rats ($p = 0.16$). CFA alone trended to decrease dopamine levels in the dorsal pons, compared to Sal/Veh rats ($p = 0.10$), but the effect was prevented by chronic galnon treatment ($p = 0.03$ compared to CFA/Veh). There was a trend toward an interaction between drug and inflammation on 5HIAA levels in the dorsal pons, $F(1, 24) = 3.20$, $p = 0.09$. There was no effect of drug, $F(1, 25) = 0.98$, $p = 0.33$, or inflammation, $F(1, 25) = 0.62$, $p = 0.44$, on serotonin levels in the dorsal pons.

Two-way (inflammation x drug) ANOVAs were performed on monoamine levels in the medial prefrontal cortex nine days after intraplantar injections in non-stressed rats (Table 3.1). There was a trend towards a significant interaction between drug and inflammation, $F(1, 26) = 3.20$, $p = 0.08$, on norepinephrine levels in the medial prefrontal cortex. There was no effect of drug, $F(1, 24) = 0.66$, $p = 0.43$, or inflammation, $F(1, 24) = 0.12$, $p = 0.73$, on DOPAC levels in the medial prefrontal cortex. There was no effect of drug, $F(1, 22) = 0.07$, $p = 0.80$, or inflammation, $F(1, 22) = 2.30$, $p = ,0.14$ on dopamine levels in the medial prefrontal cortex. There was no effect of drug, $F(1, 25) = 0.57$, $p = 0.46$, or inflammation, $F(1, 25) = 1.50$, $p = 0.24$, on 5HIAA levels in the medial prefrontal cortex. There was a trend towards a significant interaction between drug and inflammation on serotonin levels in the medial prefrontal cortex, $F(1, 25) = 3.90$, $p = 0.06$.

**Effects of CFA and Stress on Brain Monoamine Content in Vehicle-Treated Animals**

Two-way (inflammation x stress) ANOVAs were performed on monoamine levels in the ventral striatum nine days after intraplantar injections in vehicle-treated rats (Table 3.2). There was no effect of stress on norepinephrine levels in the ventral striatum, $F(1, 28) = 0.12$, $p = 0.73$,

but there was a trend toward a main effect of inflammation, $F(1, 28) = 3.90$, $p = 0.06$; CFA increased norepinephrine in the ventral striatum. There was also a trend towards a significant interaction between stress and inflammation on DOPAC levels in the ventral striatum, $F(1, 26) = 3.00$, $p = 0.10$, and a significant interaction between stress and inflammation on dopamine levels, $F(1, 27) = 5.70$, $p = 0.02$ (Figure 3.8). 5HIAA levels were not affected by stress, $F(1, 27) = 0.07$, $p = 0.80$, or inflammation, $F(1, 27) = 0.36$, $p = 0.55$. Serotonin levels were also not affected by stress, $F(1, 28) = 0.13$, $p = 0.72$, or inflammation, $F(1, 28) = 0.03$, $p = 0.86$.

Two-way (inflammation x stress) ANOVAs were performed on monoamine levels in the ventral midbrain nine days after intraplantar injections in vehicle-treated rats (Table 3.2). Norepinephrine levels in the ventral midbrain were not affected by stress, $F(1, 26) = 0.39$, $p = 0.54$, or inflammation, $F(1, 26) = 0.57$, $p = 0.46$. DOPAC levels in the ventral midbrain were also not affected by stress, $F(1, 28) = 0.13$, $p = 0.73$, or inflammation, $F(1, 28) = 2.50$, $p = 0.13$ (Figure 3.8). There was no effect of stress on dopamine levels in the ventral midbrain, $F(1, 27) = 0.004$, $p = 0.95$; however, there was a significant main effect of inflammation on dopamine levels, $F(1, 27) = 8.20$, $p = 0.01$ (Figure 3.8); CFA significantly decreased dopamine in the ventral striatum. There was no effect of stress, $F(1, 27) = 0.33$, $p = 0.57$, or inflammation, $F(1, 27) = 0.14$, $p = 0.71$, on 5HIAA levels in the ventral midbrain. There was a significant main effect of stress on serotonin levels in the ventral midbrain, $F(1, 28) = 24$, $p < 0.001$; stress significantly decreased serotonin in the ventral midbrain. There was no effect of inflammation on serotonin in the ventral midbrain, $F(1, 28) = 0.03$, $p = 0.88$.

Two-way (inflammation x stress) ANOVAs were performed on monoamine levels in the dorsal pons nine days after intraplantar injections in vehicle-treated rats (Table 3.2).

**Table 3.1**

Effects of Nine Days of Chronic Inflammatory Pain and Drug Treatment on Brain Monoamine Levels in Non-Stressed Rats

| | | **Dorsal Pons** | | **Ventral Striatum** | | **Ventral Midbrain** | | **Medial PFC** | |
|---|---|---|---|---|---|---|---|---|---|
| **NT** | **Group** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** |
| NE | Sal/Veh | 9.88 | 0.97 | 8.67 | 0.46 | $^{x}$ **11.00** | **1.08** | 6.01 | 0.15 |
| | CFA/Veh | 9.78 | 1.01 | 9.23 | 0.58 | $^{xx}$ **11.68** | **0.37** | 5.72 | 0.17 |
| | Sal/Gal | 9.69 | 0.97 | 8.11 | 0.74 | $^{xx}$ **11.89** | **1.07** | 5.68 | 0.18 |
| | CFA/Gal | 10.49 | 1.53 | 7.62 | 0.30 | 8.17 | 0.50 | 6.04 | 0.23 |
| DOPAC | Sal/Veh | 0.21 | 0.02 | 17.62 | 1.14 | 1.04 | 0.13 | 0.35 | 0.04 |
| | CFA/Veh | $^{x}$ **0.18** | **0.02** | 20.35 | 0.23 | 0.86 | 0.08 | 0.47 | 0.06 |
| | Sal/Gal | $^{x}$ **0.18** | **0.12** | 19.33 | 1.78 | 0.84 | 0.07 | 0.51 | 0.07 |
| | CFA/Gal | 0.28 | 0.07 | 18.29 | 1.57 | ***0.68** | **0.05** | 0.45 | 0.15 |
| DA | Sal/Veh | 0.52 | 0.06 | $^{\#\#}$**33.22** | **1.61** | $^{xx}$ **5.39** | **0.53** | 1.44 | 0.13 |
| | CFA/Veh | $^{x,1}$**0.38** | **0.02** | $^{\#}$**35.38** | **1.10** | 4.60 | 0.23 | 1.45 | 0.05 |
| | Sal/Gal | 0.43 | 0.03 | 41.49 | 2.39 | $^{x}$ **5.21** | **0.27** | 1.80 | 0.27 |
| | CFA/Gal | $^{2}$**0.58** | **0.11** | $^{\#}$**35.09** | **2.33** | 4.08 | 0.19 | $^{\#}$**1.20** | **0.13** |
| 5HIAA | Sal/Veh | 10.93 | 1.60 | 8.83 | 0.52 | 20.78 | 4.35 | 5.52 | 0.80 |
| | CFA/Veh | $^{3}$**14.70** | **1.75** | 9.08 | 0.46 | 19.57 | 2.53 | 4.38 | 0.49 |
| | Sal/Gal | 11.53 | 1.25 | 8.44 | 0.30 | 16.40 | 2.52 | 5.91 | 0.95 |
| | CFA/Gal | $^{\$}$**9.63** | **1.19** | 8.08 | 0.31 | 14.82 | 1.85 | 5.17 | 0.61 |
| 5HT | Sal/Veh | 18.00 | 1.75 | 11.05 | 0.50 | 31.69 | 6.06 | 9.21 | 0.59 |
| | CFA/Veh | 18.70 | 1.75 | 11.30 | 0.83 | 24.60 | 3.20 | 7.65 | 0.09 |
| | Sal/Gal | 21.93 | 1.70 | 9.83 | 0.73 | 23.27 | 3.46 | 7.89 | 0.54 |
| | CFA/Gal | 18.36 | 1.91 | 11.02 | 0.61 | ***15.57** | **1.89** | 8.55 | 0.69 |

*Note*. SEM = Standard Error of the Mean, NT = Neurotransmitter, NE = Norepinephrine, DA = Dopamine, 5HT = Serotonin, Sal = Saline, CFA = Complete Freund's Adjuvant, Veh = Vehicle, Gal = Galnon. Mean values are pg/ug protein. $^{*}p \leq 0.05$ compared to Sal/Veh, $^{\$}p \leq 0.05$ compared to CFA/Veh, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ compared to Sal/Gal, $^{x}p \leq 0.05$, $^{xx}p \leq 0.01$ compared to CFA/Gal. $^{1}p$=0.10 compared to Sal/Veh, $^{2}p$=0.10 compared to Sal/Gal, $^{3}p$ = 0.07, compared to Sal/Veh.

**Table 3.2**

Effects of Stress and Nine Days of Chronic Inflammatory Pain on Brain Monoamine Levels in Vehicle-treated Rats

| | | **Dorsal Pons** | | **Ventral Striatum** | | **Ventral Midbrain** | | **Medial PFC** | |
|---|---|---|---|---|---|---|---|---|---|
| **NT** | **Group** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** |
| NE | Sal/No | 9.89 | 0.97 | 8.67 | 0.46 | 11.00 | 1.08 | 6.01 | 0.15 |
| | CFA/No | 9.78 | 1.01 | 9.23 | 0.58 | 11.68 | 0.37 | 5.72 | 0.17 |
| | Sal/FSS | 10.03 | 0.41 | 7.59 | 0.67 | 10.35 | 0.96 | **[3]6.45** | **0.27** |
| | CFA/FSS | 8.79 | 0.56 | **[#]11.00** | **1.74** | 11.12 | 1.16 | **[4]6.48** | **0.48** |
| DOPAC | Sal/No | 0.21 | 0.02 | 17.62 | 1.14 | 1.04 | 0.13 | 0.35 | 0.04 |
| | CFA/No | 0.18 | 0.02 | 20.35 | 0.23 | 0.86 | 0.08 | 0.47 | 0.06 |
| | Sal/FSS | 0.20 | 0.02 | 20.45 | 209 | 1.05 | 0.10 | **[5]0.55** | **0.09** |
| | CFA/FSS | 0.20 | 0.01 | 17.88 | 1.27 | 0.91 | 0.09 | 0.51 | 0.65 |
| DA | Sal/No | 0.49 | 0.06 | 33.22 | 1.61 | 5.39 | 0.53 | 1.44 | 0.13 |
| | CFA/No | 0.38 | 0.02 | 35.38 | 1.10 | **[#]4.60** | **0.23** | 1.45 | 0.05 |
| | Sal/FSS | 0.47 | 0.06 | **[2]37.83** | **2.07** | 5.76 | 0.46 | 1.74 | 0.24 |
| | CFA/FSS | 0.41 | 0.03 | **[#]30.53** | **0.73** | **[#]4.28** | **0.23** | 1.78 | 0.08 |
| 5HIAA | Sal/No | 10.93 | 1.60 | 8.83 | 0.52 | 20.78 | 4.35 | 5.52 | 0.80 |
| | CFA/No | **[1]14.70** | **1.75** | 9.08 | 0.46 | 19.57 | 2.53 | 4.38 | 0.49 |
| | Sal/FSS | 12.73 | 1.34 | 8.25 | 0.29 | 23.07 | 3.67 | **[3]6.37** | **0.53** |
| | CFA/FSS | 11.81 | 1.21 | 9.09 | 0.67 | 21.50 | 4.06 | **[$]6.95** | **1.03** |
| 5HT | Sal/No | 18.00 | 1.75 | 11.05 | 0.50 | 31.69 | 6.06 | 9.21 | 0.59 |
| | CFA/No | 18.70 | 1.75 | 11.30 | 0.83 | 24.60 | 3.20 | 7.65 | 0.09 |
| | Sal/FSS | 17.04 | 0.63 | 11.20 | 11.01 | ****,[$$]28.85** | **4.84** | 9.46 | 0.77 |
| | CFA/FSS | 16.97 | 1.30 | 0.74 | 0.71 | ****,[$$]27.52** | **4.96** | 9.04 | 0.98 |

*Note.* SEM = Standard Error of the Mean, NT = Neurotransmitter, NE = Norepinephrine, DA = Dopamine, 5HT = Serotonin, CFA = Complete Freund's Adjuvant, Sal = Saline, No = No Stress, FSS = Forced swim stress. Mean values are pg/ug protein. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared to Sal/No, $^{\$}p \leq 0.05$, $^{\$\$}p \leq 0.01$ compared to CFA/No, $^{\#}p \leq 0.05$ compared to Sal/FSS, $^{1}p$=0.08 compared to Sal/No, $^{2}p$=0.09 compared to Sal/No, $^{3}p$ = 0.10 compared to CFA/No, $^{4}p$ = 0.08 compared to CFA/No, $^{5}p$ = 0.06 compared to Sal/No.

Results showed that neither stress, $F(1, 28) = 0.30$, $p = 0.59$, nor inflammation, $F(1, 28) = 0.7$, $p = 0.40$, had an effect on norepinephrine levels in the dorsal pons. DOPAC levels in the dorsal pons were also not effected by stress, $F(1, 28) = 0.28$, $p = 0.60$, or inflammation, $F(1, 28) = 1.00$, $p = 0.32$. There was no effect of stress on dopamine levels in the dorsal pons, $F(1, 28) = 0.01$, $p = 0.93$; however, there was a trend towards a main effect of inflammation, $F(1, 28) = 3.30$, $p = 0.08$; CFA reduced dopamine in the dorsal pons. There was no effect of stress on 5HIAA, $F(1, 28) = 0.13$, $p = 0.72$, or 5HT, $F(1, 26) = 0.77$, $p = 0.39$, in the dorsal pons. There was also no effect of inflammation on 5HIAA, $F(1, 28) = 0.92$, $p = 0.35$, or 5HT, $F(1, 26) = 0.04$, $p = 0.84$.

Two-way (inflammation x stress) ANOVAs were performed on monoamine levels in the medial prefrontal cortex nine days after intraplantar injections in vehicle-treated rats (Table 3.2). There were trends toward main effects of stress on norepinephrine levels, $F(1, 26) = 4.00$, $p = 0.06$, DOPAC levels, $F(1, 26) = 2.90$, $p = 0.10$, and dopamine levels, $F(1, 24) = 4.00$, $p = 0.06$, in the medial prefrontal cortex, with stress increasing levels. There was no effect of inflammation on norepinephrine, $F(1, 28) = 0.20$, $p = 0.66$, DOPAC, $F(1, 26) = 0.42$, $p = 0.52$, or dopamine, $F(1, 24) = 0.03$, $p = 0.86$, levels. Stress significantly increased 5HIAA levels, $F(1, 26) = 4.70$, $p = 0.04$, in the medial prefrontal cortex, but there was no effect of inflammation on 5HIAA, $F(1, 26) = 0.13$, $p = 0.72$. Serotonin levels in the medial prefrontal cortex were not affected by stress, $F(1, 26) = 1.20$, $p = 0.28$, or inflammation, $F(1, 26) = 1.80$, $p = 0.20$.

**Serum corticosterone**

A two-way (drug x inflammation) ANOVA was performed on serum corticosterone levels nine days after intraplantar injections in non-stressed rats (Figure 3.9). Results showed that there was no effect of drug, $F(1, 19) = 0.31$, $p = 0.58$, or inflammation, $F(1, 19) = 0.85$, $p = 0.37$, on serum corticosterone levels of non-stressed rats.

A two-way (inflammation x stress) ANOVA was performed on serum corticosterone levels nine days after intraplantar injections and one day after stress-exposure (Figure 3.9). Results showed a significant interaction between stress and inflammation, $F(1, 27) = 4.20$, $p = 0.05$. Stress increased serum corticosterone levels compared to controls in FssSal/Veh rats ($p = 0.04$), but not FssCFA/Veh rats ($p = 0.92$).

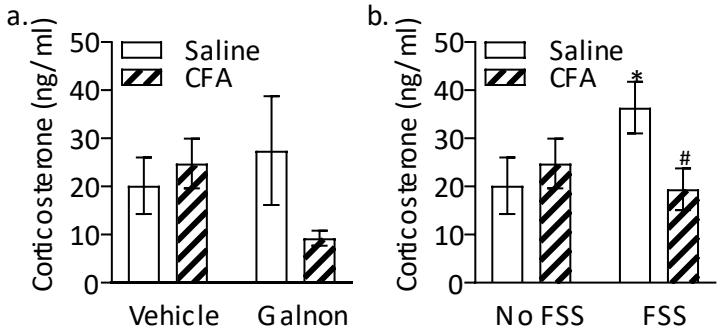


**Figure 3.9.** Effects of intraplantar administration of complete Freund's adjuvant (CFA) or saline on serum corticosterone levels after chronic treatment with galnon or saline for 9 days in non-stressed rats (a) and effects of CFA and forced swim stress (FSS) on serum corticosterone in vehicle-treated rats (b). $^{*}p \leq 0.05$ compared to Saline/No FSS, $^{\#}p \leq 0.05$ compared to Saline/FSS. Error bars represent SEM.

## Discussion

We showed that chronic inflammatory pain induced by CFA was sufficient to induce depression-related behaviors and suppress mesolimbic dopamine and that the effects were independent of acute stress. Galnon did not consistently reverse the effects of CFA, thus our results do not provide strong support for a therapeutic role for galnon in treating comorbid pain and depression.

Chronic inflammatory pain reduced sucrose consumption at one and seven days after injections and increased immobility time during forced swim stress eight days after injections. These data are consistent with other studies which showed that induction of inflammatory pain with CFA was sufficient to cause a pro-depressive phenotype (Borges et al., 2014; Gregoire et al., 2014; Hipolito et al., 2015; Leitl et al., 2014; Okun et al., 2016; Parent et al., 2012; Shi et al.,

2010; Su et al., 2015; G. F. Zhang et al., 2016). Furthermore, these data are consistent with a broader body of literature showing that other manipulations that induce acute and chronic pain, induce a depression-like phenotype (Leitl et al., 2014; Miller, Leitl, Banks, Blough, & Negus, 2015; Pozzi, Acconcia, Ceglia, Invernizzi, & Samanin, 2002).

CFA administration was also sufficient to reduce dopamine levels in the ventral midbrain. The reduction in dopamine induced by chronic inflammatory pain was not affected by galnon or stress. These results are consistent with other research showing that the dopamine system is altered after CFA administration (Cano et al., 2001; Hipolito et al., 2015), as well as, after other manipulations that induce acute or chronic pain (Miller et al., 2015; Sagheddu et al., 2015; Winland, Welsh, Sepulveda-Rodriguez, Vicini, & Maguire-Zeiss, 2017). These data support the hypothesis that dysregulation of the mesolimbic dopamine system accounts for at least some depression-like behavioral changes induced by chronic inflammatory pain; however, further research is needed to determine the mechanism.

The convergence of several lines of evidence suggests that mesolimbic dysregulation could stem from hyperactivity of the noradrenergic neurons in the locus coeruleus in response to chronic inflammatory pain. It is well-accepted that the locus coeruleus is engaged during painful stimuli and modulates both nociceptive- and affective-components of pain (Llorca-Torralba, Borges, Neto, Mico, & Berrocoso, 2016). Furthermore, chronic pain has been shown to induce neuroplastic changes to locus coeruleus activity (Alba-Delgado et al., 2013; Suto, Eisenach, & Hayashida, 2014). Also, the locus coeruleus is known to modulate dopaminergic neuron activity in the ventral tegmental area (Grenhoff, Nisell, Ferre, Aston-Jones, & Svensson, 1993). Thus, sustained activity in the locus coeruleus during chronic inflammatory pain may lead to downstream compensatory changes to catecholamine signaling in the ventral tegmental area and

nucleus accumbens. Notably, we failed to show changes to norepinephrine levels in the dorsal pons, ventral striatum, and ventral midbrain after nine days of inflammatory pain. However, NE levels were elevated in CFA-treated rats exposed to stress, which is consistent with previous reports of neuroadaptations in noradrenergic systems in response to chronic inflammatory pain. Nonetheless, the lack of changes in noradrenergic measures in non-stressed, CFA-treated rats was unexpected. It is possible that more time is necessary before changes to adrenergic signaling are evident (Alba-Delgado et al., 2013), however, there are several other possible explanations for our null results. One possibility is that firing patterns of locus coeruleus neurons and levels of extracellular norepinephrine could have been affected without changes to intracellular norepinephrine stores. Alternatively, changes in ventral midbrain adrenergic receptor expression or sensitivity could mediate effects of chronic pain on mesolimbic dysregulation. Finally, changes in expression or function of co-transmitters in locus coeruleus projections, such as galanin (Mitsi et al., 2015; Schwartz et al., 2014), could mediate effects of CFA on mesolimbic dysfunction. Future studies should further address a possible role of locus coeruleus projections in mediating mesolimbic dysfunction after chronic inflammatory pain.

The effects of chronic inflammatory pain on anxiety-like behavior were less clear. CFA administration had no effect on center time or perimeter time in the open field. The effects of CFA in the open field were not influenced by stress. However, there was a trend towards an increase in open arm time on the elevated plus maze after CFA administration for stressed and non-stressed animals. Our stress manipulation alone also increased open arm time in the elevated plus maze. “Stress inoculation” theories posit that prior exposure to a mild to moderate stressor can increase resilience to subsequent stress (Meichenbaum & Novaco, 1985). It is possible that a single exposure to forced swim stress 24 hours before testing served this function. Alternatively,

it is possible that the closed arm of our apparatus was more aversive than the open arm due pain induced by rearing, which commonly occurs during exploration of the closed arm. Therefore, our results could indicate that stress and CFA-exposure increased aversion-avoidance; however, it is unclear whether chronic inflammatory pain effects aversion learning (LaBuda & Fuchs, 2000; Moriarty, Roche, McGuire, & Finn, 2012; Refsgaard et al., 2016; Y. Wu et al., 2017). Nevertheless, these data are in contrast to a body of literature that shows anxiogenic effects of CFA. CFA administration has been shown to increase anxiety-like behaviors on the elevated plus and zero mazes (Borges et al., 2014; Gregoire et al., 2014; Parent et al., 2012; Refsgaard et al., 2016; Y. Wu et al., 2017), in the marble burying paradigm (Borges et al., 2014), and in the open field (Gregoire et al., 2014; Parent et al., 2012; Stein, Millan, & Herz, 1988). However, the effects of CFA on anxiety-related behaviors seem to be dependent on the time elapsed between administration and testing (Y. Wu et al., 2017), which may also explain our discrepant findings.

Acute stress exposure did not alter the effects of CFA, but did exert its own effects on brain monoamines and serum corticosterone. Exposure to forced swim stress increased serotonin levels in the midbrain and 5HIAA levels in the medial prefrontal cortex. Acute stress also increased norepinephrine, DOPAC, and dopamine levels in the medial prefrontal cortex 24 hours later, although the effects were statistically insignificant. These data are consistent with other research showing that acute stress, such as forced swim stress, restraint stress and foot-shock stress, are sufficient to increase monoamine levels in the medial prefrontal cortex (Ebner & Singewald, 2007; Sciolino et al., 2015; Swanson, Perry, & Schoepp, 2004) and midbrain (Emoto et al., 1993). CFA attenuated the increase in dopamine and DOPAC in the ventral striatum and blunted the increase in serum corticosterone induced by stress. There were no other interactions between stress and chronic inflammatory pain on behavior or brain monoamines. This suggests

that chronic inflammatory pain induces an aberrant response to stress, which may contribute to the comorbidity of pain and depression. Indeed, evidence shows that there is reciprocal modulation between inflammation and neuroendocrine systems (Horowitz, Zunszain, Anacker, Musaelyan, & Pariante, 2013; Raison & Miller, 2013) and that dysregulation of the stress-response increases vulnerability for depression (Horowitz et al., 2013). Further research on how the neuroendocrine system and inflammation interact to increase susceptibility for depression is needed to determine if exposure to chronic or severe stress increases vulnerability for comorbid depression in populations with chronic inflammatory pain.

The therapeutic potential of galnon for treating comorbid chronic inflammatory pain and depression remains unclear. Acute, but not chronic, administration of galnon reduced sucrose consumption in animals treated with saline. This suggests that acute galnon administration may have a pro-depressant effect on rodents, but that tolerance develops within seven days. Galnon administration did not influence the effects of CFA on sucrose consumption. However, chronic galnon did attenuate the effects of CFA on immobility time during forced swim stress. Consistent with an anxiolytic role for the galanin system (Weinshenker & Holmes, 2016), chronic galnon administration increased center time and reduced perimeter time in the open field in saline-treated animals, compared to controls. The anxiolytic effect of galnon was attenuated by CFA administration. Furthermore, galnon administration increased dopamine levels in the ventral striatum. This effect was also blunted by CFA administration. In addition, when administered together, but not alone, galnon and CFA reduced norepinephrine in the ventral midbrain. Like CFA, galnon also reduced DOPAC and serotonin levels in ventral midbrain. Rather than supporting a potential therapeutic role for galnon, these data suggest that the presence of a condition characterized by chronic inflammatory pain may decrease the efficacy of

galnon and other drugs with anti-anxiety or anti-depressant effects in treating symptoms of mood disorders. In support of this interpretation, depressed patients with moderate to severe pain were much more likely to show poor response to SSRI treatment three months later, than depressed patients without pain (Bair et al., 2004). Additional research to identify better therapeutic strategies to treat co-morbid depression and chronic inflammatory pain is still needed. Furthermore, these data also suggest that the galanin system could be involved in the effects of CFA on the mesolimbic dopamine system, therefore future research should evaluate whether endogenous galanin mediates the neurochemical effects of CFA.

This research was an investigation of the behavioral and neurochemical effects of chronic inflammatory pain. We investigated whether the effects of chronic inflammatory pain would be exacerbated by stress and/or attenuated by daily treatment with galnon. CFA administration was sufficient to induce pro-depressive-, but not anxiogenic-like behavior, and suppression of the mesolimbic dopamine system. The effects were not influenced by stress and results do not provide strong support for a therapeutic role for galnon. Further research is needed to evaluate the mechanisms underlying the effects of chronic inflammatory pain on suppression of the mesolimbic dopamine system in order to identify novel therapeutic drug targets to treat comorbid inflammatory pain and depression.

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# CHAPTER 4

# CHRONIC INFLAMMATORY PAIN ALTERS NOREPINEPHRINE AND GALANIN IN THE VTA AND DOWNREGULATES MESOLIMBIC DOPAMINE RELEASE[3]

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**Abstract**

There is a high prevalence of comorbid depression in people with disorders characterized by chronic inflammatory pain. Evidence suggests that dysregulation of the mesolimbic dopamine system may link the two disorders; however, the neural mechanisms underlying this relationship are unclear. We investigated the effects of chronic inflammatory pain induced by intraplantar complete Freund's adjuvant (CFA) injection on dopamine release in the nucleus accumbens of male Sprague-Dawley rats using microdialysis. Furthermore, we investigated the effects of CFA on total tissue concentrations of monoamines in the ventral tegmental area (VTA) and several upstream and downstream projections, including the locus coeruleus (LC) and A2 nucleus, using HPLC. We also measured galanin protein levels in the nucleus accumbens, VTA, and LC via immunoassay. CFA administration decreased extracellular dopamine levels in the nucleus accumbens, total norepinephrine levels in the VTA, and galanin protein levels in the VTA nine days later. Our results suggest that altered neurotransmission from noradrenergic inputs to the VTA and dysregulated galanin signaling in the VTA could account for downregulation of the mesolimbic dopamine system during chronic inflammatory pain.

## Introduction

There is a high prevalence of comorbid depression in people with disorders characterized by chronic inflammatory pain, such as rheumatoid arthritis (Bair, Robinson, Katon, & Kroenke, 2003). An emerging body of evidence suggests that chronic pain induces dysregulation of the mesolimbic dopamine system, which impairs motivated behavior, a hallmark of depression (Mitsi & Zachariou, 2016; Taylor, Becker, Schweinhardt, & Cahill, 2016). However, the neural events linking chronic pain to mesolimbic dysregulation remain unclear.

Intraplantar injection of complete Freund's adjuvant (CFA) is a rodent model of chronic inflammatory pain (Ren & Dubner, 1999). In addition to sustained mechanical and thermal hyperalgesia, CFA induces transient sickness behavior followed by a prolonged depression-related phenotype (Borges, Neto, Mico, & Berrocoso, 2014; Hipolito et al., 2015; Leitl et al., 2014; Pankova, Popkova, Vetrile, Basharova, & Krupina, 2001; Parent et al., 2012; Refsgaard, Hoffmann-Petersen, Sahlholt, Pickering, & Andreasen, 2016; Schwartz et al., 2014; Shi, Wang, & Luo, 2010; Stein, Millan, & Herz, 1988). Thus, CFA administration can be used to study the neurological underpinnings of comorbid pain and depression. For example, our lab and other labs have shown that CFA administration induces dysregulation of the mesolimbic dopamine system.

We previously showed that CFA administration reduced dopamine levels in the ventral midbrain nine days later (Chapter 3). Cano and colleagues (2001) showed that circadian rhythms of dopamine and norepinephrine turnover in the striatum were disrupted 18 days after CFA injection. Other known effects of CFA on the mesolimbic dopamine system and related behaviors include altered excitability of nucleus accumbens medium spiny neurons (Schwartz et al., 2014), decreased intracranial self-stimulation of the medial forebrain bundle (Leitl et al.,

2014), reduced operant responding for food (Schwartz et al., 2014) or drug reward (Hipolito et al., 2015), and blunted disinhibition of VTA neurons and dopamine release in the nucleus accumbens after heroin or DAMGO administration (Hipolito et al., 2015). However, the long-term effects of CFA on dopamine release in the nucleus accumbens are not known. Furthermore, the effects of CFA in the ventral tegmental area (VTA) and regions upstream of the mesolimbic dopamine pathway are not well understood.

Understanding the effects of chronic inflammatory pain on VTA activity will help elucidate the connection between comorbid pain and depression, but few studies have investigated this. One study showed that treatment with LPS led to decreased excitability of VTA dopamine neurons, but mechanism was not investigated (Blednov et al., 2011). Hipolito and colleagues (2015) showed that CFA altered the excitability of dopaminergic neurons and that the effect was mediated by desensitized opioid receptors in the VTA. Although the endogenous opioid system seems to play a role in the effect of inflammatory pain on mesolimbic dysregulation, it is likely that other mechanisms contribute. To our knowledge, no other labs have directly investigated the effects of CFA on VTA activity, but indirect evidence suggests that dysregulation of noradrenergic inputs to the VTA could be involved in the effects of chronic inflammatory pain.

It is well established that norepinephrine modulates dopamine neuron activity in the VTA (El Mansari et al., 2010). The locus coeruleus (LC) accounts for approximately half of the noradrenergic innervation of the VTA (Mejias-Aponte, Drouin, & Aston-Jones, 2009). Stimulation of the LC effects the firing rate of VTA dopamine neurons, dopamine release in the nucleus accumbens, and depression-like behaviors (Grenhoff, Nisell, Ferre, Aston-Jones, & Svensson, 1993; Isingrini et al., 2016; Park, Bhimani, & Park, 2017). Furthermore, the LC is

responsive to and modulates both sensory and affective components of pain (Llorca-Torralba, Borges, Neto, Mico, & Berrocoso, 2016). Therefore, noradrenergic efferents from the LC to the VTA likely play a role in mesolimbic dysregulation after chronic inflammatory pain, however this relationship has not been well-explored.

In addition to the possible effects of norepinephrine from LC projections, release of a co-transmitter could also mediate a relationship between the LC and VTA during chronic inflammatory pain. Approximately 80% of noradrenergic neurons in the LC co-express the neuropeptide galanin (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988; Melander et al., 1986; Tatemoto, Rokaeus, Jornvall, McDonald, & Mutt, 1983). Galanin receptors are expressed in the VTA and nucleus accumbens and galanin has been shown to modulate dopamine activity (Counts et al., 2002; de Weille, Fosset, Schmid-Antomarchi, & Lazdunski, 1989; Ericson & Ahlenius, 1999; Gopalan, Tian, Moore, & Lookingland, 1993; Nordstrom, Melander, Hokfelt, Bartfai, & Goldstein, 1987; Rada, Mark, & Hoebel, 1998; Skofitsch, Sills, & Jacobowitz, 1986; Tsuda, Tsuda, Nishio, Masuyama, & Goldstein, 1998; Weiss, Bonsall, Demetrikopoulos, Emery, & West, 1998). Galanin modulates anxiety-, depression-, and addiction-like behaviors as well as pain at the spinal and supraspinal levels (Lang et al., 2015; Millon et al., 2017; Picciotto, 2008; Weinshenker & Holmes, 2016). Therefore, galanin could also contribute to mesolimbic dysregulation after chronic pain. Indeed, Schwartz and colleagues (2014) showed that galanin signaling in the nucleus accumbens was necessary for the effects of CFA on mesolimbic dopamine dysregulation and motivation deficits (Schwartz et al., 2014). However, the effects of CFA on galanin in the VTA and LC remain unclear.

We investigated the effects of chronic inflammatory pain induced by CFA injection on dopamine release in the nucleus accumbens using microdialysis. Furthermore, we investigated

the effects of CFA on monoamine levels in the VTA and several regions upstream of the mesolimbic dopamine system, including the LC and A2 nucleus, another major noradrenergic nucleus with projections to the VTA. We also measured the effects of CFA on galanin expression in the nucleus accumbens, VTA, and LC. Our results suggest that altered neurotransmission from noradrenergic, serotonergic, and galanin inputs to the VTA could account for downregulation of the mesolimbic dopamine system during chronic inflammatory pain.

## Experimental Methods

### Subjects

Male Sprague-Dawley rats (N = 24; Envigo, Prattville, AL) were obtained at 175 – 200 g and given ad libitum food and water. Rats were housed individually in clear polycarbonate cages (50 x 30 x 30 cm) at 23 ± 3 °C on a 12:12 reverse light:dark cycle. All behavioral testing occurred during the dark cycle. Procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 – 23, revised 2013). Experiments were approved by University of Georgia IACUC.

### General Experimental Protocol

Rats were acclimated to the facility for 6-10 days upon arrival. Following acclimation, rats were implanted with unilateral guide cannula in the nucleus accumbens core as described below. After one week of recovery, rats were injected with saline (n = 12) or CFA (n = 12) to induce chronic inflammatory pain. Microdialysis was performed in conscious animals 9 days after intraplantar injections. Rats were euthanized via rapid decapitation following microdialysis.

**Stereotaxic Surgery**

Rats were anesthetized with isoflurane (1-5%) and unilateral guide cannula (MAB 9.10.IC; SciPro Inc) were stereotaxically implanted into the left nucleus accumbens core (+1.5 mm AP, +1.6 mm ML, and -6.0 mm DV relative to Bregma using Paxinos and Watson, 2007). Cannula were secured using surgical screws and epoxy cement. Rats were administered Meloxicam (1.0 mg/kg) immediately before and 24 hr after surgery.

**Induction of Chronic Inflammatory Pain**

Rats were briefly anesthetized with isoflurane (1-5% in O2) and the left hindpaw was swabbed with 70% ETOH. Rats were then injected with CFA (50 ul; Sigma Aldrich) or saline into the intraplantar region of the left hindpaw. Injections were delivered through a 26G needle over 30 sec. Needles were left in place for an additional 30 sec to minimize backflow. Calipers were used to measure the dorsal-ventral width (mm) of the paw at the injection site immediately before and every day after intraplantar injections for the duration of the experiment. Weights were recorded before paw measurements daily.

**In Vivo Microdialysis**

On the day of microdialysis testing, microdialysis probes (MAP 9.14.2; SciPro Inc) were primed for 10 min by placing them in clean aCSF and perfusing aCSF through them with a syringe pump at a rate of 2 ul/min, as per the manufacturer's instructions. Flow rate was then reduced to 1.5 ul/min and probes were inserted into the guide cannula of rats that were lightly anesthetized with isoflurane (1-5% in O2). The probe extended 2 mm beyond the tip of the guide cannula. Rats were then placed into a clean polycarbonate shoebox cage (50 x 30 x 30 cm) in the testing room for a 2-hour equilibration period. After equilibration, rats were placed into a novel open field chamber (43.3 cm long x 43.4 cm wide x 30.5 cm high; Med Associates, St. Albans,

VT) and allowed to freely explore for 180 min. Rats were injected with the α2 noradrenergic receptor antagonist idazoxan (20 mg/kg, i.p.; Sigma) after 60 min to stimulate noradrenergic activity. Dialysate was collected into 0.5 ml microcentrifuge tubes every 20 min. Tubes were weighed to determine dialysate volume and then 0.1 M perchloric acid was added at a ratio of 1 ul perchloric acid for every 24 ul of dialysate. Samples were placed on dry ice until microdialysis testing concluded and then were stored at -80 C until further processing.

**Tissue, Serum, and Dialysate Processing**

*Tissue collection*

Brains were extracted, frozen on dry ice, and stored at -80 C. Whole brains were sliced into 200 um coronal sections at -20 C. Cannula placement was verified during sectioning by staining 35 um sections with thionin. The following brain structures were then collected: LC, VTA, nucleus accumbens, A2 nucleus, dorsal raphe, paraventricular nucleus of the hypothalamus (PVN), and prelimbic cortex.

*Enzyme immunoassay (EIA) and high performance liquid chromatography (HPLC)*

Prior to analysis with an EIA kit, protein was extracted (Primeaux & Holmes, 2000) from the LC, VTA, and nucleus accumbens. Tissue was homogenized in 0.5 M acetic acid with 2.5% aprotinin. The homogenate was then boiled for 10 min and centrifuged at 4◦C, 3,000 rpm for 30 min. The supernatant was aspirated and the pellet discarded. The acetic acid was then evaporated from the supernatant under vacuum (20,000 mm Hg) at 60C. The samples were reconstituted in EIA buffer. Galanin protein expression was measured in the LC, nucleus accumbens, and VTA using a galanin EIA kit (Peninsula Laboratories S-1208) according to instructions provided.

Tissue from LC, VTA, nucleus accumbens, A2 nucleus, dorsal raphe, PVN, and prelimbic cortex and dialysate collected during microdialysis was analyzed for norepinephrine,

dopamine, DOPAC, serotonin, and 5-HIAA using HPLC with electrochemical detection (HPLC-EC). Tissue was homogenized in 400 ul of KRH buffer. 25 ul of the homogenate was added to 125 ul of 0.05 M perchloric acid. An aliquot of the sample was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). Samples were then centrifuged at 13,000 x g for 7 min at 25 C. The supernatant was injected with the internal standard, dihydroxybenzylamine (DHAB; 0.05 M) into the autoinjector (SIL-20AC) for HPLC analysis. Microdialysis samples were injected with DHBA and 0.05 M perchloric acid. The rest of the HPLC-EC system consisted of a Luna 5 um C18 reverse phase column (250 x 4.6 mm; Phonomenex, Torrance, CA), a CTO-20AC column oven (Shimadzu, Columbia, MD; 37 C), and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase (153.10 mM chloroacetic acid, 1.55 mM octanesulfunic acid, 0.86 mM Ultrapure EDTA, 116.30 mM sodium hydroxide, 46.90 mM acetonitrile, 1.20 mM tetrahydrofuran) was set to 1.8 ml/min using an LC-20AD pump (Shimadzu, Columbia, MD). Neurotransmitter concentration in tissue samples were expressed as pg/ug of protein.

**Statistical Analysis**

Statistics were performed using GraphPad Prism 7.03 software. Outliers were removed using the ROUT method. Furthermore, two subjects did not undergo microdialysis testing due to clogged microdialysis probes and so were not included in the dialysate analyses. Three other subjects are not included in the dialysate analyses due to HPLC equipment malfunction. Unless otherwise stated, independent t-tests were performed on all data to determine the effects of intraplantar CFA administration compared to saline administration. One-tailed tests were used when appropriate where indicated.

## Results

### Effects of Chronic Inflammatory Pain on Weight and Swelling

A two-way (day x inflammation) repeated measures ANOVA was performed to determine the effects of CFA administration on weight over the 10-day experiment (Figure 4.1). There was a significant interaction between day and inflammation, $F(9, 198) = 3.30$, $p < 0.001$. Post-hoc analysis revealed that animals injected with CFA weighed significantly less than saline-treated animals one, $p = 0.03$, and two, $p = 0.05$, days after CFA administration, but were no different on any other day.

A two-way (day x inflammation) repeated measures ANOVA was performed to determine the effects of CFA administration on paw swelling over the 10-day experiment (Figure 4.1). There was a significant interaction between day and inflammation, $F(9, 198) = 31.0$, $p < 0.001$. Post-hoc analyses revealed that there was no difference in baseline paw width, $p = 0.46$, but that CFA-treated animals had significantly larger paw widths that saline-treated animals every day after CFA administration, $p < 0.0001$.

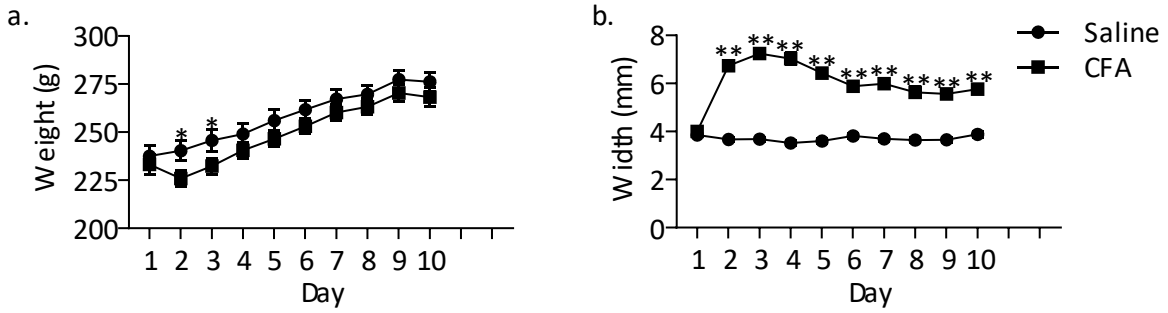


**Figure 4.1.** Effects of chronic inflammatory pain induced by intraplantar administration of compete Freund's adjuvant (CFA) on body weight (a) and paw width (b), compared to intraplantar saline administration. *p < 0.05, **p < 0.001. Error bars represent standard error of the mean.

**Effects of Chronic Inflammatory Pain on Brain Monoamines**

Two-way (inflammation x time) repeated measures ANOVAs were performed to determine whether idazoxan injections had any effects on extracellular norepinephrine, dopamine, DOPAC, or 5HIAA levels (data not shown) in the dialysate. There was no main effect of time on extracellular norepinephrine, $F(9, 108) = 0.79$, $p = 0.63$, dopamine, $F(9, 117) = 0.36$, $p = 0.95$, or 5HIAA levels, $F(9, 117) = 13$, $p = 0.23$, indicating that idazoxan did not affect extracellular levels. There was a main effect of time for extracellular DOPAC levels, $F(9, 126) = 2.2$, $p = 0.02$, with extracellular DOPAC levels decreasing over the course of testing. Post-hoc analyses were performed to evaluate differences in DOPAC levels between time points within each treatment group. Post-hoc analyses show that DOPAC levels were decreased in saline-treated animals during the last hour of testing compared to the first hour of testing. There were no such differences for CFA-treated animals. Since the changes in DOPAC were not observed until at least one hour following idazoxan injections, it is unlikely that these differences are due to this treatment, though it cannot be conclusively ruled out.

To assess the effects of CFA administration compared to saline administration on extracellular monoamine levels in the nucleus accumbens, dialysis samples were collected every 20 min during 180 min of open field exploration. HPLC was used to measure extracellular monoamine content in each sample for each rat. Average values for the entire microdialysis testing period were then calculated for each rat and independent t-tests were performed to assess the effects of CFA administration compared to saline administration on average extracellular monoamine levels (Figure 4.2). CFA administration reduced extracellular levels of DOPAC, $t(14) = 3.8$, $p < 0.001$, dopamine, $t(13) = 3.36$, $p = 0.003$, and 5HIAA, $t(13) = 3.09$, $p = 0.004$ in the nucleus accumbens, compared to saline administration. CFA administration had no effect on

norepinephrine levels in the nucleus accumbens, t(12) = 0.44, $p$ = 0.335, compared to saline administration. We were unable to measure serotonin levels in dialysate.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamines in the VTA (Figure 4.3). CFA administration significantly reduced norepinephrine, t(19) = 1.79, $p$ = 0.04 (one-tailed), and 5HIAA, t(21) = 2.25, $p$ = 0.04,  levels, in the VTA nine days later. CFA administration had no effect on DOPAC, t(19) = 0.32 (one-tailed), $p$ = 0.37, dopamine, t(22) = 1.08, $p$ = 0.15 (one-tailed), or serotonin, t(22) = 0.30, $p$ = 0.77, levels in the VTA.

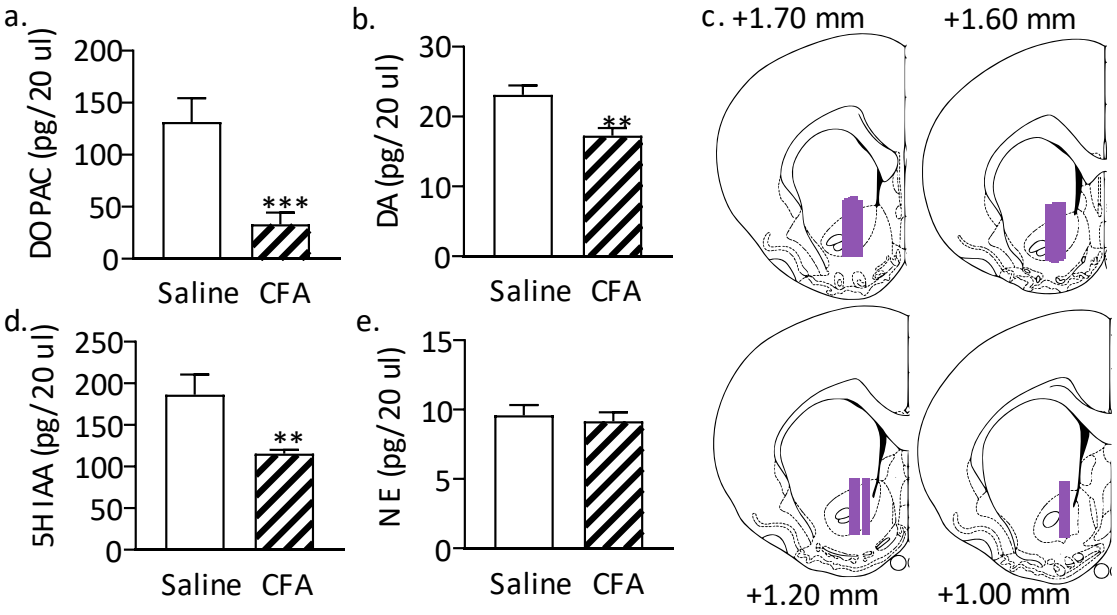


**Figure 4.2.** Chronic inflammatory pain reduced extracellular DOPAC (a), dopamine (b), and 5HIAA (d) levels in the nucleus accumbens core during microdialysis testing 9 days after intraplantar complete Freund's adjuvant (CFA) injection, compared to intraplantar saline. CFA had no effect on norepinephrine (e) levels. Probe placement in the nucleus accumbens core is shown (c). **p < 0.01, ***p < 0.001. Error bars represent standard error of the mean.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamine levels in the nucleus

accumbens (Figure 4.3). CFA administration significantly reduced DOPAC levels in the nucleus accumbens nine days later, t(20) = 1.90, $p = 0.04$ (one-tailed). CFA administration had no effect on norepinephrine, t(20) = 0.74, $p = 0.23$ (one-tailed), dopamine, t(22) = 0.90 (one-tailed), $p = 0.19$, 5HIAA, t(21) = 0.38, $p = 0.71$, or serotonin, t(16) = 0.84, $p = 0.41$, levels in the nucleus accumbens nine days after administration.

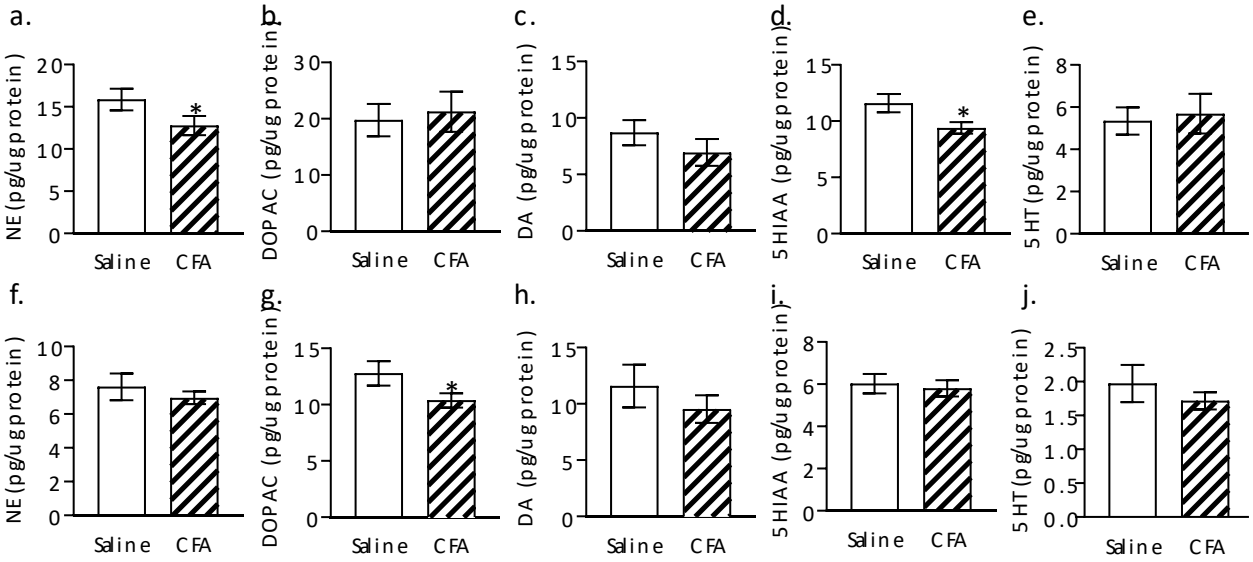


**Figure 4.3.** The effects of chronic inflammatory pain induced by intraplantar injection of complete Freund's adjuvant (CFA) on monoamine levels and their metabolites in the VTA (VTA; a-e) and nucleus accumbens (NAc; f-j). *p ≤ 0.05. Error bars represent standard error of the mean.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamine levels in the LC (Table 4.1). CFA administration had no effect on norepinephrine, t(19) = 0.06, $p = 0.48$, DOPAC, t(20) = 0.59, $p = 0.56$, dopamine, t(18) = 0.86, $p = 0.40$, or 5HIAA, t(17) = 1.35, $p = 0.19$, levels in the LC nine days later. There was a trend towards an increase in serotonin levels in the LC nine days later, t(17) = 1.96, $p = 0.07$.

A2 tissue punches were only collected from a subset of animals (saline: n = 8, CFA: n = 7). DOPAC was not detectable for three animals, dopamine was not detectable for two animals, and 5HIAA and serotonin were not detectable for one animal. Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamine levels in the A2 noradrenergic nucleus (Table 4.1). CFA administration reduced norepinephrine, $t(13) = 1.96$, $p = 0.07$, and 5HIAA, $t(11) = 1.81$, $p = 0.10$, levels in A2, although the effects were not statistically significant. CFA administration had no effect on DOPAC, $t(9) = 0.89$, $p = 0.40$, dopamine, $t(11) = 0.60$, $p = 0.56$, or serotonin, $t(12) = 1.05$, $p = 0.31$, levels in A2 nine days after administration.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentration levels of monoamine levels in the dorsal raphe (Table 4.1). CFA administration had no effect on norepinephrine $t(20) = 0.75$, $p = 0.49$, DOPAC $t(19) = 1.72$, $p = 0.10$, dopamine $t(18) = 1.01$, $p = 0.33$, 5HIAA, $t(18) = 1.64$, $p = 0.12$, or serotonin, $t(19) = 0.09$, $p = 0.93$, levels in the dorsal raphe nine days later.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamine levels in the prelimbic cortex (Table 4.1). CFA administration had no effect on norepinephrine, $t(16) = 0.08$, $p = 0.47$, DOPAC, $t(18) = 0.12$, $p = 0.45$, dopamine, $t(15) = 0.27$, $p = 0.40$, or serotonin, $t(17) = 1.25$, $p = 0.11$, levels in the prelimbic cortex nine days later. There was a trend towards an increase in 5HIAA levels in the prelimbic cortex nine days later, $t(18) = 1.35$, $p = 0.10$.

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on total tissue concentrations of monoamine levels in the PVN (Table 4.1). CFA administration had no effect on norepinephrine $t(21) = 1.02$, $p = 0.32$, 5HIAA, $t(21) =$

**Table 4.1**

Effects of Chronic Inflammatory Pain on Brain Monoamine Levels

| | | NE | | DOPAC | | DA | | 5HIAA | | 5HT | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Region** | **Group** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** |
| VTA | Saline | 15.88 | 1.29 | 19.75 | 2.86 | 8.70 | 1.11 | 11.60 | 0.81 | 5.35 | 0.65 |
| | CFA | ***12.77** | **1.14** | 21.25 | 3.56 | 6.95 | 1.19 | ***9.40** | **0.51** | 5.69 | 0.94 |
| NAc | Saline | 7.62 | 0.79 | 12.78 | 1.09 | 11.58 | 1.90 | 6.03 | 0.46 | 1.97 | 0.27 |
| | CFA | 6.97 | 0.38 | ***10.38** | **0.63** | 9.54 | 1.22 | 5.80 | 0.39 | 1.72 | 0.13 |
| LC | Saline | 17.80 | 1.54 | 3.84 | 0.55 | 1.39 | 0.20 | 8.88 | 0.59 | 2.52 | 0.27 |
| | CFA | 17.94 | 1.72 | 4.27 | 0.46 | 1.19 | 0.13 | 10.24 | 0.79 | [1]3.42 | 0.34 |
| A2 | Saline | 29.71 | 6.53 | 2.59 | 0.73 | 2.89 | 0.81 | 6.77 | 0.77 | 5.18 | 0.98 |
| | CFA | [1]14.99 | 2.91 | 1.96 | 0.26 | 2.30 | 0.47 | [2]5.16 | 0.33 | 3.96 | 0.61 |
| DR | Saline | 24.71 | 2.72 | 3.27 | 0.34 | 18.43 | 1.90 | 14.64 | 1.05 | 3.30 | 0.49 |
| | CFA | 21.97 | 2.28 | 2.56 | 0.21 | 21.36 | 2.18 | 12.52 | 0.63 | 3.36 | 0.34 |
| PL | Saline | 6.64 | 0.39 | 1.91 | 0.42 | 1.10 | 0.14 | 9.74 | 0.56 | 5.37 | 1.32 |
| | CFA | 6.60 | 0.28 | 1.97 | 0.34 | 1.06 | 0.04 | [2]11.53 | 1.10 | 3.46 | 0.84 |
| PVN | Saline | 34.03 | 2.26 | 2.84 | 0.23 | 2.42 | 0.25 | 5.26 | 0.33 | 1.80 | 0.13 |
| | CFA | 30.53 | 2.54 | ****2.13** | **0.14** | ****1.60** | **0.14** | 5.02 | 0.23 | 1.49 | 0.18 |

*Note.* SEM = Standard Error of the Mean, NE = Norepinephrine, DA = Dopamine, 5HT = Serotonin, CFA = Complete Freund's Adjuvant, VTA = Ventral Tegmental Area, NAc = Nucleus Accumbens, LC = Locus Coeruleus, PL = Prelimbic Cortex, PVN = Paraventricular Nucleus, DR = Dorsal Raphe. *$p \leq 0.05$, [1]$p = 0.07$, [2]$p = 0.10$

0.61, $p = 0.55$, or serotonin, t(19) = 1.34, $p = 0.19$, levels in the PVN nine days later. CFA administration significantly reduced DOPAC, t(20) = 2.67, $p = 0.01$, and dopamine, t(21) = 2.94, $p = 0.01$, levels in the PVN nine days later.

**Effects of Chronic Inflammatory Pain on Galanin**

Independent t-tests were performed to assess the effects of CFA administration compared to saline administration on galanin protein levels in the LC, VTA, and nucleus accumbens nine days later (Figure 4.4). CFA administration significantly reduced galanin levels in the VTA, t(11) = 2.51, $p = 0.01$, but had no effect on galanin levels in the LC, t(12) = 0.52, $p = 0.31$, or the nucleus accumbens, t(10) = 0.20, $p = 0.42$.

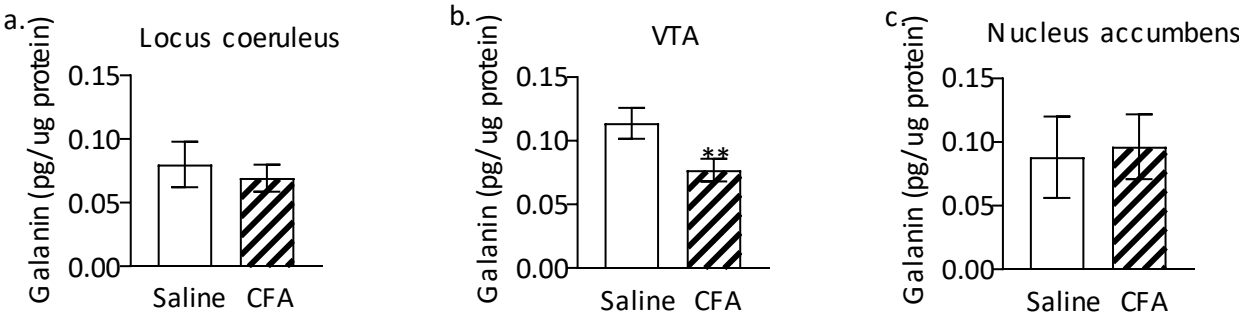


**Figure 4.4.** Chronic inflammatory pain reduced galanin protein measured by EIA in the VTA (b), but not the locus coeruleus (a), or nucleus accumbens (c). **p ≤ 0.01. Standard error bars represent SEM.

## Discussion

Chronic inflammatory pain induced dysregulation of the mesolimbic dopamine system, which is consistent with a growing body of literature implicating this neural pathway in comorbid pain and depression. Our results suggest that dysregulation of VTA dopamine neuron activity may stem from altered neurotransmission from noradrenergic and serotonergic inputs to the VTA. They also implicate dysregulated galanin signaling in the VTA in the neurobiology of comorbid pain and depression.

We showed that CFA administration decreased extracellular dopamine and DOPAC levels in the nucleus accumbens core nine days later during microdialysis testing. CFA administration also decreased whole tissue levels of DOPAC, but not dopamine, levels in the nucleus accumbens, suggesting that dopamine metabolism is reduced. Furthermore, dopamine and DOPAC levels in the VTA of CFA-treated animals were no different from those of saline-treated animals. Our results suggest that dopamine release, rather than synthesis, is decreased in the nucleus accumbens core after chronic inflammatory pain. Likewise, we showed decreased extracellular 5HIAA in the nucleus accumbens during microdialysis testing, but no change in total 5HIAA or serotonin in the nucleus accumbens or serotonin in the VTA, suggesting that serotonin release in the nucleus accumbens is also altered after chronic inflammatory pain. Our data are consistent with other evidence showing that dysregulation of the mesolimbic dopamine system is involved in the comorbidity of pain and depression (Mitsi & Zachariou, 2016; Schwartz et al., 2014); however, the cascade of events between inflammatory pain and mesolimbic dysregulation remain unclear.

Norepinephrine levels were reduced in the VTA, the major source of dopaminergic input to the nucleus accumbens (Fallon & Moore, 1978). This suggests that alterations to noradrenergic activity in the VTA could mediate the effects of CFA on dopamine release in the nucleus accumbens. However, it is unclear whether chronic inflammatory pain caused downregulation of norepinephrine pools in VTA projections or if levels were depleted due to increased activity of noradrenergic neurons during microdialysis testing. Research on the chronic effects of inflammatory pain on neurotransmission within the VTA is scant; however, one study showed LPS injections increased norepinephrine release in the VTA 90 minutes later (Sekio & Seki, 2014). Other studies have shown that treatment with LPS or CFA leads to decreased

excitability of VTA dopamine neurons (Blednov et al., 2011; Hipolito et al., 2015). This suggests that increased activity in VTA noradrenergic inputs in early stages of chronic pain could lead to dysregulation of the mesolimbic dopamine system; however, this relationship has not been directly tested. Nevertheless, our data indicate that altered noradrenergic activity in the VTA during chronic inflammatory pain could mediate downregulation of mesolimbic dopamine activity. Further testing is needed to determine whether our results reflect a compensatory downregulation of monoamine levels in VTA inputs or depletion of monoamine content due to increased activity during microdialysis testing.

The LC and A2 nucleus are two major sources of norepinephrine input to the VTA (Mejias-Aponte et al., 2009). Furthermore, both nuclei are responsive to stress, pain, and inflammation (Bonnet et al., 2009; Gaykema, Chen, & Goehler, 2007; Llorca-Torralba et al., 2016; Ressler & Nemeroff, 2001; Rinaman, 2011). Therefore, we measured whether changes in norepinephrine expression in these regions could account for the decreased norepinephrine levels in the VTA. There was no change in norepinephrine levels in the LC; however, there was a trend towards a decrease in norepinephrine levels in the A2 nucleus of CFA-treated animals. Therefore, the effects of CFA on norepinephrine activity may be restricted to projections from the LC and/or A2 to the VTA. Precisely how norepinephrine levels and release from LC, A2, and/or other noradrenergic nuclei projections into the VTA are altered remains unclear.

Although both regions project to the VTA, the LC accounts for a larger proportion of noradrenergic input to the VTA (Mejias-Aponte et al., 2009). Furthermore, the LC has been shown to directly modulate dopaminergic neuron activity. Low frequency stimulation of the LC increases the firing rate of VTA dopamine neurons, while high frequency burst firing of the LC inhibits VTA dopamine neurons (Grenhoff et al., 1993). Consistent with the inhibitory effect of

burst firing of LC neurons on VTA neurons, hyperactivity of norepinephrine neurons in the LC is associated with behavioral suppression and decreased dopamine release in the nucleus accumbens (Isingrini et al., 2016). Notably, the effects of LC hyperactivity on dopamine neurons cannot be explained by norepinephrine (Grenhoff et al., 1993). Thus, at least three non-mutually exclusive explanations could account for the effects of CFA on LC-mediated changes in mesolimbic dopamine activity. (i) CFA administration could lead to decreased noradrenergic tone in the VTA, which would decrease the stimulatory effects of LC activity on dopamine neurons. (ii) Alternatively, CFA could lead to increased burst firing of LC projections to the VTA, leading to inhibition of dopamine neuron activity by the increased release of a co-transmitter. (iii) Finally, increased norepinephrine release in the VTA during inflammatory pain could alter post-synaptic receptor expression or sensitivity, which would decrease the excitatory effects of norepinephrine on dopamine neuron activity. We investigated the merit of the second possible explanation, that the effects of CFA are mediated by a co-transmitter, galanin.

Galanin is colocalized with norepinephrine in the majority of LC neurons, including those which project to the LC (Gundlach, Rutherfurd, & Louis, 1990; Melander et al., 1986; Weiss et al., 1998; Xu, Shi, & Hokfelt, 1998). In addition, galanin is preferentially released during burst firing of LC neurons (Bartfai, Iverfeldt, Fisone, & Serfozo, 1988; Consolo et al., 1994; Karhunen, Vilim, Alexeeva, Weiss, & Church, 2001; Verhage et al., 1991). Therefore, we measured galanin protein expression in the LC, VTA, and nucleus accumbens to determine if changes in galanin expression could account for the downregulation of dopamine activity in the mesolimbic pathway. Galanin protein levels were decreased in the VTA, but not the LC or nucleus accumbens, of CFA-treated animals. These data are in keeping with those of Schwartz and colleagues (2014), further implicating galanin in CFA-induced mesolimbic changes.

Although our results indicate that galanin activity is altered in CFA-treated animals, it is unclear whether galanin protein expression in the VTA was downregulated by chronic inflammatory pain or if more galanin was released during microdialysis testing, and therefore levels were further depleted in CFA-treated animals at the time of euthanasia. Future research should measure basal galanin expression after CFA administration and galanin release in the VTA. In addition, the necessity of galanin for downregulation of the mesolimbic dopamine system should be evaluated.

We also measured monoamine content levels in several other upstream and downstream regions associated with the mesolimbic dopamine system. The dorsal raphe is a major source of serotonin input to the VTA and the nucleus accumbens (McDevitt et al., 2014), therefore we measured monoamine levels in this region. Despite showing decreased levels of the serotonin metabolite, 5HIAA, in the nucleus accumbens and VTA, we did not find any effects of CFA administration on monoamines in the dorsal raphe. This suggests serotonin release is reduced in the nucleus accumbens and VTA, without a concomitant change in synthesis. These data contribute to a mixed body of literature investigating the role of the serotoninergic system in the comorbidity of pain and depression. Some studies have shown that manipulations that induce inflammatory pain decrease serotonin (W. W. Ji et al., 2014; Yeh et al., 2015). Other studies have shown that similar manipulations increase serotonin and serotonin turnover (Gibney, McGuinness, Prendergast, Harkin, & Connor, 2013; O'Connor et al., 2009) or have no effect on serotonin (Gibney et al., 2013). Dantzer and colleagues have extensively investigated the role of the tryptophan metabolism by the kynurenine pathway in inflammation-induced depression. Their findings implicate upregulation of indoleamine 2,3, dioxygenase, the enzyme that metabolizes tryptophan to kynurenine, and quinolinic acid, the major metabolite of tryptophan,

in depression after inflammation (Remus & Dantzer, 2016). However, they have shown the effects of inflammation on this enzyme to be independent of changes to serotonin expression (O'Connor et al., 2009). Region of analysis, difference in inflammatory pain manipulations, and differences in the amount of time elapsed between experimental manipulations and tissue analysis likely explain the differences in effects of inflammatory pain on serotonin. Further research is needed to understand the role of the serotonergic system in the comorbidity of inflammatory pain and depression.

Both the prelimbic cortex and PVN of the hypothalamus receive projections from the VTA, return reciprocal projections to the VTA, and are involved in stress and pain modulation (Beier et al., 2015; Fan et al., 2018; Geisler & Zahm, 2005; Herman, Ostrander, Mueller, & Figueiredo, 2005; Herman & Tasker, 2016; N. N. Ji, Kang, Hua, & Zhang, 2018; Moench & Wellman, 2015; Palkovits, Baffi, & Pacak, 1999). Therefore, we also measured monoamine content in these areas after chronic inflammatory pain. We did not show any effects of chronic inflammatory pain on monoamines in the prelimbic cortex; however, there was a trend towards an increase in 5HIAA levels. We also did not show any effects of chronic inflammatory pain on norepinephrine, serotonin, or 5HIAA in the PVN. We did; however, show a significant decrease in dopamine and DOPAC levels in the PVN in CFA-treated animals. Dopamine activity in the PVN stimulates food intake, mediates sexual behaviors, and induces a surge in oxytocin release that leads to release of dopamine in the nucleus accumbens (Melis, Succu, Mascia, Cortis, & Argiolas, 2003; Mirmohammadsadeghi, Shareghi Brojeni, Haghparast, & Eliassi, 2018; Succu et al., 2007). Therefore, decreased PVN dopamine could in part explain the high prevalence of reduced sexual desire in chronic pain populations (Fine, 2011); however, the effects of chronic inflammatory pain on sexual activity in animal models is unexplored.

To summarize, we measured monoamine and galanin expression in the mesolimbic dopamine pathway, as well as, up- and downstream regions, after induction of chronic inflammatory pain. We showed that chronic inflammatory pain reduced extracellular dopamine in the nucleus accumbens, as well as, total tissue concentration of galanin, norepinephrine, and 5HIAA in the VTA. Our results suggest that dysregulation of VTA dopamine neuron activity may stem from altered norepinephrine, galanin, and serotonin expression or release in the VTA during states of chronic inflammatory pain. Future research is needed to tease apart the precise contributions of each of these neurotransmitters to altered VTA activity in response to chronic inflammatory pain.

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# CHAPTER 5

# EVIDENCE FOR GALANIN MODULATION OF DOPMAINE IN THE HYPOTHALAMUS AND NUCLEUS ACCUMBENS IN A MODEL OF CHRONIC INFLAMMATORY PAIN[4]

[4]Hooversmith, J. M., Lee, M. C., MohanKumar, S. M. J., MohanKumar, P. S., Miller, L. L., & Holmes P. V. To be submitted to Neuropharmacology

## Abstract

The prevalence of depression is higher in patients with rheumatoid arthritis than for the general population. Dysregulation of the mesolimbic dopamine system is implicated in the comorbidity of chronic inflammatory pain and depression. We investigated the necessity of central galanin signaling in mesolimbic dopamine dysregulation in the intraplantar complete Freund's adjuvant (CFA) model of arthritis. Male Sprague Dawley rats (N = 19) received intraplantar injections of saline or CFA. Starting on the day of intraplantar injection, rats received daily intracerebroventricular injections of aCSF or M40 (12 ug in 10 ul), a galanin receptor antagonist, for 9 days. Rats then underwent microdialysis testing and a battery of behavioral tests. We provide evidence that chronic inflammatory pain suppresses mesolimbic dopamine signaling and dopamine activity in the paraventricular nucleus of the hypothalamus. Galanin was necessary for reduced dopamine turnover in the paraventricular nucleus and levels in the nucleus accumbens. However, reduced dopamine release in the nucleus accumbens was not affected by galanin blockade, suggesting that chronic galanin signaling does not affect ventral tegmental area dopamine neuron activity directly.

## Introduction

The prevalence of depression is substantially higher in patients with rheumatoid arthritis than for the general population (16.8% versus 4.1%) (Matcham, Rayner, Steer, & Hotopf, 2013; Waraich, Goldner, Somers, & Hsu, 2004). Rheumatoid arthritis patients with comorbid depression tend to experience worse symptoms, reduced quality of life, and poorer treatment outcomes (Roubille et al., 2015). Developing a better understanding of the neurobiology of the comorbidity between conditions of depression and chronic inflammatory pain is pivotal to producing better treatment options and outcomes.

Ample evidence implicates dysregulation of the mesolimbic dopamine system in the comorbidity of chronic inflammatory pain and depression (Mitsi & Zachariou, 2016; Taylor, Becker, Schweinhardt, & Cahill, 2016). For example, intraplantar administration of complete Freud's adjuvant (CFA), a well-accepted rodent model of monoarthritis, decreases dopamine release in the nucleus accumbens during exploration and total tissue concentration of dopamine in the ventral midbrain (Chapter 3). CFA administration also disrupts circadian rhythms of dopamine turnover in the striatum (Cano et al., 2001) and blunts the effects of opioids on dopamine release in the nucleus accumbens (Hipolito et al., 2015). Excitability of medium spiny neurons in the nucleus accumbens is also altered after CFA administration (Schwartz et al., 2014). Furthermore, CFA administration disrupts behaviors that are largely controlled by the mesolimbic dopamine system including operant responding for food and drug rewards and intracranial self-stimulation (Hipolito et al., 2015; Leitl et al., 2014; Schwartz et al., 2014).

Although a role for the mesolimbic dopamine system in comorbid chronic inflammatory pain and depression is well-established, how chronic inflammatory pain disrupts this system remains unclear. Altered opioid receptor sensitization, upregulation of NF-κB, increased

proinflammatory cytokines, reduced D3 dopamine receptor expression, and increased BDNF levels in the ventral tegmental area (VTA) may explain some effects of chronic inflammatory pain on mesolimbic dysregulation (Hipolito et al., 2015; Wang et al., 2018; S. Zhang et al., 2017). Evidence suggests that altered norepinephrine and galanin activity in the VTA may also mediate the effects of CFA on mesolimbic dopamine activity (Chapter 4). The origin(s) of the dysregulated noradrenergic and galanin VTA afferents remains unknown; however, a likely candidate is the locus coeruleus (LC).

The LC provides approximately half of the noradrenergic inputs to the VTA (Mejias-Aponte, Drouin, & Aston-Jones, 2009). Furthermore, approximately 80% of noradrenergic neurons in the LC co-express galanin (Holets, Hokfelt, Rokaeus, Terenius, & Goldstein, 1988). Low frequency stimulation of the LC increases norepinephrine release onto VTA neurons, increases the firing rate of VTA neurons, and increases subsequent dopamine release in the nucleus accumbens (Grenhoff, Nisell, Ferre, Aston-Jones, & Svensson, 1993; Park, Bhimani, & Park, 2017). In contrast, high frequency stimulation of the LC suppresses VTA dopamine neuron activity, possibly via release of the neuropeptide galanin (Bartfai et al., 1988; Consolo et al., 1994; Grenhoff et al., 1993; Weiss, Bonsall, Demetrikopoulos, Emery, & West, 1998), which has a well-established modulatory effect on catecholamine neurotransmission (de Weille, Fosset, Schmid-Antomarchi, & Lazdunski, 1989; Everitt et al., 1986; Gopalan, Tian, Moore, & Lookingland, 1993; Ma et al., 2001; Nordstrom, Melander, Hokfelt, Bartfai, & Goldstein, 1987; Rada, Mark, & Hoebel, 1998; Xu, Zheng, & Hokfelt, 2005). Furthermore, LC hyperactivity and galanin administration into the VTA increase depression-like behaviors (Simson & Weiss, 1988; Weiss et al., 1998) and both neurotransmitters are implicated in supraspinal pain modulation (Lang et al., 2015; Llorca-Torralba, Borges, Neto, Mico, & Berrocoso, 2016).

Despite evidence of a role for dysregulated LC-derived norepinephrine and galanin signaling in the VTA in mesolimbic dysregulation after CFA, the necessity of these changes remains unknown. We evaluated whether galanin is necessary for the effects of CFA on mesolimbic dopamine transmission, VTA norepinephrine levels, and dopamine-sensitive behaviors. We showed that galanin mediates the effects of CFA on dopamine levels and turnover in the nucleus accumbens and PVN, but not dopamine release in the nucleus accumbens. Our results suggest that galanin's actions during chronic inflammatory pain may be independent of modulation of VTA dopamine neuron activity by LC afferents.

## Experimental Methods

### Subjects

Male Sprague-Dawley rats (N = 19; Envigo, Prattville, AL) were obtained at 175 – 200 g and given ad libitum food and water. Rats were housed individually in clear polycarbonate cages (50 x 30 x 30 cm) at 23 ± 3 °C on a 12:12 reverse light:dark cycle. All behavioral testing occurred during the dark cycle. Procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85 – 23, revised 2013). Experiments were approved by University of Georgia IACUC.

### General Experimental Protocol

Rats were acclimated to the facility for 6-10 days upon arrival. Following acclimation, rats were implanted with unilateral guide cannula in the nucleus accumbens core and the lateral ventricle as described below. After one week of recovery, rats were injected with saline (n = 7) or CFA (n = 12) to induce chronic inflammatory pain. Rats received daily intracerebroventricular (ICV) injections of artificial cerebrospinal fluid (aCSF; n = 14) or M40 (n = 5), a non-specific galanin receptor antagonist, from the day of intraplantar injection through the day of

microdialysis testing 9 days later. Following microdialysis testing rats were tested on the elevated plus maze. Rats were then euthanized via rapid decapitation. Final groups included rats treated with intraplantar saline and ICV aCSF (Saline-aCSF, n = 7), rats treated with intraplantar CFA and ICV aCSF (CFA-aCSF, n = 7), and rats treated with intraplantar CFA and ICV M40 (CFA-M40, n = 5).

**Stereotaxic Surgery**

Rats were anesthetized with isoflurane (1-5%) and unilateral guide cannula (MAB 9.10.IC; SciPro Inc) were stereotaxically implanted into the left nucleus accumbens core (+1.5 mm AP, +1.2 mm ML, -6.0 mm DV) and lateral ventricle (-0.8 mm AP, +1.2 mm ML, -3.0 mm DV) relative to Bregma using Paxinos and Watson, 2007. Cannula were secured using surgical screws and epoxy cement. Rats were administered Meloxicam (1.0 mg/kg) immediately before and 24 hr after surgery.

**Induction of Chronic Inflammatory Pain and Drug Administration**

*CFA injections*

Rats were briefly anesthetized with isoflurane (1-5% in O2) and the left hindpaw was swabbed with 70% ETOH. Rats were then injected with CFA (50 ul; Sigma Aldrich) or saline into the intraplantar region of the left hindpaw. Injections were delivered through a 26G needle over 30 sec. Needles were left in place for an additional 30 sec to minimize backflow. Calipers were used to measure the dorsal-ventral width (mm) of the paw at the injection site immediately before and every day after intraplantar injections for the duration of the experiment. Weights were recorded before paw measurements daily.

*ICV injections*

M40 (Tocris, Minneapolis MN, USA) was dissolved in aCSF (1.2 ug/ul; 6 nmol; Sciolino et al., 2015) and administered into the lateral ventricle at a rate of 1 ul/min daily over the course of 10 min. Control rats received aCSF (10 ul). For ICV injections, rats were placed in small clean shoebox cages with injector needles that extended 1mm beyond the guide cannula inserted into their guide cannulas. Injector needles were attached to tubing filled with either aCSF or M40 solution, which in turn were attached to a Hamilton syringe pump. Once injectors were inserted and the pump was started, rats could freely explore the small cage. The researcher supervised to ensure the rats did not escape the cage, injectors remained in place, and the drug or vehicle was being delivered without backflow. After 10 min, the pump was turned off and the injectors were left in place for an additional minute to minimize backflow. Rats were then returned to their home cages.

**In Vivo Microdialysis**

On the day of microdialysis testing, microdialysis probes (MAP 9.14.2; SciPro Inc) were primed for 10 min by placing them in clean aCSF and perfusing aCSF through them with a syringe pump at a rate of 2 ul/min, as per the manufacturer's instructions. Flow rate was then reduced to 1.5 ul/min and probes were inserted into the guide cannula of rats that were lightly anesthetized with isoflurane (1-5% in O2). The probe extended 2 mm beyond the tip of the guide cannula. Rats were then placed into their home cages with access to food and water in the testing room for a 2-hour equilibration period. After equilibration, rats were placed into a novel open field chamber (43.3 cm long x 43.4 cm wide x 30.5 cm high; Med Associates, St. Albans, VT) and allowed to freely explore for 160 min. Ambulatory distance, center time, and center entries were recorded throughout microdialysis testing. Rats were injected with the α2 noradrenergic receptor antagonist idazoxan (20 mg/kg, i.p.; Sigma) after 60 min to stimulate noradrenergic

activity. Dialysate was collected into 0.5 ml microcentrifuge tubes every 20 min. Tubes were weighed to determine dialysate volume and then 0.1 M perchloric acid was added at a ratio of 1 ul perchloric acid for every 24 ul of dialysate. Samples were placed on dry ice until microdialysis testing concluded and then were stored at -80 C until further processing.

**Behavioral Testing on Elevated Plus Maze**

The elevated plus maze was used to measure anxiety-like behavior 20-30 min after microdialysis testing in the open field. The wooden elevated plus maze is elevated 50cm above the floor and consisted of two opposite open arms (45 cm x 9 cm), two opposite closed arms (45 cm x 9 cm x 38 cm), and a central platform (9 cm x 9 cm). A 15 W bulb placed 1 m above maze illuminated the center platform (15 lx). Each rat was placed in the center of the maze facing an open arm of the maze opposite the experimenter. Behavior was recorded for 5 min. An experimenter blind to group assignment scored open and closed arm time and entries from a video monitor hidden behind a divider during testing.

**Tissue, Serum, and Dialysate Processing**

*Tissue collection*

Brains were extracted, frozen on dry ice, and stored at -80 C. Whole brains were sliced into 200 um coronal sections at -20 C. Cannula placement was verified during sectioning. The following brain structures were then collected: LC, VTA, nucleus accumbens, and paraventricular nucleus of the hypothalamus (PVN).

*High performance liquid chromatography (HPLC)*

Tissue from LC, VTA, nucleus accumbens, and PVN and dialysate collected during microdialysis was analyzed for norepinephrine, dopamine, DOPAC, serotonin, and 5-HIAA using HPLC with electrochemical detection (HPLC-EC). Tissue was homogenized in 180 ul of

PBS buffer. 60 ul of the homogenate was added to 2.4 ul of 0.05 M perchloric acid. An aliquot of the sample was used for protein estimation (MicroBCA assay, Pierce, Rockford, IL). Samples were then centrifuged at 13,000 x g for 7 min at 25 C. The supernatant was injected with the internal standard, dihydroxybenzylamine (DHAB; 0.05 M) into the autoinjector (SIL-20AC) for HPLC analysis. Microdialysis samples were injected with DHBA and 0.05 M perchloric acid. The rest of the HPLC-EC system consisted of a Luna 5 um C18 reverse phase column (250 x 4.6 mm; Phonomenex, Torrance, CA), a CTO-20AC column oven (Shimadzu, Columbia, MD; 37 C), and an LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase (153.10 mM chloroacetic acid, 1.55 mM octanesulfunic acid, 0.86 mM Ultrapure EDTA, 116.30 mM sodium hydroxide, 46.90 mM acetonitrile, 1.20 mM tetrahydrofuran) was set to 1.8 ml/min using an LC-20AD pump (Shimadzu, Columbia, MD). Neurotransmitter concentration in tissue samples were expressed as pg/ug of protein.

*Serum collection and processing*

Trunk blood was collected into serum tubes immediately after rapid decaptitation. After clot formation, samples were centrifuged at 1000 x g for 10 min at 4 °C. Supernatant was stored in 0.5 ml Eppendorf microcentrifuge tubes at -20 °C until further processing.

Corticosterone levels (ng/ml) were measured in serum using a corticosterone parameter assay kit (R&D Systems; KGE009) according to instructions provided with the kit. IL-1β levels (pg/ml) were measured in serum and the nucleus accumbens using an IL-1β ELISA kit (LifeSpan BioSciences; LS-F5627) according to instructions provided with the kit.

**Statistical Analysis**

Statistics were performed using GraphPad Prism 7.03 software. Outliers were removed using the ROUT method. Furthermore, one subject from the Saline-aCSF group was excluded

from all analyses because of abnormal circling behavior during behavioral testing. Unless otherwise stated, one-way ANOVAs were performed on all data to determine differences between Saline-aCSF (n = 6), CFA-aCSF (n = 7), and CFA-M40 rats (n = 5). Alpha was set to $p$=0.05 for statistical significance; however, post-hoc analyses were performed using Fishers LSD when the main effect reached $p \leq 0.10$ to avoid type-two errors.

## Results

### Effects of CFA and M40 on weight and paw inflammation

A two-way (group x time) repeated measures ANOVA was performed on weight to determine the effects of CFA and M40 on weight over the 10-day experiment (Figure 5.1). There was a significant interaction between group and time, $F(18, 135) = 2.2$, $p < 0.01$. Post-hoc analyses revealed that the interaction was primarily driven by changes in weight within groups one day after CFA injection. Average weight was no different on Day 2 from Day 1 for Saline-aCSF rats; however, both CFA-aCSF, $p < 0.001$, and CFA-M40, $p = 0.05$, weighed significantly less on Day 2 than on Day 1. To better understand the magnitude of the effects of CFA and M40 on weight loss one day after CFA injection, a one-way ANOVA was performed on the difference in weight (g) recorded on the day of and one day after CFA injection (Figure 5.1). There was a significant difference in weight change between the groups, $F(2, 15) = 8$, $p < 0.01$. Post-hoc analysis revealed that the CFA-aCSF rats had a significantly greater loss of weight compared to Saline-aCSF rats, $p = 0.001$. CFA-M40 rats lost more weight than Saline-aCSF rats, but the effect was not statistically significant, $p = 0.07$. There was no difference in weight loss between CFA-aCSF rats and CFA-M40 rats, $p = 0.10$.

A two-way (group x time) repeated measures ANOVA was performed on paw width to determine the effects of CFA and M40 on paw inflammation over the 10-day experiment (Figure

5.1). There was a significant interaction between group and time, $F(18, 144) = 16$, $p < 0.0001$, on paw width over the 10-day experiment. Post-hoc analysis revealed that there was no difference in baseline paw width of Saline-aCSF animals compared to CFA-aCSF, $p = 0.99$, or CFA-M40 animals, $p = 0.78$, but that CFA-aCSF animals and CFA-M40 animals had significantly larger paw widths than Saline-aCSF animals every day after CFA administration ($p < 0.0001$ for all comparisons). CFA-aCSF and CFA-M40 animals showed no differences in paw width on any day of the experiment.

**Food consumption**

One-way ANOVAs were performed to determine the effects of CFA and M40 on food consumption one day before, on the day of, and one and seven days after intraplantar CFA or saline administration (Figure 5.2). Baseline food consumption was similar for all groups, $F(2, 15) = 1.64$, $p = 0.28$. There was a significant difference in food consumption between groups on the day of intraplantar CFA or saline administration, $F(2, 13) = 5.88$, $p = 0.02$. Post-hoc analyses revealed that CFA-aCSF rats consumed significantly less food on the day of intraplantar injections than Saline-aCSF rats, $p < 0.01$. CFA-M40 rats consumed less food than Saline-aCSF rats on the day of intraplantar injection, but the effect was not statistically significant, $p = 0.07$. CFA-M40 rats were no different in food consumption on the day of intraplantar injection from CFA-aCSF rats, $p = 0.29$. There were no differences in food consumption between groups one, $F(2,14) = 3.62$, $p = 0.27$, or seven, $F(2, 12) = 1.32$, $p = 0.30$, days after intraplantar CFA or saline injections.

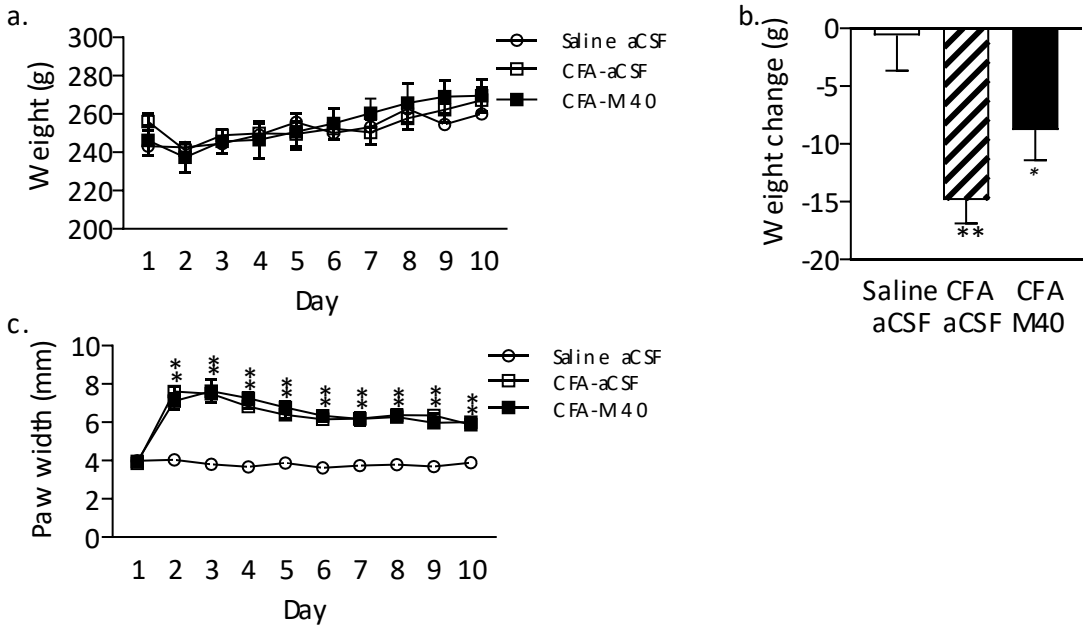


**Figure 5.1.** Effects of intraplantar CFA and daily ICV M40 on weight (a, b) and paw swelling (c). CFA was administered on Day 1. There was a significant interaction between group and time on weight over the 10-day experiment (a). CFA, but not saline, administration caused a significant decrease in weight one day later compared to baseline (b). M40 attenuated the effects of CFA on weight loss (b). *p < 0.05, **p < 0.01 compared to Saline-aCSF. CFA administration increased paw width compared to baseline (Day 1) for the duration of the experiment and the effect was not influence by M40 (c). *p<0.0001 compared to Saline-aCSF. Error bars represent SEM.

**Effects of CFA and M40 on Behavior**

One-way ANOVAs were performed to determine the effects of CFA and M40 on behavior in the open field (Figure 5.3). There were no differences between groups for amount of time spent in the center zone of the open field, $F(2, 14) = 0.55$, $p = 0.59$, or entries into the center zone, $F(2, 15) = 0.14$, $p$ 0.87. There was a significant difference between groups for total distance traveled in the open field during microdialysis testing, $F(2, 13) = 8.10$, $p = 0.01$. Post-hoc analyses showed that Saline-aCSF rats traveled a significantly greater distance than CFA-aCSF rats, $p = 0.01$, and CFA-M40 rats, $p = 0.002$. CFA-aCSF rats traveled a similar distance to CFA-M40 rats, $p = 0.24$.

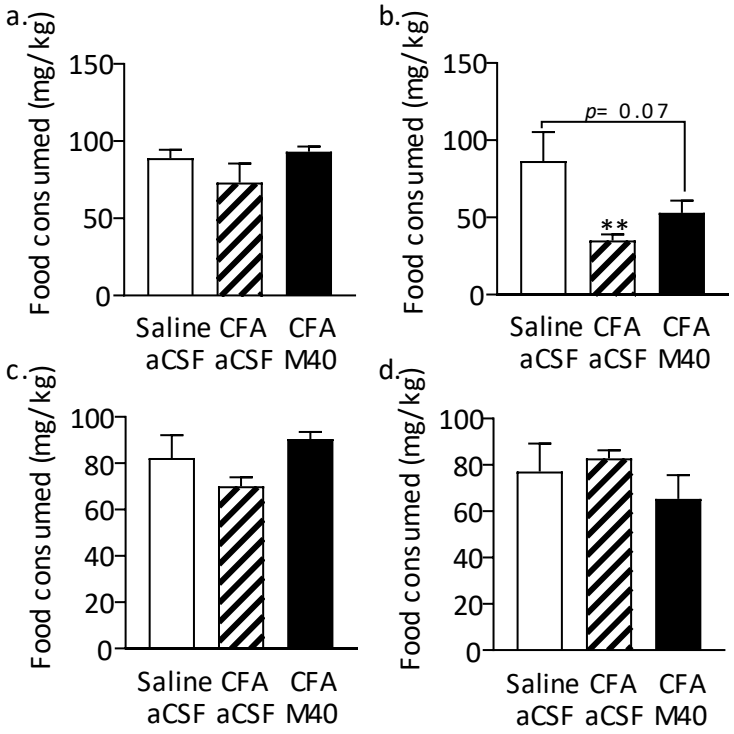


**Figure 5.2.** Effects of intraplantar CFA and daily ICV M40 on baseline food consumption (a), and food consumption on the day of (b), one day after (c), and seven days after (d) intraplantar saline or CFA injections. Error bars represent SEM. **p < 0.01 compared to Saline-aCSF.

A 3x2 (group x time) two-way repeated measures ANOVA was performed on distance traveled during the 20 min preceding and 20 min following idazoxan injection to determine if idazoxan injection affected exploration (Figure 5.3). Idazoxan injection significantly reduced distance traveled, $F(1, 14) = 16.00$, $p = 0.001$, compared to distance traveled before injection. There was also a significant effect of group, $F(2, 14) = 4.10$, $p = 0.04$, and a trend towards an interaction between group and time, $F(2, 14) = 3.00$, $p = 0.08$. Post-hoc analyses revealed that CFA-M40 rats explored significantly less than saline-aCSF, $p = 0.02$, and CFA-aCSF, $p = 0.03$ rats, during the 40-min interval analyzed. Post-hoc analyses also revealed that CFA-aCSF rats explored significantly less after idazoxan injection than before injection, $p < 0.001$, but that

idazoxan injection did not affect total distance traveled for saline-aCSF, $p = 0.09$, or CFA-M40, $p = 0.35$, rats.

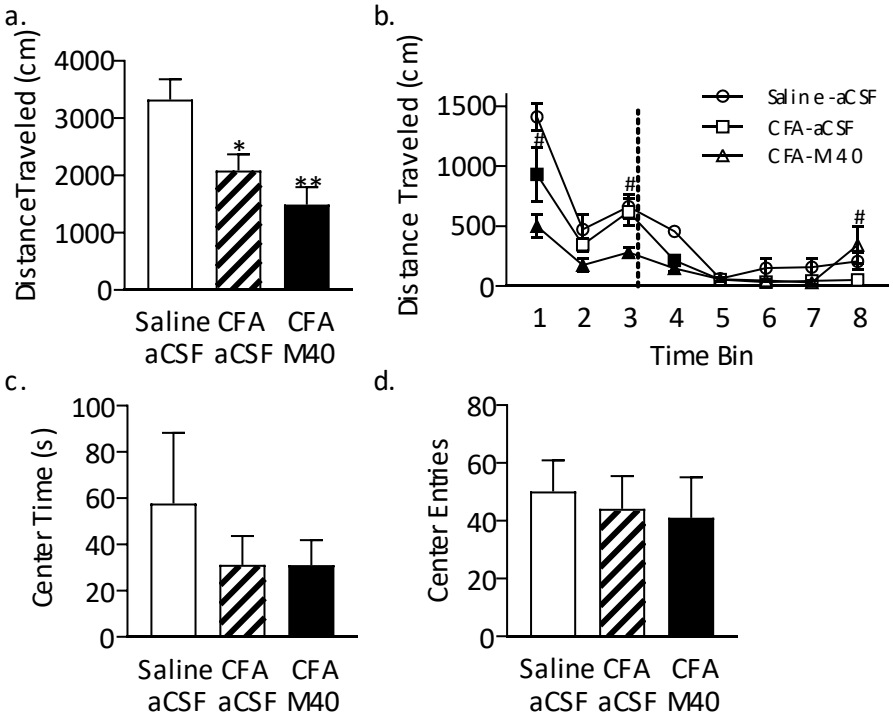


**Figure 5.3.** Effects of intraplantar CFA and daily ICV M40 on total exploration of the open field (a) and exploration during 20-min intervals (b) during microdialysis testing nine days later. The dotted line represents the time-point of idazoxan injection. Shaded symbols indicate a statistical difference compared to Saline-aCSF. *$p \leq 0.05$, **$p \leq 0.01$ compared to Saline-aCSF; $^{\#}p \leq 0.05$, compared to CFA-M40. Effects on total time spent in the center zone of the open field (c) and total number of entries into the center zone of the open field (d) during testing are also shown. Error bars represent SEM.

One-way ANOVAs were performed to determine the effects of CFA and M40 on behavior on the elevated plus maze (Figure 5.4). There were no differences between groups for total exploration of the maze, measured by the total number of arm entries, $F(2, 13) = 1.79$, $p = 0.21$. There was a trend toward a significant difference between groups for number of open arm entries, $F(2, 13) = 3.19$, $p = 0.07$, and amount of time spent in the open arm, $F(2, 13) = 3.10$, $p = 0.08$. Post-hoc analyses revealed that CFA-aCSF rats entered the open arm significantly more

than Saline-aCSF rats, $p = 0.04$, and CFA-M40 rats, $p = 0.05$. Post-hoc analyses also revealed that CFA-aCSF rats spent significantly more time in the open arm than Saline-aCSF rats, $p = 0.03$. CFA-aCSF rats also trended to spend more time in the open arm than CFA-M40 rats, but the effect did not reach statistical significance, $p = 0.11$. There was a significant difference between groups for number of closed arm entries, $F(2, 13) = 8.05$, $p = 0.01$, and amount of time spent in the closed arm, $F(2, 13) = 3.98$, $p = 0.04$. Post-hoc analyses revealed that CFA-aCSF rats, $p = 0.002$, and CFA-M40 rats, $p = 0.03$, entered the closed arm significantly less than Saline-aCSF rats. Post-hoc analyses also revealed that CFA-aCSF rats spent less time in the closed arm than Saline-aCSF rats, $p = 0.02$, and CFA-M40 rats, $p = 0.04$. CFA-M40 rats were no different on closed arm time than Saline-aCSF rats, $p = 0.76$.

**Effects of CFA and M40 on Brain Monoamines**

Dialysate samples were collected from the nucleus accumbens every 20 minutes during the 160-min microdialysis session. Extracellular monoamine levels in each dialysate sample were determined for each rat using HPLC. Average extracellular monoamine levels were then calculated for each rat from the eight samples collected. 3x2 (group x vial) two-way repeated measures ANOVAs were performed on average monoamine levels from dialysate collected at minutes 60 and 80 to determine if idazoxan injections had any effect on monoamine activity in the nucleus accumbens (data not shown).There was no effect of group, $F(2, 15) = 1.7$, $p = 0.22$, or idazoxan on extracellular norepinephrine in the nucleus accumbens, $F(1, 15) = 0.79$, $p = 0.39$. There was also no effect of group on extracellular DOPAC, $F(2, 14) = 1.9$, $p = 0.18$, or dopamine, $F(2, 14) = 2.00$, $p = 0.17$, in the nucleus accumbens. Nor was there an effect of idazoxan on extracellular DOPAC, $F(1, 14) = 0.02$, $p = 0.89$, or dopamine, $F(1, 14) < 0.01$, $p = 0.99$, in the nucleus accumbens. There was no effect of group on extracellular 5HIAA levels in

the nucleus accumbens, F(2, 15) 1.30, $p = 0.30$. However, idazoxan injection significantly reduced extracellular 5HIAA levels in the nucleus accumbens, F(1, 15) = 8.6, $p = 0.01$. Post-hoc analyses revealed that the effect of idazoxan only manifested in saline-aCSF animals, $p = 0.01$, not CFA-aCSF, $p = 0.35$, or CFA-M40, $p = 0.32$, animals.

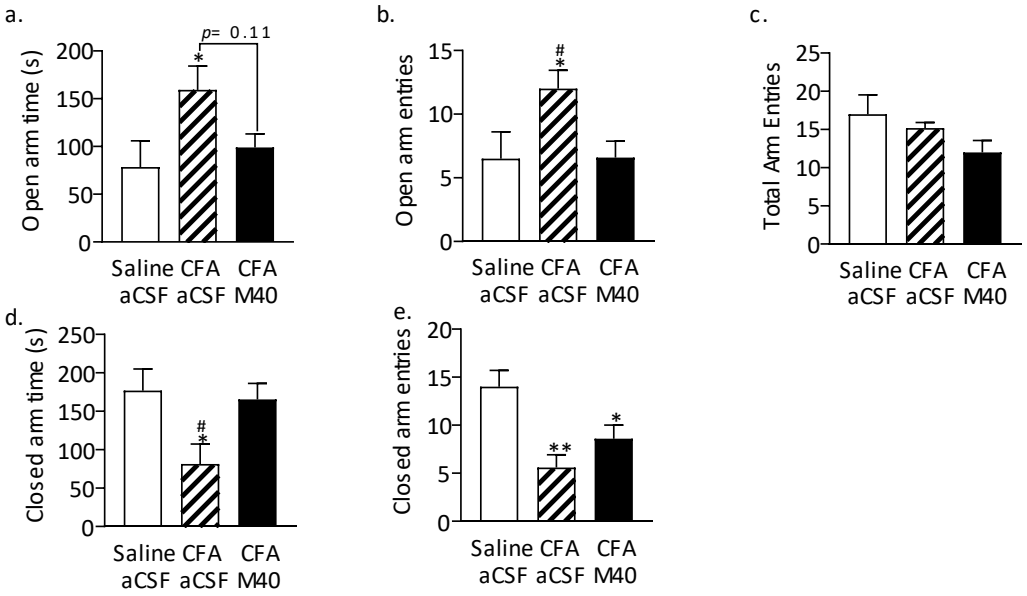


**Figure 5.4.** Effects of intraplantar CFA and daily ICV M40 on elevated plus maze behavior nine days later. Error bars represent SEM. *p ≤ 0.05, **p ≤ 0.01 compared to Saline-aCSF; #p ≤ 0.05 compared to CFA-M40.

One-way ANOVAs were performed to determine the effects of CFA and M40 on average extracellular levels of monoamines in the nucleus accumbens core (Figure 5.5). There were significant differences between groups for extracellular nucleus accumbens levels of norepinephrine, F(2, 15) = 4.71, $p = 0.03$, dopamine, F(2, 15) = 4.09, $p = 0.04$, and 5HIAA, F(2, 15) = 6.85, $p = 0.01$. There was also a trend towards significant group differences for DOPAC levels, F(2, 13) = 3.28, $p = 0.07$. Serotonin levels were below the threshold of detection. Post-hoc analyses showed that CFA administration significantly reduced extracellular norepinephrine levels in the nucleus accumbens in rats treated with aCSF, $p = 0.02$, and rats treated with M40, $p$

= 0.02, compared to Saline-aCSF rats. CFA administration also significantly reduced extracellular DOPAC levels in the nucleus accumbens in rats treated with aCSF, $p = 0.05$, and rats treated with M40, $p = 0.05$, compared to Saline-aCSF rats. Likewise, CFA administration significantly reduced extracellular dopamine levels in the nucleus accumbens in rats treated with aCSF, $p = 0.03$, and rats treated with M40, $p = 0.02$, compared to Saline-aCSF rats. In addition, CFA administration significantly reduced extracellular 5HIAA levels in the nucleus accumbens in rats treated with aCSF, $p = 0.01$, and rats treated with M40, $p = 0.01$, compared to Saline-aCSF rats. CFA-aCSF rats and CFA-M40 rats were no different for extracellular norepinephrine, $p = 0.89$; DOPAC, $p = 0.77$; dopamine, $p = 0.76$; or 5HIAA, $p = 0.89$, levels in the nucleus accumbens.

One-way ANOVAs were performed to determine the effects of CFA and M40 on total tissue concentrations of monoamines in the VTA (Table 1; Figure 5.6). There was a significant difference between groups for norepinephrine levels in the VTA, $F(2, 15) = 6.92$, $p = 0.01$. Post-hoc analyses revealed that CFA-M40 rats had significantly more norepinephrine in the VTA compared to Saline-aCSF rats, $p = 0.003$, and CFA-aCSF rats, $p = 0.02$. Saline-aCSF rats were no different from CFA-aCSF rats, $p = 0.31$. There was also a significant difference between groups for DOPAC levels in the VTA, $F(2, 14) = 5.13$, $p = 0.02$. Post-hoc analyses revealed that CFA-M40 rats had significantly less DOPAC in the VTA compared to Saline-aCSF rats, $p = 0.01$, and CFA-aCSF rats, $p = 0.03$. Saline-aCSF rats were no different from CFA-aCSF rats, $p = 0.37$. There were no differences between groups for dopamine, $F(2, 14) – 0.51$, $p = 0.61$, 5HIAA, $F(2, 15) = 1.43$, $p = 0.27$, or serotonin, $F(2, 15) = 0.28$, $p = 0.76$, levels in the VTA. One-way ANOVAs were also performed on the ratio of DOPAC to dopamine levels in the VTA and the ratio of 5HIAA to serotonin levels in the VTA to determine the effects of CFA and M40 on

dopamine and serotonin metabolism. There was no difference between groups for DOPAC/DA levels, $F(2, 15) = 0.48$, $p = 0.63$, or 5HIAA/serotonin levels, $F(2, 15) = 0.09$, $p = 0.91$, in the VTA.

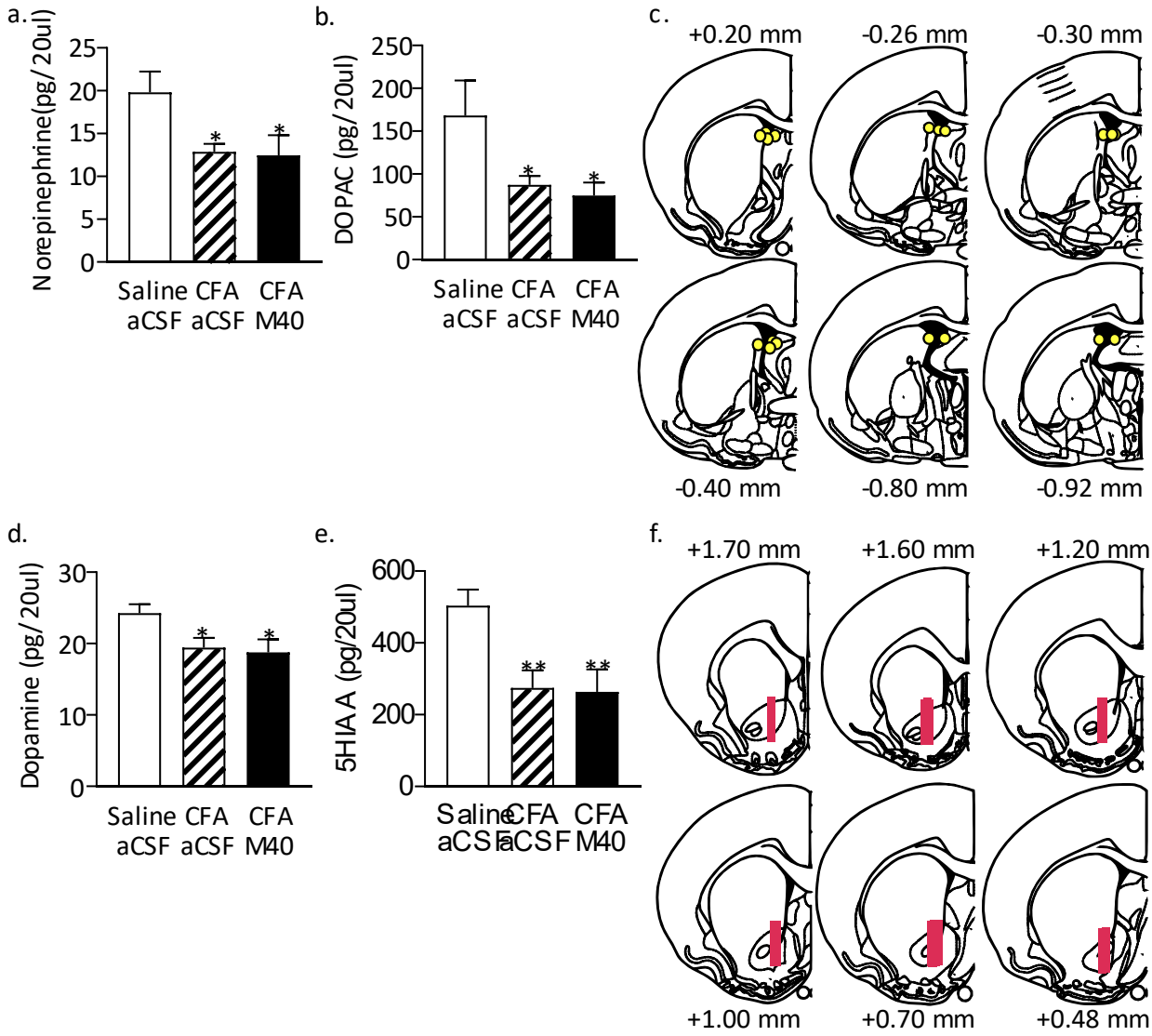


**Figure 5.5.** Effects of intraplantar CFA and daily ICV M40 on average extracellular monoamine levels collected from the nucleus accumbens using microdialysis nine days later (a, b, d, e). Cannula placement for microdialysis probes in the nucleus accumbens (c) and drug infusion in the lateral ventricle (f) are also shown. Error bars represent SEM. *$p \leq 0.05$, ** $p \leq 0.01$ compared to Saline-aCSF.

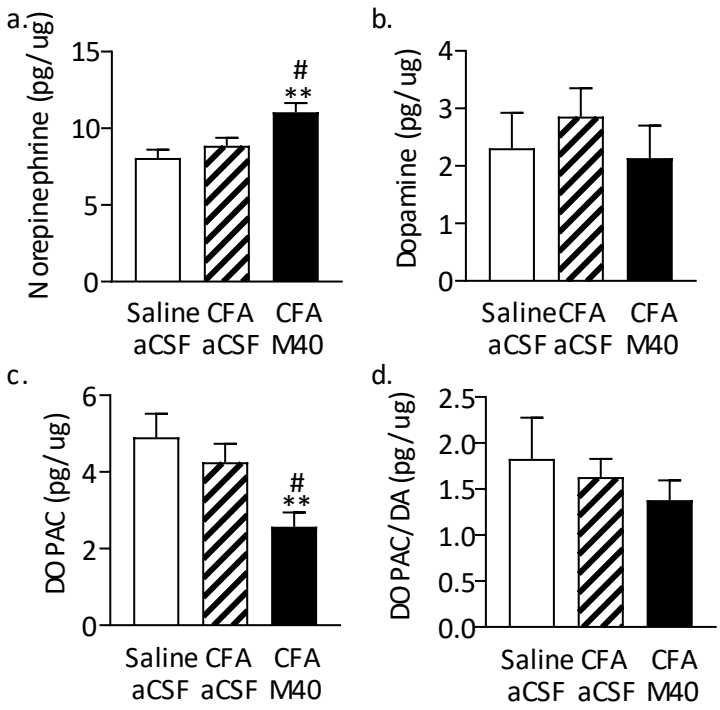


**Figure 5.6.** Effects of intraplantar CFA and daily ICV M40 on average total tissue levels of catecholamines (a - c) and dopamine turnover (d) in the VTA nine days later. Error bars represent SEM. **$p \leq 0.01$ compared to Saline-aCSF; $^{\#}p \leq 0.05$ compared to CFA-aCSF.

One-way ANOVAs were performed to determine the effects of CFA and M40 on total tissue concentrations of monoamines in the nucleus accumbens (Table 5.1; Figure 5.7). There were no significant differences between groups for norepinephrine, $F(2, 14) = 0.68$, $p = 0.53$, or serotonin, $F(2, 15) = 0.53$, $p = 0.60$, levels in the nucleus accumbens. There was a significant difference between groups for DOPAC levels in the nucleus accumbens, $F(2, 13) = 3.88$, $p = 0.05$. There were trends toward significant differences between groups for dopamine, $F(2, 13) = 3.18$, $p = 0.08$, and 5HIAA levels, $F(2, 14) = 3.35$, $p = 0.07$, in the nucleus accumbens. Post-hoc analyses showed that CFA-aCSF rats had reduced DOPAC, $p = 0.06$, and dopamine, $p = 0.14$, levels in the nucleus accumbens compared to Saline-aCSF rats, but the trend was not significant. CFA-M40 rats had significantly more DOPAC, $p = 0.02$, and dopamine, $p = 0.02$, levels in the nucleus accumbens compared to CFA-aCSF rats. CFA administration reduced 5HIAA in the nucleus accumbens in aCSF-treated rats compared to Saline-aCSF, $p = 0.04$, and aCSF-M40 rats,

$p = 0.06$. One-way ANOVAs were also performed on the ratio of DOPAC to dopamine levels and the ratio of 5HIAA to serotonin levels in the nucleus accumbens to determine the effects of CFA and M40 on dopamine and serotonin metabolism. There was no difference between groups for DOPAC/DA levels, $F(2, 15) = 0.85$, $p = 0.45$, or 5HIAA/serotonin levels, $F(2, 15) = 0.15$, $p = 0.86$, in the nucleus accumbens.

One-way ANOVAs were performed to determine the effects of CFA and M40 on total tissue concentrations of monoamines in the LC (Table 5.1). There were no differences between groups for norepinephrine, $F(2, 15) = 0.91$, $p = 0.42$, DOPAC, $F(2, 14) = 0.33$, $p = 0.73$, dopamine, $F(2, 15) = 1.19$, $p = 0.33$, 5HIAA, $F(2, 15) = 0.07$, $p = 0.93$, or serotonin, $F(2, 15) = 0.36$, $p = 0.70$, levels in the LC. One-way ANOVAs were also performed on the ratio of DOPAC to dopamine levels in the LC and the ratio of 5HIAA to serotonin levels in the LC to determine the effects of CFA and M40 on dopamine and serotonin metabolism. There was no difference between groups for DOPAC/DA levels, $F(2, 15) = 0.478$, $p = 0.63$, or 5HIAA/serotonin levels, $F(2, 15) = 0.97$, $p = 0.40$, in the LC.

One-way ANOVAs were performed to determine the effects of CFA and M40 on total tissue concentrations of monoamines in the PVN (Table 5.1; Figure 5.8). There were no significant differences between groups for norepinephrine, $F(2, 14) = 1.48$, $p = 0.26$, DOPAC, $F(2, 13) = 1.12$, $p = 0.36$, dopamine, $F(2, 13) = 0.01$, $p = 0.99$, or serotonin, $F(2, 13) = 0.72$, $p = 0.34$, levels in the PVN. There was a trend towards a significant differences between groups for 5HIAA levels in the PVN, $F(2, 13) = 2.92$, $p = 0.09$. One-way ANOVAs were also performed on the ratio of DOPAC to dopamine levels in the PVN and the ratio of 5HIAA to serotonin levels in the PVN to determine the effects of CFA and M40 on dopamine and serotonin metabolism. There was a significant difference between groups for DOPAC/dopamine in the PVN, $F(2, 12) =$

6.13, $p = 0.01$. CFA-aCSF rats had a significantly lower ratio of DOPAC to dopamine in the PVN compared to Saline-aCSF rats, $p = 0.04$, and compared to CFA-M40 rats, $p = 0.01$. Saline-aCSF rats and CFA-M40 rats were no different from each other, $p = 0.27$. There was no difference between groups for 5HIAA/serotonin levels in the PVN, $F(2, 12) = 0.86$, $p = 0.45$.

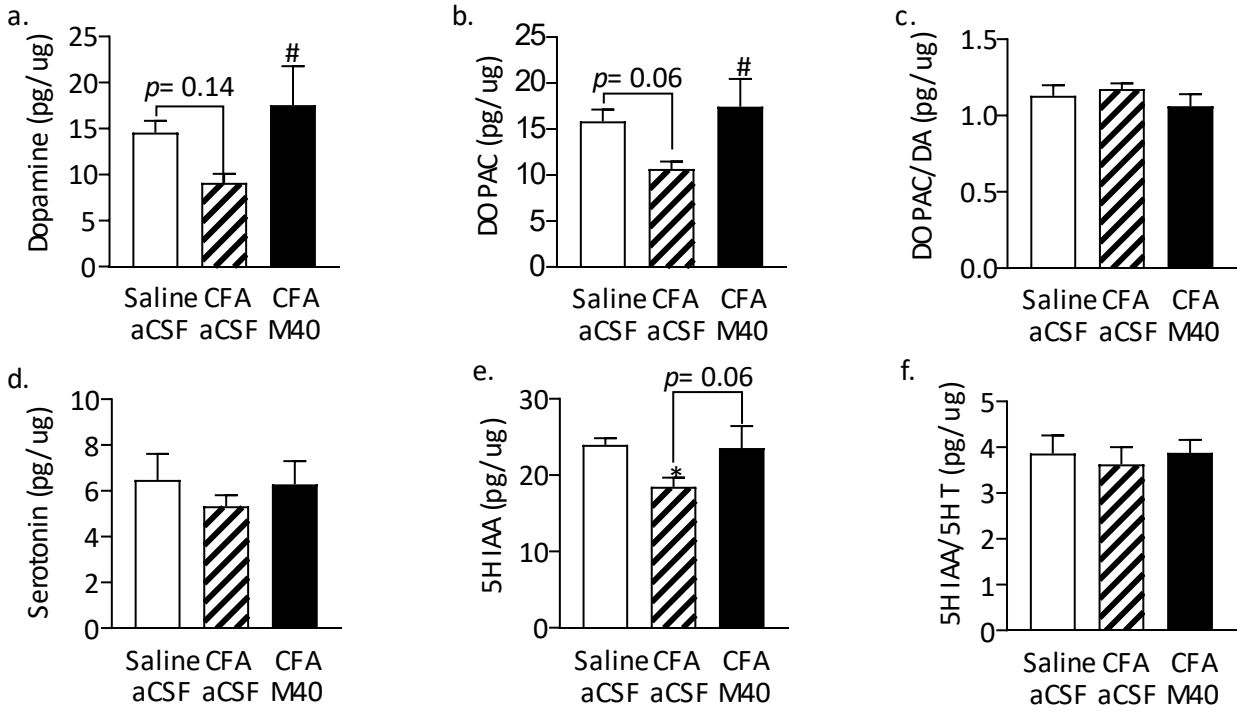


**Figure 5.7**. Effects of intraplantar CFA and daily ICV M40 on average total tissue levels of monoamines (a, b, d, e), as well as, dopamine (c) and serotonin turnover (f), in the nucleus accumbens nine days later. Error bars represent SEM. *$p \leq 0.05$ compared to Saline-aCSF; $^{\#}p \leq 0.05$ compared to CFA-aCSF.

**Corticosterone and IL-1β**

One-way ANOVAs were performed to determine the effects of CFA and M40 on serum levels of corticosterone and IL-1β (Figure 5.9). There were no differences between groups for serum levels of corticosterone, $F(2, 14) = 0.56$, $p = 0.58$. There were no differences between groups for serum levels of IL-1β, $F(2, 14) = 1.53$, $p = 0.25$. A one-way ANOVA was also

performed to determine the effects of CFA and M40 on IL-1β levels in the nucleus accumbens (Figure 5.9). There were no differences between groups on nucleus accumbens IL-1β, $F(2, 14) = 0.14$, $p = 0.87$.

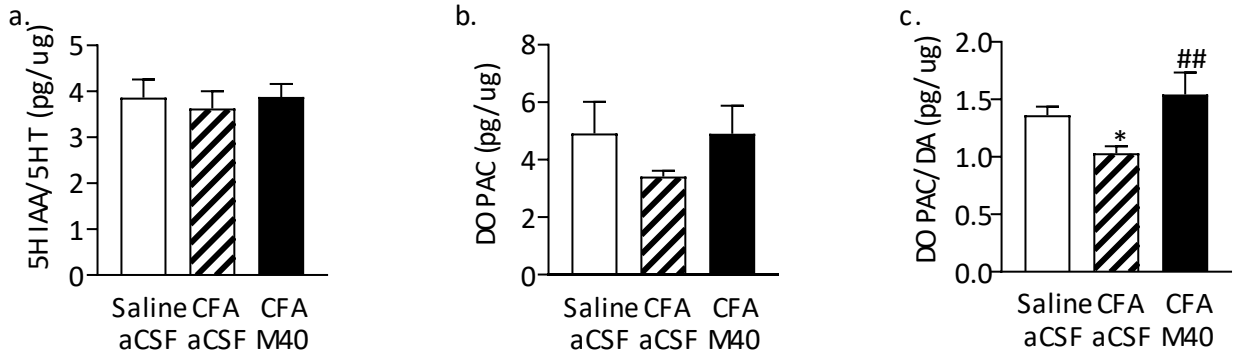


**Figure 5.8.** Effects of intraplantar CFA and daily ICV M40 on average total tissue levels of dopamine (a), DOPAC (b), and dopamine turnover (c) in the PVN nine days later. Error bars represent SEM. *$p \leq 0.05$ compared to Saline-aCSF; $^{\#\#}p \leq 0.01$ compared to CFA-aCSF.

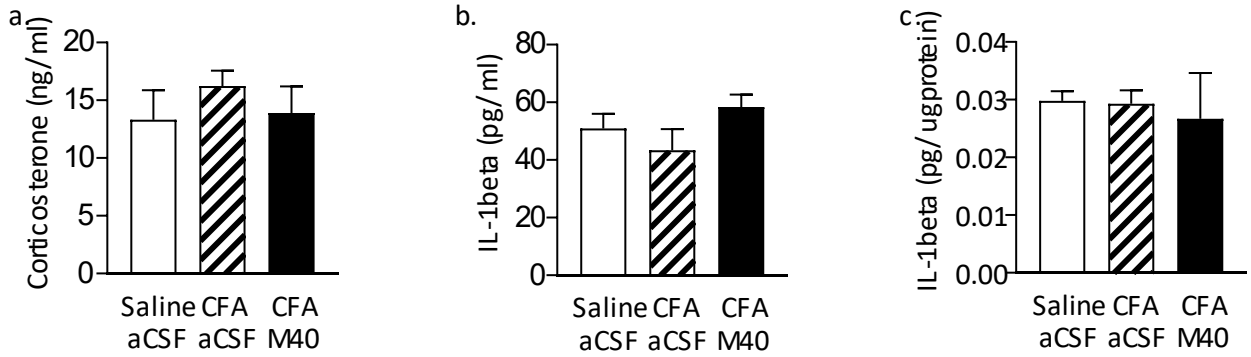


**Figure 5.9.** Effects of intraplantar CFA and daily ICV M40 on average serum levels of corticosterone (a) and IL-1β in serum (b) and the nucleus accumbens (c) nine days later. Error bars represent SEM.

## Discussion

We investigated the role of galanin in mediating the effects of chronic inflammatory pain induced by intraplantar CFA on mesolimbic dopamine dysregulation, which is thought to

underlie some of the motivational deficits associated with comorbid depression. We provide evidence that galanin effects local synthesis of dopamine in the nucleus accumbens, rather than basal VTA firing rate and subsequent dopamine release during chronic inflammatory pain. We also show that galanin activity mediates the reduced dopamine turnover in the PVN after CFA, suggesting that hypothalamic galanin contributes to effects of chronic inflammatory pain.

CFA administration decreased extracellular concentrations of dopamine and DOPAC levels in the nucleus accumbens during open field exploration, as well as total tissue concentrations. M40 administration did not alter the effects of CFA on extracellular dopamine activity, but M40 administration did prevent the effects of CFA on dopamine levels in the nucleus accumbens. Our results suggest galanin mediates the effects of intraplantar CFA on downregulation of dopamine stores in the nucleus accumbens. Our results do not support the hypothesis that LC-derived galanin mediates the effect of chronic inflammatory pain on mesolimbic dopamine release; however, several limitations make it difficult to draw clear conclusions about galanin's precise role in mesolimbic dopamine regulation from our data. It is possible that chronic administration of M40 led to compensatory upregulation or sensitization of galanin receptors on dopamine neurons or noradrenergic nerve terminals in the VTA. If the effects of galanin on dopamine release are acute, then tolerance to M40 may have precluded its acute effects. Future research should measure galanin receptor expression in regions of interest and evaluate the effects of acute M40 administration before microdialysis testing on extracellular dopamine levels to rule out this possibility. Furthermore, evidence suggests that inflammatory cytokines increase monoamine reuptake transporter activity (Zhu, Blakely, & Hewlett, 2006). Increased rate of clearance of monoamines from the extracellular milieu could also preclude the effects of galanin receptor blockade on dopamine release. This could also explain why

norepinephrine and 5HIAA were also reduced in the extracellular space of the nucleus accumbens and not effected by chronic M40. Using a technique with better temporal resolution than microdialysis, such as voltammetry, or blocking the dopamine transporter during microdialysis, might be superior approaches to determining the effects of CFA on catecholamine release. Finally, it is noteworthy that we previously showed reduced galanin protein levels in the VTA nine days after CFA administration (Chapter 4). It is unclear whether the reduction reflects decreased galanin synthesis or depletion after microdialysis testing; however, decreased galanin availability in the VTA could also explain why M40 did not affect dopamine release. Consistent with this, we previously showed that galanin levels were not affected in the nucleus accumbens after CFA administration (Chapter 4). Therefore, chronic, but not acute, galanin activity in the nucleus accumbens during inflammatory pain may decrease local dopamine synthesis in nerve terminals independent of VTA activity. Indeed, Schwartz and colleagues (2014) showed that stimulation of the GalR1 receptor in the nucleus accumbens is necessary for the effects of CFA on altered medium spiny neuron excitability, suggesting that galanin activity is increased in the nucleus accumbens during chronic inflammatory pain. Which galanin afferents in the nucleus accumbens are involved in the effects of chronic inflammatory pain remains unclear, but possible candidates are the basolateral amygdala, PVN, and arcuate nucleus (Schwartz et al., 2014).

How galanin effects dopamine stores in the nucleus accumbens remains unknown. Galanin has been previously shown to inhibit tyrosine hydroxylase (TH) expression in midbrain dopamine neuron cultures. The precise mechanism of inhibition is unclear; however, evidence suggests that stimulation of GalR1 leads to a post-transcriptional modification that decreases TH expression by opening G protein-gated inwardly rectifying K+ channels (Counts et al., 2002).

**Table 5.1**

Effects of Chronic Inflammatory Pain and M40 on Total Brain Tissue Concentration of Monoamine Neurotransmitters

| | | **NE** | | **DOPAC** | | **DA** | | **DOPAC/DA** | | **5HIAA** | | **5HT** | | **5HIAA/5HT** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Region** | **Group** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** | **Mean** | **SEM** |
| VTA | Saline-aCSF | 8.07 | 0.56 | 4.90 | 0.62 | 2.31 | 0.62 | 1.83 | 0.45 | 14.53 | 1.02 | 5.36 | 0.61 | 2.82 | 0.29 |
| | CFA-aCSF | 8.87 | 0.51 | 4.26 | 0.48 | 2.86 | 0.49 | 1.63 | 0.20 | 15.87 | 1.34 | 5.82 | 0.46 | 2.88 | 0.44 |
| | CFA-M40 | ****,#11.05** | **0.60** | ****,#2.57** | **0.37** | 2.14 | 0.57 | 1.38 | 0.21 | 17.38 | 0.70 | 5.84 | 0.42 | 3.04 | 0.24 |
| NAc | Saline-aCSF | 7.71 | 0.86 | 15.87 | 1.26 | 14.55 | 1.29 | 1.13 | 0.07 | 23.99 | 0.88 | 6.48 | 1.14 | 3.87 | 0.40 |
| | CFA-aCSF | 6.71 | 0.49 | ***10.67** | **0.81** | **[2]9.11** | **0.98** | 1.17 | 0.04 | **[*]18.46** | **1.22** | 5.33 | 0.48 | 3.63 | 0.37 |
| | CFA-M40 | 6.89 | 0.53 | **[#]17.43** | **3.03** | **[#]17.57** | **4.22** | 1.06 | 0.08 | **[3]23.56** | **2.89** | 6.28 | 1.02 | 3.88 | 0.29 |
| LC | Saline-aCSF | 14.27 | 1.70 | 4.67 | 1.77 | 1.57 | 0.22 | 2.71 | 0.90 | 16.81 | 0.91 | 4.69 | 0.71 | 3.87 | 0.39 |
| | CFA-aCSF | 14.13 | 0.20 | 3.71 | 0.73 | 1.84 | 0.12 | 2.70 | 0.67 | 16.97 | 0.69 | 5.07 | 0.44 | 3.54 | 0.40 |
| | CFA-M40 | 16.27 | 1.41 | 3.34 | 0.37 | 2.00 | 0.25 | 1.81 | 0.36 | 16.48 | 1.15 | 5.44 | 0.68 | 3.11 | 0.20 |
| PVN | Saline-aCSF | 33.75 | 2.32 | 4.92 | 1.10 | 3.10 | 0.51 | 1.36 | 0.08 | 11.22 | 1.28 | 1.81 | 0.32 | 7.95 | 1.17 |
| | CFA-aCSF | 45.98 | 6.53 | 3.42 | 0.20 | 3.12 | 0.21 | ***1.03** | **0.06** | **[1]15.21** | **1.63** | 1.79 | 0.25 | 9.12 | 1.19 |
| | CFA-M40 | 38.35 | 5.72 | 4.90 | 0.98 | 3.17 | 0.53 | **[##]1.54** | **0.19** | ***16.70** | **1.48** | 2.17 | 0.53 | 6.71 | 0.66 |

*Note.* SEM = Standard Error of the Mean, NE = Norepinephrine, DA = Dopamine, 5HT = Serotonin, CFA = Complete Freund's Adjuvant, VTA = Ventral Tegmental Area, NAc = Nucleus Accumbens, LC = Locus Coeruleus, PL = Prelimbic Cortex, PVN = Paraventricular Nucleus, DR = Dorsal Raphe. $^{*}p \leq 0.05$, $^{**}p \leq 0.05$, $^{1}p = 0.08$, $^{2}p = 0.14$ compared to Saline-aCSF; $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{3}p = 0.06$ compared to CFA-aCSF,

Alternatively, galanin could also decrease TH transcription by inhibiting adenylyl cyclase production of cAMP through stimulation of the $G_{i/o}$-coupled GalR1 receptor subtype, as TH transcription is facilitated by the binding of CREB to the cAMP responsive element (CRE) site on the rat TH promotor (Fossom, Sterling, & Tank, 1992; Kim, Lee, Carroll, & Joh, 1993; Kumer & Vrana, 1996; Leviel, Guibert, Mallet, & Faucon-Biguet, 1991; Piech-Dumas et al., 2001). Another possible mechanism by which galanin could reduce dopamine levels is through dysregulation of tetrahydrobipterin (BH4). BH4 is an essential cofactor for tryptophan hydroxylase and TH, which are the rate limiting enzymes for serotonin, dopamine, and norepinephrine synthesis. BH4 also serves as a cofactor for nitric oxide synthase (Sumi-Ichinose et al., 2001; Thony, Auerbach, & Blau, 2000). Inflammation causes BH4 to be preferentially used for conversion of arginine to nitric oxide, thereby reducing monoamine synthesis. Dysregulated BH4 activity is implicated in the comorbidity of chronic inflammation and depression (Miller, Haroon, Raison, & Felger, 2013; Vancassel, Capuron, & Castanon, 2018). To our knowledge, there is no direct evidence linking galanin to BH4 activity; however, the links between inflammation, galanin, and dopamine activity suggest that a relationship is possible. Furthermore, nitric oxide synthase activity is induced by galanin administration and both transmitters are upregulated in response to injury, inflammation, colchicine treatment, and nicotine withdrawal (Abot et al., 2018; Brecht, Buschmann, Grimm, Zimmermann, & Herdegen, 1997; Consolo, Uboldi, Caltavuturo, & Bartfai, 1998; Okere & Waterhouse, 2006, 2013; Xu & Hokfelt, 1997; X. Zhang et al., 1996), lending further support for this hypothesis. It should be noted that neither CFA nor M40 effected IL-1β levels in the nucleus accumbens. Therefore, the effects on dopamine are likely not mediated by direct chronic action of inflammatory cytokines in the nucleus accumbens.

We also investigated a potential role for galanin in modulating monoamine levels in the VTA and the LC, which is a major source of noradrenergic and galanin input to the VTA (Mejias-Aponte et al., 2009). CFA administration did not affect any monoamine levels in the VTA or LC. This is in opposition to our previous finding that CFA reduced norepinephrine in the VTA (Chapter 4). CFA combined with M40 increased norepinephrine levels and deceased DOPAC levels in the VTA compared to the other groups but had no other effects in the VTA or LC. Without a saline-M40 group, it is impossible to know if these effects are due to blocking galanin alone or reflective of a synergistic effect of CFA and galanin blockade. We previously showed that CFA administration reduced galanin levels in the VTA. If the effect of CFA on VTA galanin was due to decreased galanin availability (rather than depletion during testing) then further reducing galanin's autoinhibitory functions could lead to increased norepinephrine levels in noradrenergic terminals. However, it is important to note that all catecholamine levels in the VTA were much lower for this cohort of animals, compared to previous cohorts (e.g., 15.88 pg/ug versus 8.07 pg/ug of norepinephrine for control animals; Chapter 4). Therefore, VTA data from this experiment should be interpreted with caution.

We previously showed that CFA administration reduced dopamine and DOPAC levels in the PVN (Chapter 4), therefore we investigated whether the effect is mediated by galanin. Although the reduction in PVN dopamine and DOPAC levels after CFA was not statistically significant in this experiment, we did show a significant decrease in dopamine turnover. The effect was blocked by chronic M40 administration, which suggests that galanin mediates the effects of chronic inflammatory pain on dopamine activity in the PVN. The functional consequences of these changes are unclear. Neither CFA nor M40 effected glucocorticoid levels in the serum, therefore the changes to dopamine signaling do not seem to influence basal HPA-

axis activity. However, we previously showed that CFA treatment blunted an acute stress-induced increase in serum corticosterone levels 24 hr later, compared to saline treatment (Chapter 3). Whether altered galanin and dopamine activity in the PVN contributes to the aberrant HPA-axis stress response remains unknown. Another possible consequence of reduced dopamine activity in the PVN is altered regulation of oxytocin activity, which may have implications for sexual behaviors and mesolimbic dopamine activity (Melis & Argiolas, 2011). Consistent with this, galanin also modulates oxytocin activity (Wodowska & Ciosek, 2014) and oxytocin is upregulated by chronic inflammatory pain (Matsuura et al., 2015). Alternatively, these changes may have implications for changes in feeding, metabolism, hormone secretion, reproductive behaviors, and general circadian disruption during chronic inflammatory pain (Cardinali & Esquifino, 2003; Selgas, Arce, Esquifino, & Cardinali, 1997; Tejas-Juarez et al., 2014). Future research should focus on elucidating the functional effects of galanin and dopamine interactions in the PVN and other hypothalamic nuclei during chronic inflammatory pain.

We also investigated galanin's role in mediating CFA-induced hypophagia, a sickness behavior commonly observed after injury or infection. CFA administration decreased food consumption on the day of injection and caused rats to lose a significant amount of weight one day later. These effects are consistent with sickness behavior that occurs after induction of inflammatory pain or systemic inflammation. Administration of M40 did not alter the effects of CFA on weight and food consumption. Therefore, galanin is not necessary for the induction of sickness-induced hypophagia. This is consistent with the known orexigenic effects of galanin on feeding (Barson, Morganstern, & Leibowitz, 2010). Although we did not measure galanin levels in the hypothalamus, evidence suggests that downregulation of hypothalamic galanin may

contribute to hypophagia during sickness (Sergeyev, Broberger, & Hokfelt, 2001). However, others have shown upregulation of galanin in hypothalamic nuclei in chronic neuropathic and visceral pain models (Imbe et al., 2004; Nishii, Nomura, Aono, Fujimoto, & Matsumoto, 2007), suggesting that hypothalamic galanin may mediate a variety of functions altered by chronic inflammatory pain. Future research is needed to understand the role of hypothalamic galanin in other aspects of sickness behaviors and depression-like characteristics that are under hypothalamic regulation, such as fever, increased sleep, reduced reproduction, and aberrant HPA-axis function.

The functional consequences of galanin blockade on mesolimbic dopamine dysregulation after CFA remain unclear as we did not measure behaviors under strong control of mesolimbic signaling, such as operant responding for drug or food reward. However, we did measure other behaviors that are sensitive to dopamine activity. Rats treated with CFA and aCSF showed reduced exploration of the open field during the first 20 min of microdialysis testing, compared to saline-treated rats. This result is consistent with evidence of the necessity of mesocorticolimbic dopamine for normal locomotor and exploratory behavior in a novel open field (Fink & Smith, 1980a, 1980b). Furthermore, it suggests possible involvement of dopamine reduction in the substantia nigra, which is critical for volitional movement (Amalric & Koob, 1993). Although it is tempting to assume that decreased exploration in the open field is due to decreased behavioral activation in response to novelty or impaired motivation, it is impossible to rule out the influence of allodynia without further testing. It is unclear why daily administration of M40 exacerbated the effect of CFA on the first 20 min of exploration, particularly because central galanin administration reduces open field exploration (Ericson & Ahlenius, 1999). Galanin is also involved in supraspinal suppression of pain (Lang et al., 2015), so perhaps M40

administration further reduced exploration after CFA by blocking analgesic properties of central galanin signaling. It is difficult to interpret this effect without first evaluating the effects of galanin and M40 on other pain-related behaviors after CFA administration.

CFA administration increased open arm time and decreased closed arm time on the elevated plus maze. The effects of CFA were prevented by chronic M40 administration. Canonical interpretation suggests that CFA administration has an anxiolytic-like effect, which is dependent on galanin. This is consistent with a role for galanin in reducing anxiety-like behavior; however, it is inconsistent with other studies showing an anxiogenic effect of CFA (Borges, Neto, Mico, & Berrocoso, 2014; Gregoire, Wattiez, Etienne, Marchand, & Ardid, 2014; Parent et al., 2012; Refsgaard, Hoffmann-Petersen, Sahlholt, Pickering, & Andreasen, 2016; Stein, Millan, & Herz, 1988; Wu et al., 2017). Blocking the D4 dopamine receptor subtype in the medial prefrontal cortex increases open arm time on the elevated plus maze and decreases burying in the shock probe defensive burying test (Shah, Sjovold, & Treit, 2004), so it is possible that this effect is driven by mesocorticolimbic dopamine dysregulation. One possible alternative explanation is that the CFA-treated animals show increased avoidance of aversion (LaBuda & Fuchs, 2000; Moriarty, Roche, McGuire, & Finn, 2012; Refsgaard et al., 2016; Wu et al., 2017). Rodents tend to rear when in the closed arm of the elevated plus maze. CFA-treated animals may associate the closed arm with the pain evoked by rearing and therefore avoid the arm. While CFA-treated animals did rear less than saline-treated animals overall, the proportion of time spent rearing relative to time in the closed arm was the same for saline- and CFA-treated animals. Further research is needed to determine if the long-term effect of CFA on elevated plus maze behavior is truly anxiolytic or if we are measuring a non-specific effect of the manipulation.

In summary, we provide evidence that chronic inflammatory pain suppresses mesolimbic dopamine signaling and dopamine activity in the PVN. Furthermore, we show that chronic inflammatory may also affect nigrostriatal dopamine systems. The effects of chronic inflammatory pain seem to be mediated by multiple mechanisms. Galanin is necessary for reduced dopamine turnover in the PVN and levels in the nucleus accumbens. However, reduced dopamine release in the nucleus accumbens was not affected by galanin blockade, suggesting that chronic galanin signaling does not affect VTA dopamine neuron activity directly. Several limitations of our research may explain the null effect of galanin blockade on dopamine release and therefore further research is warranted before a role for galanin plasticity in the VTA during chronic inflammatory pain is dismissed. Future research should also be directed at understanding the role of galanin in the PVN during chronic inflammatory pain due to the relevance in understanding potential neurobiological substrates linking rheumatoid arthritis, endocrine dysfunction, and depression.

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# CHAPTER 6

## SUMMARY

The research presented provides evidence supporting the emerging hypothesis that dysregulation of mesolimbic dopamine underlies motivational deficits associated with chronic inflammatory pain. Furthermore, it provides further support for the role of supraspinal galanin in modulating behavioral changes associated with stress and chronic inflammatory pain. We also show that galanin mediates alterations to dopamine activity in the paraventricular nucleus (PVN) after chronic inflammatory pain; however, the functional consequences of this remain unclear.

In Chapter 1, we showed that galanin administration into the prelimbic cortex impaired the consolidation and expression of contextual fear conditioning, a model of post-traumatic stress disorder. The mechanism of galanin action in the prelimbic cortex is unclear from our research; however, galanin's effects are likely mediated by inhibition of norepinephrine release in the cortex. This interpretation is consistent with the known stress-responsive role of galanin in the brain and the necessity of norepinephrine signaling in memory formation and fear conditioning. Further research is needed to clarify the role of endogenous locus coeruleus (LC)-derived galanin in fear-motivated learning paradigms. Nevertheless, this experiment, coupled with other data presented in this dissertation, supports a broad role for galanin in effecting stress- and catecholamine-sensitive behaviors related to anxiety- and depression-like phenotypes.

In Chapter 2, we characterized the effects of intraplantar complete Freund's adjuvant (CFA) in our hands. We replicated other research which shows that CFA administration induces a depression-like phenotype, including decreased sucrose consumption and increased immobility

time in the forced swim test. CFA administration also consistently decreased dopamine levels and release in the nucleus accumbens in Chapters 2, 3, and 4. This is in keeping with the hypothesis that mesolimbic dopamine is dysregulated in conditions of chronic inflammatory pain. In Chapter 3, we provided evidence that disrupted neurotransmission of norepinephrine and galanin in the ventral tegmental area (VTA) could mediate the effects of CFA on mesolimbic dopamine dysregulation. Specifically, CFA administration decreased norepinephrine and galanin levels in the VTA at the time of euthanasia. It remains unclear if this reflects decreased galanin availability or galanin depletion after increased release. We favor the interpretation of increased galanin activity because it is consistent with literature showing upregulation of galanin in the brain and spinal cord after pain and stress, as well as, literature showing pro-depressant effects of galanin, inhibitory modulation of catecholamine activity, and the role of galanin in addiction. However, chronic galanin blockade by M40 did not affect dopamine release in the nucleus accumbens. These results suggest that galanin signaling in the VTA is not necessary for the effects of CFA on dopamine neuron activity, but tolerance to M40 may have precluded the effects of acute galanin blockade. A summary of key findings is shown in Figure 6.1.

Several additional experiments are needed to clarify our findings and draw conclusions about galanin activity in the VTA. (i) Galanin protein and mRNA in the VTA and LC need to be systematically measured at several timepoints after CFA administration without additional manipulations that might alter expression. (ii) GalR2 and α2 receptor expression should be measured in the VTA and LC after chronic M40 or aCSF administration to determine if there are compensatory changes that might have precluded the effects of acute galanin blockade on dopamine release. (iii) Effects of acute M40 administration in the VTA before microdialysis in CFA and saline treated rats should be evaluated to determine if galanin has an acute effect on

mesolimbic dopamine activity during chronic inflammatory pain. Moreover, additional research is needed to elucidate the role of LC-VTA projections in chronic inflammatory pain. For example, microdialysis should be performed in the VTA to measure basal norepinephrine activity and norepinephrine release in response to noxious stimulation and during an operant task in healthy animals and CFA-treated animals to determine if this pathway plays a role in supraspinal modulation of pain and pain-related behaviors. It would also be useful to measure galanin release in the VTA and LC in a model of chronic inflammatory pain; however, galanin is notoriously difficult to sample *in vivo* and therefore appropriate collection methods would need to be optimized.

Although our results suggest that galanin may not be necessary for CFA-induced changes to mesolimbic dopamine release, galanin was necessary for CFA-induced decreases in total tissue concentration of dopamine in the nucleus accumbens and decreased dopamine turnover in the PVN in Chapter 4. Galanin protein levels in the nucleus accumbens were not altered after CFA in Chapter 3 though, suggesting that the local effects in this region might be due to chronic stimulation, rather than acute galanin release during activity. The functional consequences of these changes remain unclear but may have broad implications for circadian, metabolic, and endocrine changes associated with chronic inflammatory pain and depression. Furthermore, galanin inputs to the nucleus accumbens from the PVN and indirect activation of the mesolimbic pathway via the PVN provide alternate pathways by which galanin could mediate mesolimbic dopamine activity. Therefore, future research should be directed toward elucidating the role of galanin in the PVN in conditions of chronic inflammatory pain.

Although our data are preliminary and only tenuous conclusions can be drawn, we propose the following model to explain these results and a potential pathway to comorbid

depression-like motivational deficits. CFA administration induces a rapid and prolonged increase in swelling of the ipsilateral hind paw. Swelling is accompanied by allodynia and hyperalgesia that last for at least 6 weeks, as well as a non-evoked chronic pain state. Like other salient stimuli and stressors, noxious stimuli activate noradrenergic neurons in the LC and dopamine neurons in the VTA. This likely serves to enhance attention and vigilance, encode information about salience, and mediate motivation-related behavioral responses to the stimulus. Allodynia and hyperalgesia of the hind paw presumably corresponds to increased daily frequency of acute noxious stimulation, as well as, a sustained non-evoked chronic pain state. Repeated activation of the LC by the same stimulus leads to compensatory changes to LC signaling and norepinephrine release in supraspinal target areas, such as the VTA. We propose that galanin activity is increased in LC-VTA projections in response to the increased LC activation. Increased LC-derived galanin activity in the VTA could then suppress norepinephrine release in the VTA, decreasing stimulatory input onto dopamine neurons. Galanin release could also directly inhibit dopamine neuron activity. Alternatively, chronic galanin receptor stimulation could alter VTA dopamine neuron excitability through changes in gene expression. Regardless of the precise mechanism, persistent alterations to LC-VTA circuitry might then account for decreased dopamine release in the nucleus accumbens and deficits to motivational behaviors, manifesting as a depression-like state. Our current data do not provide strong support for galanin's role in this model; however, additional research is needed before revisions can be made.

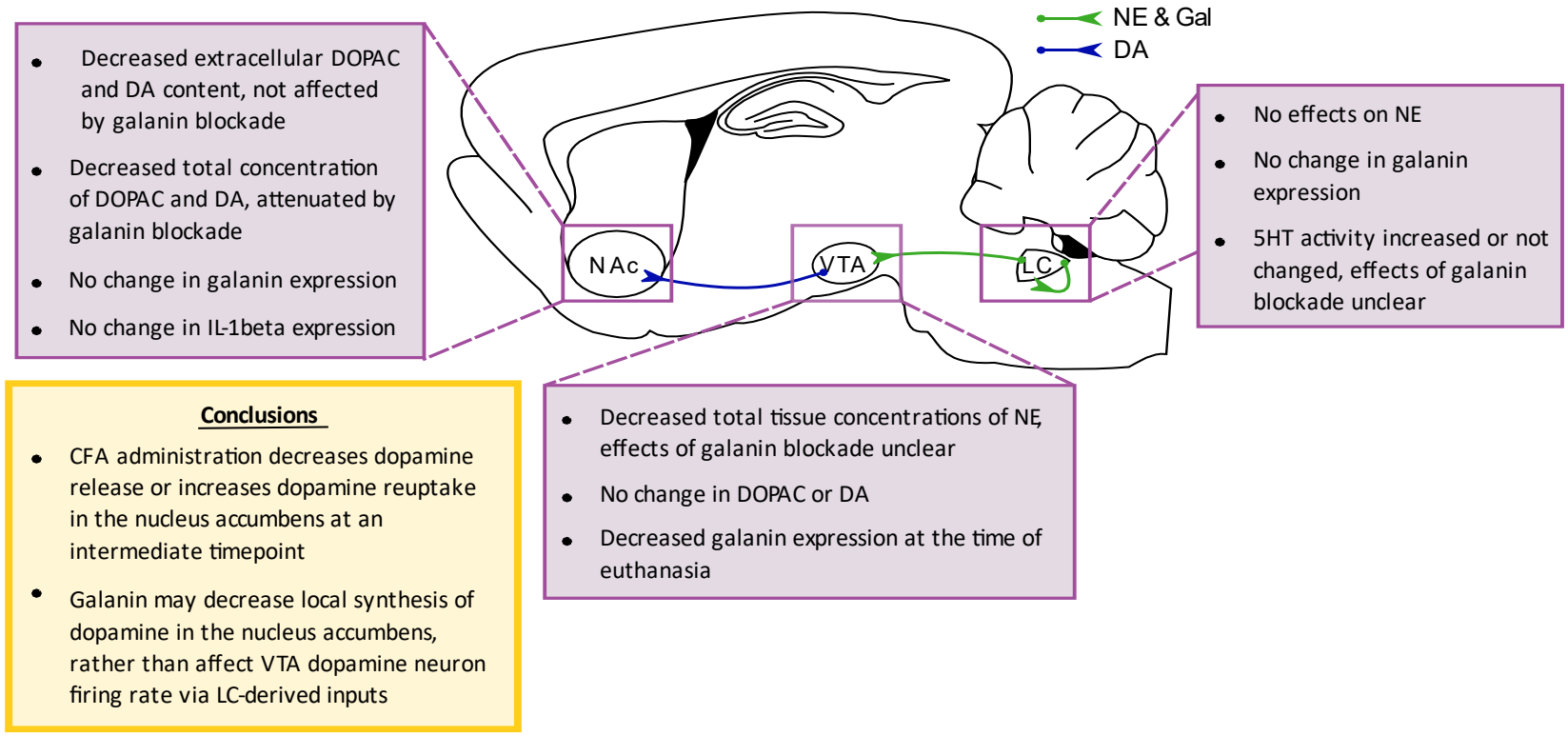


**Figure 6.1.** A summary of key findings related to the role of galanin in mediating changes to mesolimbic dopamine (DA) signaling after induction of chronic inflammatory pain using intraplantar complete Freund's adjuvant. Our data suggest that galanin activity during chronic inflammatory pain may affect DA synthesis in the nucleus accumbens, rather than ventral tegmental area (VTA) DA neuron activity. Whether locus coeruleus (LC)-derived norepinephrine and/or galanin affect VTA activity after chronic inflammatory pain remains unclear.