A ROLE FOR THE NEUROPEPTIDE GALANIN IN THE NEUROPROTECTIVE AND NEUROMODULATORY EFFECTS OF PHYSICAL ACTIVITY

by

JESSICA LEE GROVES-CHAPMAN

(Under the Direction of Philip V. Holmes)

ABSTRACT

Galanin expression has been shown to be upregulated in rats given free access to running wheels for three weeks. Upregulation of galanin and activation of specific galanin receptor subtypes have been linked to neuroprotection and enhanced cognitive functions. The signaling pathways through which these effects are mediated remain unknown. The first series of experiments were designed to elucidate the galanin receptor subtypes involved in neuroprotection, utilizing an intracerebroventricular (ICV) kainic acid excitotoxicity model in conjunction with receptor specific agonists. The results from these experiments indicate that the galanin receptor GALR1, and not GALR2, is required for the decrease in cell death within the Cornu Ammonis region 3 (CA3) of the hippocampus. However, none of the GALR agonists were observed to reduce seizure behavior. The purpose of the second series of experiments was to evaluate the endogenous upregulation of galanin within the locus coeruleus (LC) using an exercise model, and how this might influence behavior in a cocaine-primed condition place preference (CPP) model. The results indicate that physical activity influences cocaine-primed and stress-primed reinstatement to drug addiction in different ways. Access to a running wheel enhanced reinstatement to the drug-paired chamber following a cocaine priming injection, while

reducing reinstatement to the drug-paired chamber following uncontrollable footshock. *In situ* hybridization analysis revealed elevated levels of galanin mRNA expression in activity animals compared to sedentary counterparts for the stress-primed reinstatement test. Additionally, it was observed that sedentary animals in the cocaine-primed reinstatement test had elevated levels of cFos mRNA expression in cortical areas compared to activity animals.

INDEX WORDS: galanin; neuroprotection; excitotoxicity; voluntary wheel running; condition place preference; cocaine-primed reinstatement; stress-primed reinstatement

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JESSICA LEE GROVES-CHAPMAN

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MS, University of Georgia 2010

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Major Professors:	Philip V. Holmes Rod K. Dishman
Committee:	Gaylen Edwards John Wagner

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

This work is dedicated to the memory of my grandfather, Dr. Barney R. Groves; and in honor of my parents, Gregory and Patricia Groves, and my husband, Marvin E. Chapman III. Without the unconditional love and support of my family this journey and the achievement of this dream would never have occurred. I owe everything I have accomplished to their belief in me.

I would also like to dedicate this work to my children, may they always know that hard work and determination will take you anywhere you want to go.

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CHAPTER 1

INTRODUCTION

Physical activity and exercise have become the focus of many areas of research within neuroscience over the past few decades. Evidence, from both human and animal studies now suggests that exercise is a viable therapeutic intervention for psychological and neurological disorders. In human studies, exercise has been associated with decreases in reported and observed depression and anxiety symptomology, enhanced cognitive functioning, prevention of the development of Alzheimer's disease, and protection from traumatic brain insult and injury (Buckworth, Dishman, O'Connor, & Tomporowski, 2013; Dishman et al., 2006; Radak et al., 2010). Animal studies utilizing voluntary wheel running have provided evidence that suggests physical activity yields increased neurogenesis, enhanced synaptic plasticity, and protection from neuronal insult and seizure (Dishman et al., 2006; Duman, Schlesinger, Russell, & Duman, 2008; Dunn & Dishman, 1991; Eisenstein & Holmes, 2007; Farmer et al., 2004; Garcia-Capdevila, Portell-Cortes, Torras-Garcia, Coll-Andreu, & Costa-Miserachs, 2009; Gobbo & O'Mara, 2005; Greenwood et al., 2011; Greenwood, Strong, Foley, & Fleshner, 2009; Griffin, Bechara, Birch, & Kelly, 2009; Holmes, Yoo, & Dishman, 2006; Murray et al., 2010; Olson, Eadie, Ernst, & Christie, 2006; Reiss, Dishman, Boyd, Robinson, & Holmes, 2009; Sartori et al., 2009; Sciolino, Dishman, & Holmes, 2012; Van Hoomissen, Chambliss, Holmes, & Dishman, 2003). Further support of the neuroprotective role that exercise and physical activity have on the brain comes from rodent models of psychopathology that indicate modulation of signaling pathways involved within the limbic system structures that regulate mood and cognition (Chaouloff, 1989; Cotman

& Berchtold, 2002; Duman et al., 2008; Dunn & Dishman, 1991; Greenwood et al., 2011; Hillman, Erickson, & Kramer, 2008). Emphasis on catecholamine and indolamine systems regulation, and the influence of activity on neurotrophic factor and neuropeptide induction, indicates that the neurophysiological effects of exercise are widespread (Buckworth et al., 2013; Dishman et al., 2006; Duman et al., 2008; Dunn & Dishman, 1991; Eisenstein & Holmes, 2007; Farmer et al., 2004; Garcia-Capdevila et al., 2009; Gobbo & O'Mara, 2005; Greenwood et al., 2011; Greenwood et al., 2009; Grace Sophia Griesbach, Gomez-Pinilla, & Hovda, 2004; Griffin et al., 2009; Groves-Chapman et al., 2011; Ma, 2008; Murray et al., 2010; Olson et al., 2006; Reiss et al., 2009a; Sartori et al., 2009; Sciolino et al., 2012; Tong, Shen, Perreau, Balazs, & Cotman, 2001; van Praag, 2009).

A neuropeptide directly influenced by exercise is the 29 amino acid peptide galanin. A number of studies have provided evidence that galanin expression within the locus coeruleus is influenced by physical activity, specifically three weeks of uninterrupted voluntary wheel running (Holmes et al., 2006; Murray et al., 2010; Reiss et al., 2009a; Sciolino et al., 2012). Galanin serves as a key neuromodulator of the catecholamines, indolamines, and various other transmitters involved in both excitatory and inhibitory signaling, and is colocalized with many of these throughout the brain (Ash & Djouma, 2011). The mechanisms by which galanin produces these neuromodulatory effects on signaling pathways associated with mood, cognition, appetitive behaviors, etc. remain unknown. However, three primary galanin receptor subtypes have been identified, GALR1 – GALR3. GALR1 and GALR2 have previously been implicated as being directly associated with the neuroprotective attributes of galanin, with widespread distribution throughout the brain; including dense populations within the hippocampal formation (Branchek, Smith, Gerald, & Walker, 2000a; Hökfelt & Tatemoto, 2008; Runesson, Robinson, Sollenberg,

& Langel, 2009; Waters & Krause, 1999; Webling, Runesson, Bartfai, & Langel, 2012). GALR3 is less established as a primary receptor in rodent models, and appears to more limited expression in the rat brain (Webling, Runesson, Bartfai, & Langel, 2012). Understanding the signaling cascade of each receptor subtype continues to be a topic of great interest in excitotoxic models, such as Alzheimer's disease, stroke and seizure.

Galanin has also been shown to produce modulatory effects within the mesocorticolimbic dopamine system. Specifically, galanin has been shown to produce inhibition of VTA signaling and reduce dopamine in striatal slices (Ericson & Ahlenius, 1999; Grenhoff, Nisell, Ferre, Aston-Jones, & Svensson, 1993; Tsuda, Tsuda, Nishio, Masuyama, & Goldstein, 1998; Weiss et al., 2005), as well as enhance dopamine release in the ventral striatum following direct injections into the PVN (Rada, Mark, & Hoebel, 1998). Galanin has also been determined to influence a variety of drug-seeking and addiction-related behaviors, with most evidence indicating suppressive effects (Ericson & Ahlenius, 1999; Ogbonmwan; Picciotto, Brabant, Einstein, Kamens, & Neugebauer, 2010; Rada et al., 1998; Tsuda et al., 1998; Weiss et al., 2005). In a more recent experiment conducted in our laboratory, it was established that administration of the galanin agonist galnon yielded reductions in cortical dopamine and altered the time course of striatal dopamine post-cocaine in an *in vivo* model (Ogbonmwan). Additionally, it was observed that administration of galnon reduced the stimulatory effect of cocaine on locomotor behavior and on reinstatement of drug-seeking behavior in a self-administration model (Ogbonmwan). This evidence supports a modulatory role for galanin.

Conditioned place preference (CPP) is a behavioral model of drug seeking behavior that measures the capacity for drug-paired contextual cues to elicit appetitive behavior. Since the CPP paradigm depends on contextual learning as well as changes in motivations states,

experimental manipulations that differentially influence these processes may produce conflicting results. This confounding of motivation and learning may account for the mixed and conflicting results reported for the effects of exercise on CPP (Lynch, Peterson, Sanchez, Abel, & Smith, 2013). Conflicting evidence also suggests that the type of exercise is key, with treadmill running manipulations producing results that are often contrary to results from voluntary wheel running studies (Lynch et al., 2013). This may be due to the stressful nature of forced exercise models, in which animals are often prodded or shocked to continue running on the treadmill (Arida, Scorza, da Silva, Scorza, & Cavalheiro, 2004; Yanagita, Amemiya, Suzuki, & Kita, 2007). In conjunction with reports on galanin's role in stress, resilience, and anxiety (Picciotto et al., 2010; Sciolino et al., 2012), these findings suggest that stress may play a role in how exercise-induced galanin modulates the mesocorticolimbic dopamine systems. A further consideration of the activity and CPP model lies in the connection between physical activity and enhanced performance on learning and memory tasks (Cotman & Berchtold, 2002; Dishman et al., 2006; Eisenstein & Holmes, 2007; Etnier & Chang, 2009; Garcia-Capdevila et al., 2009; Gobbo & O'Mara, 2005; Griffin et al., 2009; Sartori et al., 2009). Galanin has also been associated with protection from the cognitive impairments produced by Alzheimer's, as well as functioning as an important neuropeptide within hippocampal-specific learning models (Ding, MacTavish, Kar, & Jhamandas, 2006a; Heneka et al., 2010; Heneka, O'Banion, Terwel, & Kummer, 2010; Hobson et al., 2008; Runesson, Robinson, et al., 2009).

For the study presented in Chapter 3 it was hypothesized that either galanin receptors 1, 2, or both would result in protection from kainic acid-induced neuronal cell death in the CA3. Additionally, it was predicted that this protection would extend to a reduction in the observed motor seizure behaviors. In the experiments utilizing the cocaine-primed CPP model, Chapter 4,

it was hypothesized that activity-dependent galanin (induced by three weeks of voluntary wheel running to avoid any potential stress influence associated with forced activity) would reduce cocaine and stress-primed reinstatement to drug seeking (Rozeske, Greenwood, Fleshner, Watkins, & Maier, 2011; Smith, Pennock, Walker, & Lang, 2012). It was also hypothesized that wheel running would produce an enhancement in the acquisition of CPP, and yield faster extinction of this association based upon evidence for enhanced cognitive performance in exercising animals (Eisenstein & Holmes, 2007; Garcia-Capdevila et al., 2009; Greenwood et al., 2009; Ploughman, 2008).

CHAPTER 2

LITERATURE REVIEW

Exercise and Physical Activity: A role in Neuroprotection

It is widely accepted that exercise and a physically active lifestyle provide many health benefits and protections against the development of disease. Exercise is associated with improvements in cardiovascular function, enhanced insulin sensitivity, and weight management; as well as being associated with the reduction in mortality across the lifespan (Garber et al., 2011). Physical activity in human and rodent models indicate that physical activity also influences cognitive function, providing protection against the onset of dementia and cognitive deficits associated with Alzheimer's disease, protection from neurological insult such as stroke, and the reduction and prevention of mood disorders (Dishman et al., 2006; Ganguly & Guha, 2010; Garber et al., 2011; Garza, Ha, Garcia, Chen, & Russo-Neustadt, 2004; Hillman et al., 2008; Ma, 2008; Middleton et al., 2013; Ploughman, 2008; van Praag, 2009). Evidence from animal studies indicates that physical activity provides robust effects throughout the central nervous system, including the modulation and maintenance of neurotransmitter and neuropeptide systems (Chaouloff, 1989; Dishman et al., 2006; Hillman et al., 2008; Ma, 2008; Sciolino & Holmes, 2012; van Praag, 2009).

In rodent models of exercise, voluntary activity such as wheel running has been shown to confer the benefits of physical activity without the added confound of stressing an animal through forced running (Burghardt, Fulk, Hand, & Wilson, 2004; Sciolino & Holmes, 2012). Reports also indicate that physical activity offers protection from stress in the form of promoting

stress resilience and reductions in anxiety (Greenwood et al., 2003; Picciotto et al., 2010; Sciolino et al., 2012). Voluntary wheel running is an exercise model for rodents that provides a natural mechanism by which to induce neurogenesis, neuroprotection, and influence neurotransmitter synthesis, release, reuptake, and receptor densities. Wheel running has also been linked to alterations in the synthesis and release of other trophic factors and neuropeptides (Chaouloff, 1989; Cotman & Berchtold, 2002; Dishman et al., 2006; Duman et al., 2008; Dunn & Dishman, 1991; Farmer et al., 2004; Garcia-Capdevila et al., 2009; Garza et al., 2004; Gobbo & O'Mara, 2005; Gomez-Pinilla, Vaynman, & Ying, 2008; Gomez-Pinilla, Ying, Roy, Molteni, & Edgerton, 2002; Greenwood et al., 2003; Greenwood et al., 2011; Greenwood et al., 2009; Griesbach, Hovda, Gomez-Pinilla, & Sutton, 2008; Griesbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004; Griffin et al., 2009; Holmes et al., 2006; Lynch et al., 2013; Lynch, Piehl, Acosta, Peterson, & Hemby, 2010; Marais, Stein, & Daniels, 2009; Neeper, Gomez-Pinilla, Choi, & Cotman, 1996; Sarbadhikari & Saha, 2006; Sciolino et al., 2012).

While the duration wheel access required for behavioral and neurobiological effects of running has not been well established in the literature, evidence from the our laboratory promotes a three week, 24-hour access to a running wheel model that has been established as necessary for inducing certain neuropeptide and neurotrophic factor expression (Eisenstein & Holmes, 2007; Groves-Chapman et al., 2011; Holmes et al., 2006; Murray et al., 2010; Reiss et al., 2009; Sciolino et al., 2012; Van Hoomissen, Holmes, Zellner, Poudevigne, & Dishman, 2004). One neuropeptide in particular, galanin, reflects elevations in both mRNA expression and protein levels following three weeks of voluntary wheel running (Holmes et al., 2006; Murray et al., 2010; Reiss et al., 2009; Sciolino et al., 2012). Exercise-induced upregulation of galanin has been associated with in enhancements in learning, reductions in depression and anxiety

behaviors, neurogenesis, and protection from neuronal insult such as seizure (Dishman et al., 2006; Eisenstein & Holmes, 2007; Holmes et al., 2006; Reiss et al., 2009; Sciolino et al., 2012).

Galanin: A Complex Neuropeptide

Galanin is a 29 amino-acid peptide that is primarily produced in the brain stem A6 and A2 regions, with most cell bodies located in the locus coeruleus (LC). Galanin has an been established as providing neuromodulatory, neurotrophic, and neuroprotective effects which are evident from the widespread projections throughout the brain (Runesson, Saar, Lundström, Järv, & Langel, 2009). The LC is the location of primary projections of the galaninergic system, with other important projections of galanin extending to the frontal cortex, ventral striatum, ventral tegmental area, and hippocampus. To date, 3 primary galanin receptors have been established, GALR1 – GALR3, all of which are G-protein coupled receptors associated with excitatory and inhibitory signaling. All three subtypes are G protein-coupled and can activate Gi and Go proteins (Lang, Gundlach, & Kofler, 2007). More is presently known about the distribution and signaling properties of GALR1 and GALR2, which exist in higher densities throughout the rat brain than GALR3 (Webling et al., 2012), and therefore the rest of this section will focus on these two receptors only.

GALR1 expression is regulated by cyclic adenosine monophosphate (cAMP) and has been identified in many brain regions, including the hippocampus, hypothalamus, amygdala, thalamus, cortex, and brain stem. This receptor produces inhibitory signaling through Gi/Go G-protein inhibition of adenylyl cyclase (AC), which in turn produces a decrease in the production of cAMP, yielding an inhibition in phosphorylated cAMP response element binding protein (CREB). This signaling cascade leads to the opening of G-protein inward rectifying potassium channels (GIRKs), producing the inhibitory effects observed post-activation. Finally, GALR1

activation has been established as stimulating mitogen activating protein kinase (MAPK) due to the influence of the βγ subunit of Gαi, in a protein kinase C-independent mechanism (PKC) (Lang et al., 2007; Webling et al., 2012).

GALR2 in the rat brain is expressed in the hypothalamus, dentate gyrus, amygdala, piriform cortex, and mammillary nuclei (K. E. Webling et al., 2012). GALR2 activation influences multiple classes of G-proteins, including Gi/o, thus producing inhibitory effects on downstream CREB through inhibition of cAMP. However, unlike GALR1, GALR2 also activates G-proteins such as Gq/11 that yield stimulatory effects by inducing an increase in Ca2+ release and the opening of Ca2+-dependent chloride channels following initiation of phospholipase C activation (Lang et al., 2007; Webling et al., 2012). Finally, GALR2 activates MAPK in a PKC-dependent mechanism, presumably through Gαo G-proteins (Webling et al., 2012).

Due to the extensive influence of galanin in many brain regions and neurotransmitter systems, via both excitatory and inhibitory signaling, it is not surprising that galanin serves as a neuromodulator, trophic factor, and confers neuroprotection throughout the brain (Mitsukawa, Lu, & Bartfai, 2008; Runesson, Robinson, et al., 2009). As the next sections will indicate, galanin modulation serves to fine tune the critical systems of the brain, essential to responses optimal to positive health outcomes.

Galanin and Neuroprotection

Galanin has had a complex history as a neuropeptide involved in neuroprotection with evidence supporting galanin as providing protection from neuronal insults and injury, as well as promoting resilience in mood and stress-related disorders; however other reports indicate that

galanin is a causal factor in cognitive deficits associated with Alzheimer's Disease (Counts, Perez, Ginsberg, & Mufson, 2010; Ding et al., 2006; Einstein, Asaka, Yeckel, Higley, & Picciotto, 2013; M.T. Heneka et al., 2010; Hobson et al., 2008a; Holmes & Picciotto, 2006; Kapur, 2011; Kuteeva, Wardi, Lundström, Sollenberg, Langel, Hökfelt, & Ogren, 2008; Lerner, Sankar, & Mazarati, 2010; Lu et al., 2010; Lundstrom, Elmquist, Bartfai, & Langel, 2005; Malin et al., 1992; A. M. Mazarati, 2004; Mitsukawa, Lu, & Bartfai, 2010; Ögren et al., 1998; Ögren, Kuteeva, Elvander-Tottie, & Hökfelt, 2010; Ögren, Pramanik, & Schött, 1996; Picciotto et al., 2010; Robinson & Brewer, 2008; Runesson, Robinson, et al., 2009; Schött, Bjelke, & Ogren, 1998; Sciolino et al., 2012; Tsuda et al., 1998; Vrontakis, 2002; Yoshitake et al., 2011; Zhao, Seese, Yun, Peng, & Wang, 2013). For example, the evidence suggesting a causal role in the dementia and cognitive deficits associated with Alzheimer's Disease through inhibition of acetylcholine remains abundant and well substantiated, as galanin directly inhibits acetylcholine signaling in the ventral hippocampus and other regions associated with cognition and memory (Mitsukawa et al., 2010; Vrontakis, 2002). Additionally, other evidence indicates that galanin infusion produces impairments in learning and memory models (Mitsukawa et al., 2010; Ogren, Kehr, & Schött, 1996; Schött et al., 1998). However, other reports indicate that galanin signaling provides protective modulatory effects in neurotransmitter systems like norepinephrine, serotonin, and dopamine that are directly linked to cognition, mood, and stress regulation. Evidence strongly supports galanin's involvement in anticonvulsant mechanisms, neurogenesis, and providing antidepressant and anxiolytic effects (Elliott-Hunt et al., 2004; Elliott-Hunt, Pope, Vanderplank, & Wynick, 2007; Ericson & Ahlenius, 1999; Hobson et al., 2008; Holmes & Picciotto, 2006; Kapur, 2011; Kong, Lorenzana, Deng, McNeill, & Schauwecker, 2008; Kuteeva, Wardi, Lundström, Sollenberg, Langel, Hökfelt, & Ogren, 2008; Lerner et al., 2010;

Lundstrom et al., 2005; Mahoney et al., 2003; A. Mazarati & Lu, 2005; Andrey Mazarati et al., 2004; Mazarati, Lu, Shinmei, Badie-Mahdavi, & Bartfai, 2004; Mazarati et al., 2006b; Mazarati, 2004; Mazarati et al., 2000; Ogbonmwan; Reiss et al., 2009; Robinson & Brewer, 2008; Wrenn & Crawley, 2001; Zhao et al., 2013). In order to better understand the complexities in galanin's neuroprotective features a better understanding of the differential signaling cascades of the galanin receptor subtypes and their associated distributions, needs to be established.

Literature on galanin and neuroprotection from neuronal insult (i.e. excitotoxicity) has yielded evidence suggesting the differential actions of GALR1 and GALR2 activation in providing protection from excitotoxicity, with most evidence supporting activation of GALR2 (Dobolyi et al., 2014; Elliott-Hunt et al., 2007; Lerner et al., 2010; Lu et al., 2010; Mazarati et al., 2004; Pirondi et al., 2005a; Runesson, Robinson, et al., 2009; Wang, Hashemi, Fried, Clemmons, & Hawes, 1998). Yet most of the evidence supporting GALR2 activation in inhibiting excess glutamatergic signaling in excitotoxic models predates the development of GALR1 specific agonists (Mazarati, 2004). Much of the evidence on galanin indicates that it does not differentiate between GALR1 and GALR2, and does not selectively bind to one over the other (Mazarati, 2004). Combined with more recent evidence on the involvement of GALR1 in epilepsy and seizure models, this suggests that it is plausible that GALR1 is also essential for the observed neuroprotective effects of galanin in excitotoxicity models (Lerner et al., 2010).

One particular excitotoxicity model utilizes the toxin kainic acid (KA) to yield excess glutamatergic signaling throughout the brain (Sperk, 1994). Evidence suggests that intracerebroventricular (ICV) administration of kainic acid yields robust neuronal cell death in the CA3 region of the hippocampus, thus allowing for a direct measure of neuroprotection via cell counts (Reiss et al., 2009; Sperk, 1994). In one study involving three weeks of running

wheel access prior to KA-excitotoxicity, it was determined that running activity protected against both neuronal apoptosis in the CA3 region, as well as a reduction in seizure severity (Reiss et al., 2009). This same study also measured galanin mRNA in the LC and found that animals with reduced neuronal apoptosis and seizure activity also had increased levels of galanin mRNA (Reiss et al., 2009). However, the mechanism by which this protective effect of galanin occurs is still unknown. While the role of GALR1 and GALR2 activation in neuroprotective models has been somewhat evaluated (Burazin & Gundlach, 1998; Einstein et al., 2013; Elliott-Hunt et al., 2007; Mahoney et al., 2003; Mitsukawa et al., 2010; Runesson, Robinson, et al., 2009), it is still unclear to what extent each contributes to the association between galanin and protection from excitotoxicity. To determine the specific galanin receptor involvement in protection from KA-excitotoxicity, experiments were conducted in Paper 1 (Chapter 3) to evaluate the efficacy of galanin receptor agonists with different affinities for both GALR1 and GALR2. Contrary to other findings on the necessary activation and expression of GALR2 within the hippocampus for neuroprotective effects of galanin, Paper 1 presents evidence that GALR1 is essential for these effects in a KA-excitotoxicity model.

Galanin and Addiction

Adding to the complexity of this neuropeptide's role in the central nervous system, galanin projects to and is colocalized with many neurotransmitters throughout the brain (Ash & Djouma, 2011). Galanin serves as a neuromodulator for various systems, including but not limited to the dopaminergic mesolimbic and mesocortical systems (Ash & Djouma, 2011). The role of neuromodulators, like Galanin, is to fine tune neurochemical signaling responses to stimuli. Evidence indicating galanin's involvement in suppressing dopamine (DA) release in striatal slices and inhibiting ventral tegmental area neurons following bath application is not in

direct contradiction with other reports of galanin enhancing DA release in the ventral striatum following injection into the paraventricular nucleus (Ericson & Ahlenius, 1999; Rada et al., 1998; Tsuda et al., 1998; Weiss et al., 2005). In a more recent investigation, intraperitoneal (IP) injections of a galanin agonist, Galnon, yielded altered DA overflow in the prefrontal cortex (PFC) and ventral striatum (VS) differentially (Ogbonmwan). Due to the interactions with the dopaminergic systems galanin has also been indicated to influence a variety of drug-seeking and addiction-related behaviors (Holmes & Picciotto, 2006; Picciotto et al., 2010). Most of the evidence on galanin's role in addiction models reflects suppressive effects on drug-seeking and reinstatement to drug-seeking (Brabant, Alleva, Quertemont, & Tirelli, 2010; Einstein et al., 2013; Holmes & Picciotto, 2006; Ogbonmwan; Picciotto et al., 2010; Rada et al., 1998; Tsuda et al., 1998; Zhao et al., 2013), yet galanin the neurochemical mechanisms that underlie the effects of galanin agonists are largely unidentified. It is presumed that these effects would arise from inhibition of dopamine release within regions like the PFC and/or VS, which are involved directly in addiction-related behaviors (Ash & Djouma, 2011).

In a recent study conducted in our laboratory, IP administered galnon was determined to not only alter in-vivo catecholamine release within the PFC and VS following cocaine administration but subsequently reduced the stimulatory effect of cocaine on open field behavior (Ogbonmwan). It was also observed that while galnon dose-dependently decreased dopamine overflow in the cortex, the affect in the ventral striatum was quite different. The study found that galnon dose-dependently enhanced the time course of dopamine release within the ventral striatum. In a separate group of animals, galnon reduced drug-seeking behavior in a self-administration, cocaine-primed reinstatement model, suggesting that galanin's suppression of

behavioral effects may be the result of multifaceted signaling modulation in the dopaminergic systems (Ogbonmwan).

The LC's projections to various brain regions involved in addiction, such as the PFC and VS, provide further evidence in support of galanin's modulatory role in addiction-related behaviors (Ash & Djouma, 2011). While the development of galanin receptor agonists is a component of the research on therapeutic options for addiction treatment (Ash & Djouma, 2011), another area of interest utilizes exercise (Greenwood et al., 2011; Lynch et al., 2013; Lynch et al., 2010; Ma, 2008; Malin et al., 1992; Rozeske et al., 2011; Smith, Gergans, Iordanou, & Lyle, 2008; Smith et al., 2012). As indicated previously, voluntary wheel running in rodents induces a natural upregulation in galanin mRNA expression and protein levels within the LC, making this a viable area to explore the effects of naturally induced galanin on addiction-related behaviors. However, to assess running effects on addiction, self-administration models are not the best option due to the complications of surgical manipulations and activity restriction during administration of cocaine (Bardo & Bevins, 2000; Tzschentke, 2007). Condition place preference (CPP) provides a mechanism by which to evaluate addiction-related behaviors at various stages, from acquisition to reinstatement of drug seeking, all while providing a simpler behavioral model that is less-invasive than self-administration models (Carboni & Vacca, 2003). The current literature on CPP and running is fraught with contradictions, with many reports that running enhances CPP and also reduces acquisition (Smith et al., 2008). Furthermore, the evidence on exercise effects on reinstatement to drug-seeking behavior suggests that exercise confers protection in both cocaine- and stress-primed reinstatement models (Smith et al., 2012). Confounding these findings is the immense amount of evidence supporting exercise as promoting performance on cognitive tasks involving learning and memory (Buckworth et al.,

2013; Dishman et al., 2006). Additionally, as was previously addressed, galanin has been associated with cognitive decline in Alzheimer's disease. This leaves many conflicting questions left to be addressed. Does exercise promote learning or protect against addiction in a learning and memory task like CPP? Does galanin inhibit learning, or can it be associated with cognitive enhancements observed in exercising animals?

In an attempt to tease out the answers to these questions, the experiments presented in Paper 2 were conducted to evaluate the effects of three weeks of voluntary wheel running on cocaine-primed CPP. The results indicate that voluntary wheel running does not enhance or inhibit acquisition, yet it does influence reinstatement to drug-seeking behavior. Consistent with previously mentioned reports of galanin's effects on reinstatement behavior, running reduced stress-primed reinstatement in a galanin-dependent manner. However, as will be discussed later, running was also observed to enhance cocaine-primed reinstatement, in a galanin-independent manner.

Summary

The following studies aim to elucidate mechanisms by which physical activity, via upregulation of galanin, may confer neuroprotective and neuromodulatory effects within identified regions of the brain. The first experiment (Chapter 3) investigates the role of specific galanin receptors in protection from excitotoxicity with the CA3 region of the hippocampus. The second experiment (Chapter 4) provides evidence in support of activity-dependent upregulation of galanin and the associated modulation of the mesolimbic dopamine system. The role of galanin and the hypothesized neuromodulatory effects were evaluated through changes in reinstatement to drug-seeking behaviors as measured in a cocaine-primed CPP model of addiction. To elucidate the neurochemical changes associated with these hypothesized

behavioral difference galanin and activity-dependent genes were measured within the locus coeruleus and nucleus accumbens regions.

CHAPTER 3

PHARMACOLOGICAL STIMULATION OF GALANIN RECEPTOR 1 BUT NOT GALANIN RECEPTOR 2 ATTENUATES KAINIC ACID-INDUCED NEURONAL CELL DEATH IN THE RAT HIPPOCAMPUS[1]

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[1]Groves-Chapman, J. L.*, Webling, K.*, Runesson, J., Saar, I., Lang, A., Sillard, R., Jakovenko, E., Kofler, B., Holmes, P.V., Langel, Ü. To be submitted to *Peptides*.

Abstract

The neuropeptide galanin is widely distributed in the central and peripheral nervous systems and part of a bigger family of bioactive peptides. Galanin exerts its biological activity through three G-protein coupled receptor subtypes, GALR1-3. Throughout the last 20 years, data has accumulated that galanin can have a neuroprotective effect that probably is mediated through the activation of GALR1 and GALR2. In order to test the pharmaceutical potential of application of galanin receptor subtype selective ligands to inhibit excitotoxic cell death, the first step involved the development of an additional galanin receptor ligand, M1154, which is a GALR1/2 selective ligand in a binding assay. We thereafter adapted a single protocol to use as a signaling assay for all three galanin receptors, by utilizing a non-labeled real time instrument, to ease classification of novel ligands according to their ability to activate the different galanin receptor subtypes.

The novel M1154 peptide was compared to earlier published selective ligands, namely M617 and M1145, in its ability to reduce the excitotoxic effects of kainic acid. Though there was no significant difference in the time course or severity of the kainic acid-induced seizure behavior, administration of either M617 or M1154 before KA administration significantly attenuated the neuronal cell death in the hippocampus. Our results clearly indicate the potential therapeutic value of agonists selective for GALR1 in the prevention of neuronal cell death.

Keywords: GPCR, galanin, neuropeptide, galanin receptors, selective galanin receptor ligands, GALR1, label-free real time technology, excitotoxicity, M1154

Introduction

Excitotoxicity is involved in a variety of acute and chronic neurodegenerative conditions in the central nervous system (CNS) such as hypoxia–ischemia, status epilepticus, Alzheimer's disease, Parkinsons disease, amyloid lateral sclerosis (ALS) and multiple sclerosis (MS). Neuronal damage by glutamate excitotoxicity was identified as early as 1969 (Olney, 1969) and has been extensively studied since then. Despite more than 50 years of research, there are currently no pharmacological interventions in the clinical settings of acute neurodegenerative conditions.

One of the most well documented excitotoxins is kainic acid (KA), an analog of glutamic acid, of which the pathological changes partially mimic neurodegeneration. Thus, KA-induced neurodegeneration in rodents has been used as a model for exploring relevant pharmacological treatment of excitotoxicity in neurodegenerative disorders. Furthermore, central administration of KA has been known to produce convulsions through activation of the excitatory amino acid receptors. Therefore, KA has also been used to make a model for the study of epilepsy (Ben-Ari & Cossart, 2000). Centrally administered KA induces secondary damage in a number of structures and areas associated with the epileptic seizure, i.e. the cell damage is not caused by a direct KA-induced excitotoxic mechanism (reviewed in Jarrard, 2002; Sperk, 1994). It can therefore be difficult to dissociate direct and indirect neuroprotection effects in paradigms where seizures are induced, since an indirect neuroprotection through anticonvulsant properties of any pharmaceutical might be interpreted as a direct neuroprotective action of the drug (reviewed in (Mazarati et al., 2004). Subsequently, in this study, KA was administered intracerebroventricular (ICV) in a dose that was earlier optimized to ensure neuronal cell death with a direct excitotoxic mechanism (Reiss et al., 2009), thus allowing us to study the neuroprotective components of the compounds used.

There are numerous compounds that have been proposed to have either or both neuroprotective and anticonvulsant activities. One of these is the neuropeptide galanin. Galanin is a 29 amino acid (30 in humans) bioactive peptide distributed broadly in the brain, spinal cord and gut and which has been ascribed involvement in a diversity of physiological actions such as food intake (Saar et al., 2011), mood (Kuteeva, Wardi, Lundström, Sollenberg, Langel, Hökfelt, & Ögren, 2008), nociception (Jimenez-Andrade et al., 2006), modulating the effects of drug abuse (Jackson, Chen, Miles, Harenza, & Damaj, 2011) and recently in regulation of hair growth (Holub et al., 2012). The expression of galanin is highly plastic and is markedly up-regulated in many areas under certain physiological conditions. Galanin conducts its effects via three members (GALR1-3) of the G-protein-coupled receptor (GPCR) superfamily. The receptors have distinct distribution patterns as well as signaling pathways. GALR1 and GalR3 has been reported to predominantly signal through Gi/o, leading to reduced cAMP-levels, whereas GALR2 mainly signal through Gq/11, leading to inositol phosphate accumulation and an increase in intracellular [Ca^{2+}] (Branchek, Smith, Gerald, & Walker, 2000b).

It has been shown that galanin serves in developmental survival and functions as a trophic factor to adult neurons (Abbosh, Lawkowski, Zaben, & Gray, 2011). David Wynick and colleagues have shown that GALR2 mediates this effect by utilizing several transgenic mice lines and the selective GALR2/GalR3 ligand, galanin(2-11) (reviewed in Hobson et al., 2008). Several lines of evidence reveal that galanin functions as an endogenous neuroprotective factor for hippocampal neurons. A recombinant adeno-associated viral (AAV) system that overexpress galanin together with the fibronectin secretory signal sequence succeeded to attenuate the neuronal death of centrally administered KA (Haberman, Samulski, & McCown, 2003). Transgenic galanin knock-out mice display a greater cell death than wildtype littermates when

KA is administered i.p., in concordance with the reduction of cell death seen in galanin overexpressing (OE) mice (Elliott-Hunt et al., 2004). In vitro studies in hippocampal cultures from these transgenic mice have confirmed that endogenous galanin diminishes excitotoxicity and apoptosis (Elliott-Hunt et al., 2004a). The authors proposed that this neuroprotection is mediated primarily through the regulation of hippocampal excitability. A recent study showed that administration of M15, a non-selective subtype galanin receptor antagonist (see (Lang, Gundlach, & Kofler, 2007)), significantly increases cell death in several hippocampal areas after systemic administration of KA (Schauwecker, 2010).

There is conflicting data regarding which receptor subtype mediates this neuroprotective role of galanin. Studies utilizing organotypic and dispersed primary hippocampal cultures have shown the importance of GALR2 in galanin-mediated neuroprotection, when cultured cells are exposed to staurosporine (Elliott-Hunt et al., 2004), glutamate (Pirondi et al., 2005b) and Amyloid-β (Ding, MacTavish, Kar, & Jhamandas, 2006b). In contrast, Elyse Schauwecker and colleagues have shown in a series of publications that there is a correlation between expression level of GALR1 and the neuronal cell death after excitotoxic assaults by systemic administration of KA (Kong et al., 2008; Schauwecker, 2010). Furthermore, it has been reported that GALR1 knock-out mice have an enhanced susceptibility to excitotoxin-induced neuronal injury (Mazarati et al., 2004; Mazarati, 2004).

Currently there are few subtype selective ligands available in the galanin field. To successfully engineer GPCR selective ligands, correct characterization is obviously a critical limitation in development, and may be the primary limitation in receptor specific ligands. Recently, label-free real-time technologies have emerged as a powerful tool to study GPCR signaling. These have numerous advantages which include that neither the ligand nor the

receptor are required to be labeled; simplifying assay design and minimizing artifacts or liabilities created by the labeling process (reviewed in Minor, 2008; Nayler, Birker-Robaczewska, & Gatfield, 2010; Scott & Peters, 2010). Cellular impedance based technology detects small changes in the contact area between cells and electrode due to changes in cell numbers and cell morphology, which can be affected by GPCRs. Advantages include a single detection system that can quantify responses to several GPCRs that are known to couple to different signaling pathways. This is particularly true when screening for subtype selective ligands of galanin receptors, since the galanin receptor subtypes have been shown to signal through different G-proteins.

The N-terminal part of galanin, residue 1-14, is highly conserved among species and is vital for receptor interaction and biological activity. Exemplifying the importance of the N-terminal portion of galanin for receptor binding, galanin(1-16), despite lacking half the galanin sequence, retains high affinity binding (Table 2). All three receptors display high affinity for galanin but are distinguishable by the fact that GALR1 do not tolerate N-terminal deletions of the galanin peptide in comparison to the other two receptors. This has successfully been explored in the design of several peptides, including the M871 (Sollenberg, Lundström, Bartfai, & Langel, 2006; Sollenberg, Runesson, Sillard, & Langel, 2010) and the M1145 peptide (Runesson, Saar, Lundström, Järv, & Langel, 2009), which both bind less to GALR1 compared to GALR2. The advancement in the galanin field is dependent on receptor subtype selective ligands or other means that selectively modify the expression of the different galanin receptor subtypes. An important advance in the field was the publication of the galanin fragment, galanin(2-11), as a non-GALR1 ligand (Liu et al., 2001; Lu, Lundström, & Bartfai, 2005). Two GALR1 preferential ligands have also been published, the M617 and the Gal-B2, with a modest, 25-fold and 15-fold

selectivity (respectively) for GALR1 compared to GALR2 (Bulaj et al., 2008; Lundström et al., 2005). The GalR3 interaction for M617 has also been characterized, showing a 200-fold difference when compared with GALR1 (Sollenberg et al., 2010).

In this study, we present a novel peptide with GALR1/GALR2 selective binding, namely M1154 (for sequence see Table 1). Furthermore, we introduce a single protocol for screening new ligands for subtype selective receptor signaling for all three galanin receptor subtypes, which is likely to promote the research effort to develop galanin receptor subtype specific ligands. We also demonstrate that M1154 is an agonist for both GALR1 and GALR2. Our data indicate, that M617 and M1154, but not M1145, significantly reduces neuronal cell death in a KA-excitotoxicity model. These findings suggest that the neuroprotective effect of pharmacological stimulation of galanin receptors *in vivo* after ICV administration of KA in the CA3 region is mediated through GALR1.

Materials and Methods

Peptide sequence design

The novel peptide M1154 (for sequence refer to Table 3.1) was designed to minimize biological response from GalR3, as a mixed GALR1/GALR2 ligand has been implicated as a putative therapeutic in several cases (Mitsukawa et al., 2008). For the design of M1154, particular interest was turned to the M617 peptide and its high affinity for GALR1. Deletion of Gly12 in several galanin analogous severely affect the interaction with GalR3 relative to GALR1 and GALR2 (Runesson et al., unpublished data), possibly due to the narrow binding pocket in GalR3 (Runesson et al., 2010) which does not tolerate the relative movement of the kink induced by Pro13 in these galanin analogs. Furthermore, we have shown earlier that the Ala21Arg mutation in M617 reduces the GalR3 affinity six-fold (Sollenberg et al., 2010). M1154 combines

these two modifications known to reduce GalR3 affinity relative to the other two galanin receptor subtypes, creating a GalR3 non-interacting galanin receptor ligand.

Cell culture

Bowes human melanoma cells (American type Culture Collection CRL-9607) expressing human GALR1 were cultured in Eagle's minimal essential medium with Glutamax-1 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Chinese Hamster Ovary (CHO) K1 cells stably expressing human GALR2 (a kind gift from Kathryn A. Jones and Tiina P. Iismaa, Sydney, Australia) were cultured in Dulbecco's modified essential medium F-12 with Glutamax supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Human embryonic kidney (HEK) 293 cells stably expressing rat GALR2 (a kind gift from Xiaoying Lu and Tamas Bartfai, La Jolla, USA) were cultured in Dulbecco's modified essential medium supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Lu et al., 2010). SHSY5Y cells stably expressing GALR1 or GALR2 were grown in minimum essential media supplemented with 10% fetal bovine serum, 1% sodium puruvate, 1% non-essential amino acids and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Flp-In T-REx 293 GalR3 cell line (a kind gift from F. Hoffman-La Roche Ltd, Basel, Switzerland) were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 15 μg/ml blasticidin S and 150 μg/ml Hydromycin B (J. Runesson, I. Saar, et al., 2009). Cell cultures were all grown at 37 °C in a 5% CO_2 incubator.

Peptide synthesis

The peptides were synthesized in a stepwise manner using small scale (0.1 mmol) 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis strategy on an automated Syro multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany). Fmoc-L-amino acids (Iris Biotech GmbH, Marktredwitz, Germany) were also coupled as hydroxybenzotriazole (HOBt) esters to a p-methylbenzylhydrylamine (MBHA) resin. The peptides were finally cleaved from the resin using 95% trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% H_2O solution for 3 h. All peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Discovery® C-18 Supelco® column (Sigma-Aldrich, Stockholm, Sweden) using a gradient of acetonitrile/water containing 0.1% TFA. The identity of the purified products was verified by Perkin Elmer prOTOF™ 2000 matrix-assisted laser desorption ionization time-of-flight mass-spectrometer (Perkin Elmer, Upplands Väsby, Sweden). The mass-spectra were acquired in positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix (Sigma-Aldrich, Stockholm, Sweden) (10 mg/ml, 7:3 acetonitrile:water, 0.1% TFA).

Galanin receptor binding studies

Cells for ^{125}I-galanin-receptor displacement studies were seeded in 150 mm cell culture dishes and cultured 2–4 days until confluent. Cell dishes were washed and scraped into phosphate-buffered saline (PBS) and centrifuged twice at 4 °C, 3000 x g for 5 min. The pellet was resuspended in assay buffer (20 mM HEPES, 5 mM $MgCl_2$, pH 7.4) supplemented with EDTA (5 mM) and incubated on ice for 45 min before centrifugation at 4 °C, 8500 x g for 15 min. After washing the pellet in assay buffer and repeated centrifugation the obtained pellet was resuspended in assay buffer supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich,

St. Louis, MO, USA) and stored at -80 degrees centigrade until used. Protein concentration was determined according to Bradford (BioRad, Stockholm, Sweden).

Displacement studies on cell membranes were performed in a final volume of 200 µl, containing 0.1-0.12 nM porcine-[^{125}I]-galanin (2200 Ci/mmol, Perkin–Elmer Life Science, Boston, MA, USA), 30 µg cell membrane, and various concentrations of peptide (10^{-5}-10^{-11} M). Peptide solutions were made in assay buffer supplemented with 0.3% BSA using silanized (dichlorodimethylsilane, Sigma-Aldrich, St. Louis, MO, USA) tubes, 96-well plates and pipette tips. Samples were incubated at 37 °C for 30 min while shaking after which the samples were transferred and filtered through a MultiScreen-FB filter plate (Millipore, Billerica, MA, USA) pre-soaked in 0.3% polyethylenimine solution (Sigma-Aldrich, St. Louis, MO, USA) using vacuum. The filters were washed trice with HM-buffer and the retained radioactivity was determined in a β-counter (Tri-Carb Liquid Sqintillation Analyzer, model 2500 TR, Packard Instrument Company, Meriden, CT, USA) using OptiPhase Supermix Cocktail (Perkin–Elmer Life Science, Boston, MA, USA) as scintillation fluid. IC_{50} values for the peptides were calculated using Prism 5.0(GraphPad Software Inc., San Diego, CA, USA) and converted into K_i values using the equation of Cheng–Prusoff (Cheng & Prusoff, 1973).

Galanin Receptor Signaling Studies - cAMP measurements

SHSY5Y cells stably expressing GALR2 were seeded in a 48 well plate and grown to confluency. Media was then changed to DMEM containing [3H]adenine (3 uCi/ml) purchased from Perkin Elmer (Waltham, MA, USA) for 2 h. The cells were washed twice with Hank's balanced salt solution, HBSS, before stimulated by 20 uM forskolin and/or M1154 for 30 minutes in the presence of 3-isobutylmethylxanthine, IMBX (1 mM), BAY 60-7550 (1 uM) and rolipram (10 uM) or with HBSS alone. The buffer were then removed and cells lysed with 5%

(w/v) trichloroacetic acid followed by 250 uL trichloroacetic acid supplemented with cAMP (0.1 mM) and ATP (0.1 mM) for 20 minutes at 4 degrees centigrade. The lysates were stored at -80 degrees centigrade until analysed and applied to Dowex 50W-X4 colums (200-400 mesh) and washed with water and subsequently placed over alumina colums and washed with water before placed over scintillation vials and eluted with 6 mL imidazole (0.1 M). 10 mL scintillation liquid (Ultima Gold XR) was added to each vial and analyzed in a liquid scintillation counter (Tri-Carb model 1600 TR; Packard Instrument Company).

Galanin Receptor Signaling Studies – label-free real time technology

The xCelligence system (Roche Diagnostics, Sweden) is based on the ACEA RT-CES cell sensor electrodes which allow monitoring and analysis of the kinetic aspects of cellular behavior. The technology is described in detailed elsewhere (Yu et al., 2006). Briefly, this assay is based on the principle that activated GPCRs will cause a change in cell morphology, regardless of which G-protein signaling cascade used. The change in cell morphology influences the contact area between cells and the electrodes on which the cells are grown and this small change is measured in real time as impedance. Experiments were performed using the EVIEW-Plates™ from ACEA (San Diego, CA, USA). One day before the experiment, 100 µL of medium was added to each well and background recorded. Following background measurement, 100 µL of media containing the cell suspension was seeded on the EVIEW-Plate™, incubated at room temperature for 30 min and then placed on the device station, and hosted in an incubator at 37°C with 5% CO_2. The cells were allowed to equilibrate for 24 h and impedance were constantly monitored every minute during the whole experiment. To evaluate the effect of cell density on impedance responses and to identify the better signal-to-noise ratio, we tested cell densities ranging from 5000-40000 cells/well. Optimal density was 40000 cells/well for GALR1 cells,

25000 cells/well for GALR2 cells and 20000 cells/well for GalR3 cells. Prior to the experiment, cell media was replaced with 180 μL low-FBS (1%) containing cell media, according to the manufactures instruction, and incubated for one hour. Then, 20 μL of the compounds diluted in PBS, at 10x the desired concentration, was gently added. In experiments aimed at evaluating antagonism, both compound and galanin were added simultaneously. In the experiments performed with the tetracycline induced hGalR3 cell line, cells were treated with 1 ng mL^{-1} tetracycline for 24 h and then processed as described above. Data were analyzed using the integrated software package expressing changes in cell electrode impedance as changes in CI (Yu et al., 2006) and normalized to the cell index at the time of ligand addition. Concentration effect curves were generated by calculation of the area under curve (AUC) between 0 and 3600 s and plotted against ligand concentration. The subsequently calculation of EC_{50} values were determined by non-linear regression using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Behavioral experiments and Hippocampal cell counts

Adult, male Sprague-Dawley rats (total of 41, weighing 250-300g) were obtained from Harlan Inc. (Indianapolis, IN, USA) and housed individually throughout the experiment in 42L x 22W x 20H cm polycarbonate cages. Animals were maintained in a temperature and humidity-controlled environment on a 12-hour light/dark schedule. Food and water were available ad libitum. Rats were allowed a one week adaption to the animal facility prior to cannulation surgeries and were randomly assigned to one of six groups (www.randomizer.org): Low dose M1145 (n = 6); High Dose M1145 (n = 8); M1154 (n = 9); M617 (n = 7); Saline Control (n = 8); Naïve Control (n = 3). All procedures were conducted in accordance with NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Georgia Animal Care

and Use Committee. Rats were anesthetized with a 1-3% isoflorane/oxygen mixture delivered through a vaporizer and nose cone and mounted in a stereotactic frame. A longitudinal incision was made along the scalp and overlaying connective tissue and periosteum was scraped away from the scalp. Cannulae (1 cm) were surgically implanted into the right lateral ventricle at the following coordinates (from Bregma): posterior 1.0 mm, lateral 1.5 mm, and ventral 3 mm according to the rat atlas of Paxinos & Watson (Paxinos & Watson, 1986). Cannulae were secured to the skull using 3 stainless steel screws and epoxy. All rats received 1 mg/kg meloxicam post- surgery. Cannulae placement was verified at the end of all procedures by injecting 2 mg/ml of fast green dye following euthanasia and verifying its presence in the ventricles.

One week following surgeries, rats were injected with one of five compounds plus 0.2 µg of kainic acid. Doses of compounds were as follows: low dose M1145 -10 µg, high dose M1145 - 20µg, M1154 - 20µg, M617 -20µg, Saline - 20µg. All drugs were dissolved in deionized water and injected in a volume of 5 µl. Naïve animals did not undergo cannulation surgery or receive any drugs.

Seizure behavior was scored live by a rater blind to group assignment for 30 minutes post microinjections and video recorded for additional ratings. The rating scale was adapted from the Racine scale (Racine, 1972; Reiss, Dishman, Boyd, Robinson, & Holmes, 2009b) and averages of seizure rating scores were compiled for statistical analysis. Animals were perfused transcardially with PBS followed by a 4% formalin solution 48 hours post-ICV injections and seizure rating. Brains were extracted, fixed in 4% formalin solution, and kept frozen at -20ºC until sectioned. Sections from both the dorsal and ventral hippocampus (20 µm) were thaw

mounted on slides. One slide per region, dorsal and ventral hippocampus, was nissl-stained and cells of the CA3 region were counted under a microscope and recorded.

A two-way contingency table analysis of high and low seizure scores was conducted to assess effect of treatment on seizure ratings. A Kruskal-Wallis test was conducted to evaluate differences among the treatment conditions; pairwise comparisons were conducted to evaluate differences between treatment groups and saline group. These statistical analyses were conducted using PASW Statistics Windows version 18.0 (IBM Corporation, Armonk, NY, USA).

Results

Galanin receptor binding studies

^{125}I-galanin-receptor displacement experiments with galanin, galanin(1-16) and M1154 were performed on cell membranes from human Bowes melanoma cells endogenously expressing hGALR1, CHO cells stably transfected with hGALR2 and Flp-In TREx 293 cells with inducible expression of hGalR3. Displacement of ^{125}I-galanin by M1154 at GALR1 showed a relatively high affinity with a Ki of 11.7 ± 7.2 nM, whereas galanin(1-16), had a Ki of 0.78 ± 0.26 nM in the same experiments. When tested on GALR2, M1154 had a Ki of 14.4 ± 4.1 nM whereas galanin(1-16) had a Ki of 2.44 ± 0.57 nM on the same receptor. When tested on GalR3 M1154 showed no binding at the highest tested concentration, 10000 nM, whereas galanin(1-16) had a Ki of 8.98 ± 3.8 nM (Table 3.2, Figure 3.1b). These results reveal that M1154 is selective for GALR1 and GALR2 and the difference towards GalR3 is greater than 1000 times (Table 3.2, Figure 3.1a).

cAMP measurements

GALR1and GALR2 expressing SHSY5Y cells were treated with M1154 at different concentrations together with 20 μM forskolin. The GALR1/R2-selective agonist M154 decreases

forskolin stimulated cyclic adenosine-monophosphate production significantly in a dose-dependent manner in GALR1 expressing cells with an EC_{50} of 159.6 nM (Table 3.3, Figure 3.8A). In SH-SY5Y-R2 cells it increases the cAMP production also in a dose-dependent fashion with an EC_{50} of 1.53 µM (Table 3.3, Figure 3.8B).

Galanin receptor signalling studies

To assess if galanin receptor stimulation could be detected in the impedance based technology, cells were challenged with the full length galanin. Compound addition induced a fast concentration-dependent response in normalized cell index (NCI) with different concentration dependent profiles for each receptor subtype (Figure 3.2a-d). Two different GALR2 cell lines was characterized, CHO cells expressing hGALR2 and HEK cells expressing rGALR2, displaying similar profiles for galanin (Figure 3.2b-c), although the signal to noise ratio was significantly higher in the HEK cells and was therefore continuously used. Because the instrument monitors the impedance changes elicited by receptor activation in real time, is it possible to generate concentration-activity curves in a variety of ways, dependent on the time point or period of time chosen and if peak values or AUC is used. We wanted to generate a robust protocol that could be used independent of the type of G-protein that is activated and eliminate the need for a clear peak maximum, since that is often not seen in the literature (Schröder et al., 2010; Scott & Peters, 2010). Qualification of NCI signals for concentration effect curves and the subsequently calculation of EC_{50} was therefore performed by calculation of the AUC between 0 and 3600s.

Galanin shows similar EC_{50} values to GALR1 and GALR2, 1.1 ± 0.11 nM and 8.26 ± 1.9 nM, respectively (Table 3.3, Figure 3.2), while the EC_{50} towards GalR3 was 412 ± 38 nM. The xCELLigence system has similar or improved sensitivity when compared to traditional endpoint

assays performed in the same laboratory (Table 3.3). The relative low EC_{50} for galanin at GalR3 could reflect intrinsic receptor properties or might be related to the utilize cell clone. We have earlier shown a similar EC_{50}-value for galanin, 530 nM (Table 3.3), in the commonly used GTPγ-assay (J. Runesson, I. Saar, et al., 2009). It has been hypothesized in other studies that transfection of cell lines with GalR3 yield very low and variable receptor expression, which affect the possibility to detect the signal efficacy at this receptor (Berger et al., 2004; Lang et al., 2005; Ohtaki et al., 1999); it may account for the low EC_{50} for galanin seen at the GalR3 cell clone, although, we have earlier showed that the GalR3 cell clone used in this study has similar receptor expression as the GALR1 and GALR2 cell lines (J. Runesson, I. Saar, et al., 2009). Unfortunately, signaling properties of the GalR3 and the pharmacological profiles of common galaninergic ligands are still ill-defined and more studies are needed to characterize the properties of GalR3.

Lundström et al. showed that M617 has agonistic properties at both GALR1 and GALR2, tested through cAMP and IP assays, with an EC_{50}-value of 104 and 304 nM, respectively (Lundström et al., 2005). In the present study, M617 display an EC_{50}-values that are considerable lower at GALR1 and GALR2, 11.4 ± 0.67 nM and 24.6 ± 3.8 nM, respectively (Table 3.3, Figure 3.2), although the relative three time GALR1 selectivity was observed in both studies. A later publication ascribed the M617 peptide the ability to mediated activation of GalR3, measured as the ability to inhibit forskolin produced cAMP, at a similar concentration as for GALR1 and GALR2, with an EC_{50}-value of 121 ± 48 nM (Sollenberg et al., 2010). In the present setup, M617 display an EC_{50}-value of 2840 ± 1090 nM which results in a signaling profile at the galanin receptor subtypes more in concordance with the binding profile (Table 3.2 and Table 3.3).

We have previously shown the agonistic properties at GALR2 of M1145 in an immunoprecipitation accumulation assay, with an EC_{50} value of 38 nM (Runesson, Saar, et al., 2009). In this study, a slightly lower EC_{50}-value was obtained with the xCELLigence system, with an EC_{50} value of 16 ± 4.7 nM. Here, we also characterize for the first time the ability of M1145 to activate GALR1 and GalR3. M1145 acts as an agonist at all receptor subtypes, although the potency varies significantly, with a more than 70 times difference in the calculated EC_{50}-values (Table 3.3, Figure 3.2).

The novel peptide, M1154, displayed a slightly lower potency than galanin at GALR1 and GALR2 with an EC_{50}-value of 124 ± 47nM and 26 ± 1.1 nM, respectively. M1154 had no effect at any concentration tested when tested on GalR3 (Table 3.3, Figure 3.2). To address the efficacy of M1154, the maximal response induced by M1154 were compared to the maximal response (Emax normalized to 100 percentage) induced by galanin. M1154 behaves as a full agonist, with a similar Emax value as galanin (data not shown). Application of M1154 (10 nM) induced a shift to the left of the concentration activity curve of galanin with a consistent modification of the EC_{50} (EC_{50} = 21 nM), confirming a M1154-mediated agonist effect (Figure 3.3a). Application of galanin (1 or 10 nM) induced a similar shift to the left of the concentration activity curve of M1154 (EC_{50} = 10-90 nM) (Figure 3.3b), confirming again the both galanin and M1154-mediated an agonist effect.

Behavioral experiments and Hippocampal cell counts

Kainic acid, 0.2 µg, administered ICV induced seizures-typical behaviors to a similar extent as previous studies (Reiss et al., 2009b). A Pearson chi square test revealed no significantly changes in the seizure rating scores for any of the administered galanin receptor ligands, $\chi^2(4, N = 38) = 1.839$, $p = .765$ (Figure 3.4). Administration of KA resulted in a dramatic

cell loss in the CA3 region (Figure 3.6) for both dorsal and ventral hippocampus. When the cell number was quantified, a trend towards a reduction of cell death was seen for all co-administrated galanin receptor ligands (Figure 3.5). The results from an independent samples Kruskal-Wallis tests for cell count differences among treatments indicated significance after KA administration for both the dorsal hippocampus, $\chi^2(5, N = 41) = 18.248$, $p = 0.003$; and the ventral hippocampus, $\chi^2(5, N = 38) = 15.702$, $p = 0.008$. Follow-up pairwise comparisons indicated that cell counts in the dorsal hippocampus were significantly different from the Vehicle treatment for two groups, M1154 + KA ($p = 0.038$) and M617 + KA ($p = 0.001$). Additionally, pairwise comparisons indicated that cell counts in the ventral hippocampus were significantly different from the Vehicle treatment in the M617 + KA group ($p< 0.001$) (Figure 3.5).

Discussion

In the present study, we investigated the pharmaceutical potential of galanin receptor subtypes to block KA-induced neurodegeneration as a model for excitotoxicity. This study was motivated by the fact that galanin has been shown to modulate excitability in the hippocampus and administration of galanin has been shown to affect neurodegeneration in several setups, including different epilepsy models (A. Mazarati et al., 2006a). Several attempts have been made to characterize the contribution of each galanin receptor subtype at different aspects of neuroprotection; and both GALR1 and GALR2 have been shown to have neuroprotective effects (C. R. Elliott-Hunt, R. J. Pope, P. Vanderplank, & D. Wynick, 2007; A. Mazarati & Lu, 2005; Schauwecker, 2010), while very few studies have addressed the contribution of GalR3 in galanin-mediated neuroprotection.

To address the contribution of the individual receptor subtypes, we developed and characterized a novel GALR1/GALR2 agonist, the M1154 peptide. To further improve the

development of galanin receptor ligands, we present here a robust protocol for receptor signaling, utilizing a label-free instrument, to be able to test the receptor activation signature of our receptor ligands on all three galanin receptor subtypes in a single setup. This is highly motivated since each galanin receptor subtype has its own unique capacity to activate the different G-protein subtypes which makes it difficult to address receptor subtype selectivity for novel ligands. Furthermore, very few studies address the contribution of GalR3, mostly because we still lack reliable pharmacological signatures at GalR3 for commonly used galaninergic tools. In the present study, we demonstrate the generation of a novel galanin analogue, M1154, with binding affinities very similar to galanin and the galanin fragment, galanin(1-16), towards GALR1 and GALR2. However, M1154 display no ability to displace galanin at GalR3 in binding studies up to 10 μM (Figure 3.1, Table 3.2). In addition, galanin and galanin (1-16) were tested for comparison.

The galanin fragment, galanin(1-16), display a 10 times lower K_i in this study compared to earlier publication (K. E. Smith et al., 1998), now more in concurrence with the binding affinity of the full length peptide towards GalR3, giving a similar binding profile for the galanin and the galanin(1-16) peptide on all three receptor subtypes (Table 3.2). We found that M1154, in concurrence with galanin, to produce a clear stimulation through both GALR1 and GALR2 in the xCELLigence system and in concordance with the binding results, no activity was seen when tested at GalR3 (Figure 3.3). Consistent with previous studies, evaluation of the signal characteristics of M617 and M1145 in the present study, revealed these ligands to be GALR1 and GALR2 selective, respectively (Lundström et al., 2005; J. Runesson, I. Saar, et al., 2009; Sollenberg et al., 2010).

Administration of KA has been utilized as a model of both neurodegeneration and status epilepticus (Ben-Ari & Cossart, 2000; Wang, Yu, Simonyi, Sun, & Sun, 2005). We and others have shown that CA3 pyramidal cells are most vulnerable to KA treatment (Ben-Ari & Cossart, 2000; Reiss et al., 2009b). Thus, KA provides the opportunity to test the neural circuits that protect CA3 neurons from hyperexcitability. Even so, there are other obvious effects of ICV KA-administration, such as induction of seizure-typical behaviors. In the present study, ICV administration was utilized to insure a robust excitotoxic effect within the CA3 region, which is mediated through a direct excitotoxic effect of KA. Therefore, compounds that effectively reduce KA-induced cell death in CA3 in this model most likely reflect neuroprotective effects not involved in anticonvulsant activity. We previously reported that ICV administration of a galanin receptor antagonist had no effect on seizure-typical behaviors induced by ICV administrated KA (Reiss et al., 2009). In the present study none of the ICV administrated galanin receptor agonists had an effect on the seizure-typical behaviors induced by KA, depicted in Figure 3.5. Thus, it can be speculated that the seizure-typical behaviors induced by KA are galanin-independent, and most likely due to overflow of KA and hyperexcitability in the motor cortices (Sperk, 1994). Therefore we cannot exclude that these ligands have anticonvulsant properties.

ICV administration of KA induces rigorous neuronal cell death in the CA3 region of both ventral and dorsal hippocampus (Figure 3.6). Animals treated with the GALR2 selective ligand, M1145, were equally affected in the CA3 region by KA-administration compared to vehicle treated animals. In contrast, administration of the mixed GALR1/GALR2 agonist reduced cell loss significantly. Furthermore, animals treated with the GALR1 preferential ligand displayed further reduced cell death. These results indicate that the Galanin-mediated neuroprotective

effect occurs through GALR1 and not GALR2. This is in concordance with earlier studies on inbred mice where a lower expression of GALR1 correlated with a larger cell loss than wildtype littermates in the CA3 region when exposed to KA (Kong et al., 2008; Schauwecker, 2010). Similar to this study, these animals had no alteration in seizure-typical behaviors, separating neuroprotective and anticonvulsant effects of the galaninergic system (Schauwecker, 2010).

Taken together, these results present a novel GALR1/2 selective ligand and demonstrate its ability to significantly reduce the excitotoxicity of KA, a common model of excitotoxicity. The comparison of these results with the effect of already published M617 and M1145 indicate that GALR1 mediates the neuroprotective effect of galanin. This suggests that a GALR1 agonist could potentially be used for treatment of exposure to excitotoxic compounds.

Acknowledgements

We thank Kathryn A. Jones and Tiina P. Iismaa (Neurobiology Program, Garvan Institute of Medical Research, Sydney, Australia) for CHO cells stably transfected with hGALR2, Xiaoying Lu and Tamas Bartfai (Scripps Research Institute, La Jolla, USA) for HEK293 cells stably transfected with rGALR2, Linda Lundström and Silvia Gatti-McArthur (F. Hoffmann-La Roche AG, Basel, Switzerland) for Flp-In T-REx 293 cell line stably transfected with the hGalR3. This work was supported by grants from the Olle Engkvist Byggmästares Foundation, Helge Ax:son Johnsons foundation and Sven & Dagmar Saléns foundation (JR), Swedish Science Foundation (VR-Med), the Knut and Alice Wallenberg Foundation and Swedish Center for Biomembrane Research (ÜL).

Table 3.1 Amino acid sequences of peptides used or discussed in this study.

Name	**Sequence**
Galanin(1-29), rat	GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-amide
Galanin(1-16)	GWTLNSAGYLLGPHAI-amide
Galanin(2-11)	WTLNSAGYLL-amide
M617	GWTLNSAGYLLGPQPGFSPFR-amide
M1145	RGRGNWTLNSAGYLLGPVLPPPALALA-amide
M1154	GWTLNSAGYLLPQPGFSPFA-amide

Table 3.2 Experimental K_i determined by displacement studies with Porcine-[^{125}I]-Galanin on Bowes Melanoma Cells expressing hGalR1, CHO cells stably transfected with hGalR2 or Flp-In T-REx 293 cells expressing hGalR3.

Name	K_i (nM) GalR1	K_i (nM) GalR2	K_i (nM) GalR3	K_i GalR1/ K_i GalR2	K_i GalR3/ K_i GalR2
Galanin	1.75 ± 1.7[a]	2.98 ± 1.4[a]	4.49 ± 0.8[a]	0.6	1.5
Galanin(1-16)	0.78 ± 0.26	2.44 ± 0.57	8.98 ± 3.8	0.3	3.7
M617	0.23 ± 0.1[b]	5.71 ± 1.3[b]	49 ± 9.4[c]	25	8.6
M1145	587 ± 250[a]	6.55 ± 2.7[a]	497 ± 150[a]	90	76
M1154	11.7 ± 7.2	14.4 ± 4.1	>15000	0.8	>1000

[a] (Runesson et al., 2009), [b] (Lundström et al., 2005), [c] (Sollenberg et al., 2010)

Table 3.3 Obtained EC_{50}-values for rat galanin in galanin receptor signaling studies utilizing either the impedance (RT-CES) system GalR1-3) or traditional end-point assays, cAMP (GalR1), cAMP or IP-production (GalR2) and GTPγS (GalR3). Cells used where Bowes Melanoma Cells expressing GalR1, CHO, SHSY5Y or HEK cells stably transfected with GalR2 or Flp-In T-REx 293 cells expressing GalR3.

Name	EC_{50} (nM)		
	GalR1	**GalR2**	**GalR3**
RT-CES	1.1	8.3	412
Galanin(1-29)	31.6 [a]	173 [a]	530 [b]
M1145	n.t	38[b]	n.t
M617	104[a]	304[a]	121[c]
M1154	159	1530	n.t

[a] (Lundström et al., 2005), [b] (Runesson et al., 2009), [c] (Sollenberg et al., 2010),

Table 3.4 Potency of Galanin Receptor Ligands in the Impedance (RT-CES) system. Cells used where Bowes Melanoma Cells expressing hGalR1, HEK cells stably transfected with rGalR2 or Flp-In T-REx 293 cells expressing hGalR3.

Name	EC_{50} (nM)			EC_{50} GalR1/ EC_{50} GalR2	EC_{50} GalR3/ EC_{50} GalR2
	GalR1	GalR2	GalR3		
Galanin(1-29)	1.1 ± 0.11	8.26 ± 1.9	412 ± 38	0.13	50
M617	11.4 ± 0.67	24.6 ± 3.8	2840 ± 1090	0.46	120
M1145	1260 ± 119	16 ± 4.7	2670 ± 502	79	170
M1154	124 ± 47	26 ± 1.1	>31600	4.8	>1210

Figure 3.1 Galanin receptor binding studies. Displacement of porcine-[^{125}I]-galanin from membranes by peptide M1154 (A) and the galanin fragment, galanin(1-16) (B). Membranes were from human Bowes melanoma cells expressing GalR1 (open circle), CHO cells expressing GalR2 (closed square) and Flp-In T-REx 293 cells expressing GalR3 (closed triangle). The data is from three representative experiments performed in duplicates, presented as mean ± SEM. Calculated K_i values are summarized in Table 3.2.

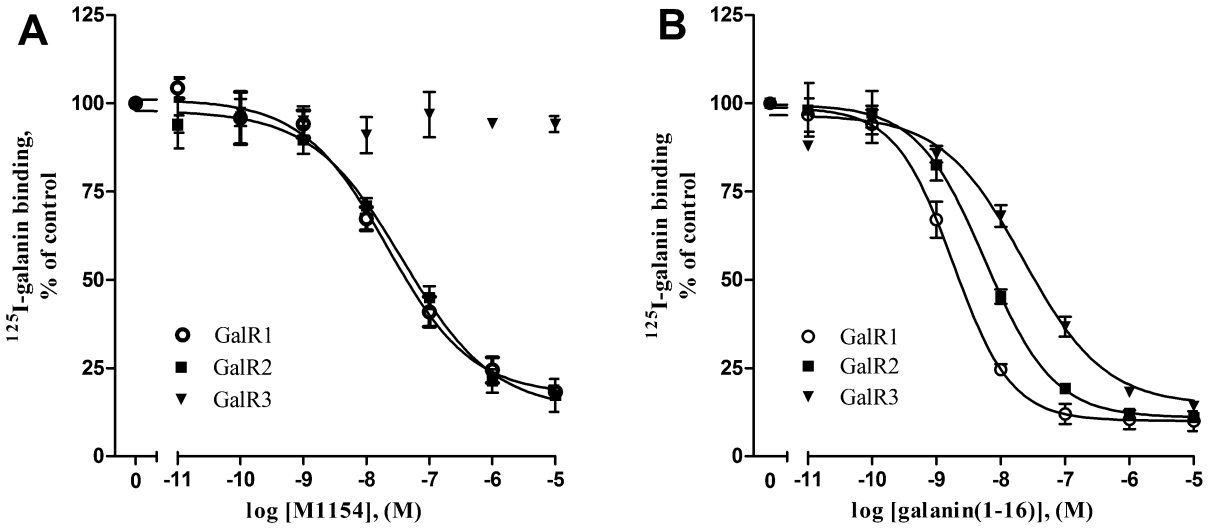
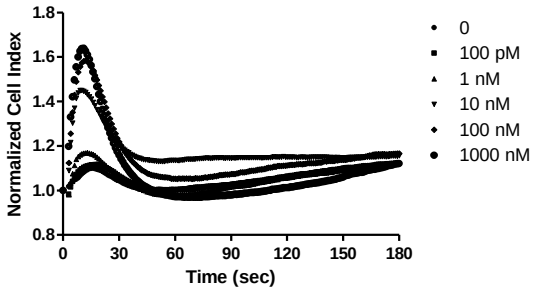
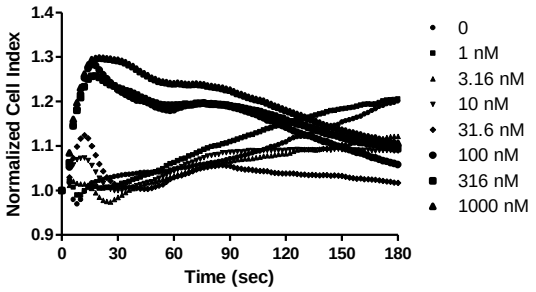
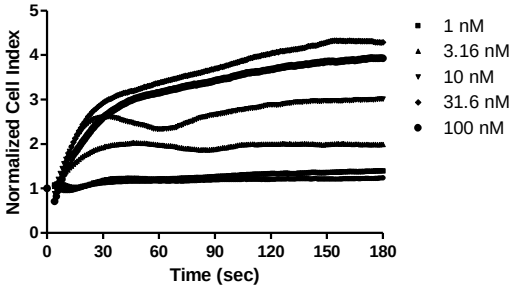

A
125
100
75
50
25
0
^{125}I-galanin binding,
% of control
GalR1
GalR2
GalR3
0
-11
-10
-9
-8
-7
-6
-5
log [M1154], (M)
B
125
100
75
50
25
0
^{125}I-galanin binding
% of control
GalR1
GalR2
GalR3
0
-11
-10
-9
-8
-7
-6
-5
log [galanin(1-16)], (M)

Figure 3.2 Galanin receptor signaling studies. Potency of Galanin Receptor Ligands in the Impedance (RT-CES) system. Concentration dependent profiles of galanin and the galanin fragment, galanin(1-16), in (A) Bowes melanoma cells expressing hGalR1 (B) CHO cells expressing hGalR2 (C) HEK cells expressing rGalR2 (D) Flp-In T-REx 293 cells expressing hGalR3.


Normalized Cell Index
1.8
1.6
1.4
1.2
1.0
0.8
0
30
60
90
120
150
180
Time (sec)
0
100 pM
1 nM
10 nM
100 nM
1000 nM


Normalized Cell Index
1.4
1.3
1.2
1.1
1.0
0.9
0
30
60
90
120
150
180
Time (sec)
0
1 nM
3.16 nM
10 nM
31.6 nM
100 nM
316 nM
1000 nM


Normalized Cell Index
5
4
3
2
1
0
0
30
60
90
120
150
180
Time (sec)
1 nM
3.16 nM
10 nM
31.6 nM
100 nM

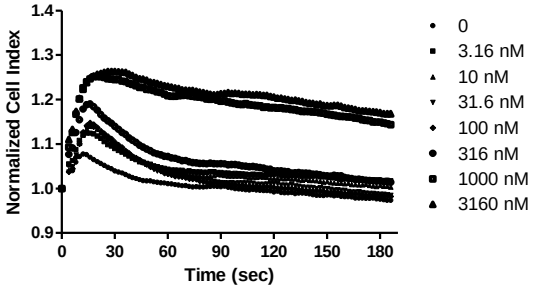

Normalized Cell Index
1.4
1.3
1.2
1.1
1.0
0.9
0
30
60
90
120
150
180
Time (sec)
0
3.16 nM
10 nM
31.6 nM
100 nM
316 nM
1000 nM
3160 nM

Figure 3.3 Galanin receptor signaling studies. Potency of Galanin Receptor Ligands in the Impedance (RT-CES) system; Galanin (A-C), M617 (D-F), M1145 (G-I) and M1154 (J-L). Cells used where Bowes Melanoma Cells expressing hGalR1, HEK cells stably transfected with rGalR2 or Flp-In T-REx 293 cells expressing hGalR3. The data is from three representative experiments performed in duplicates, presented as mean ± SEM. Calculated EC_{50} values are summarized in Table 3.4.

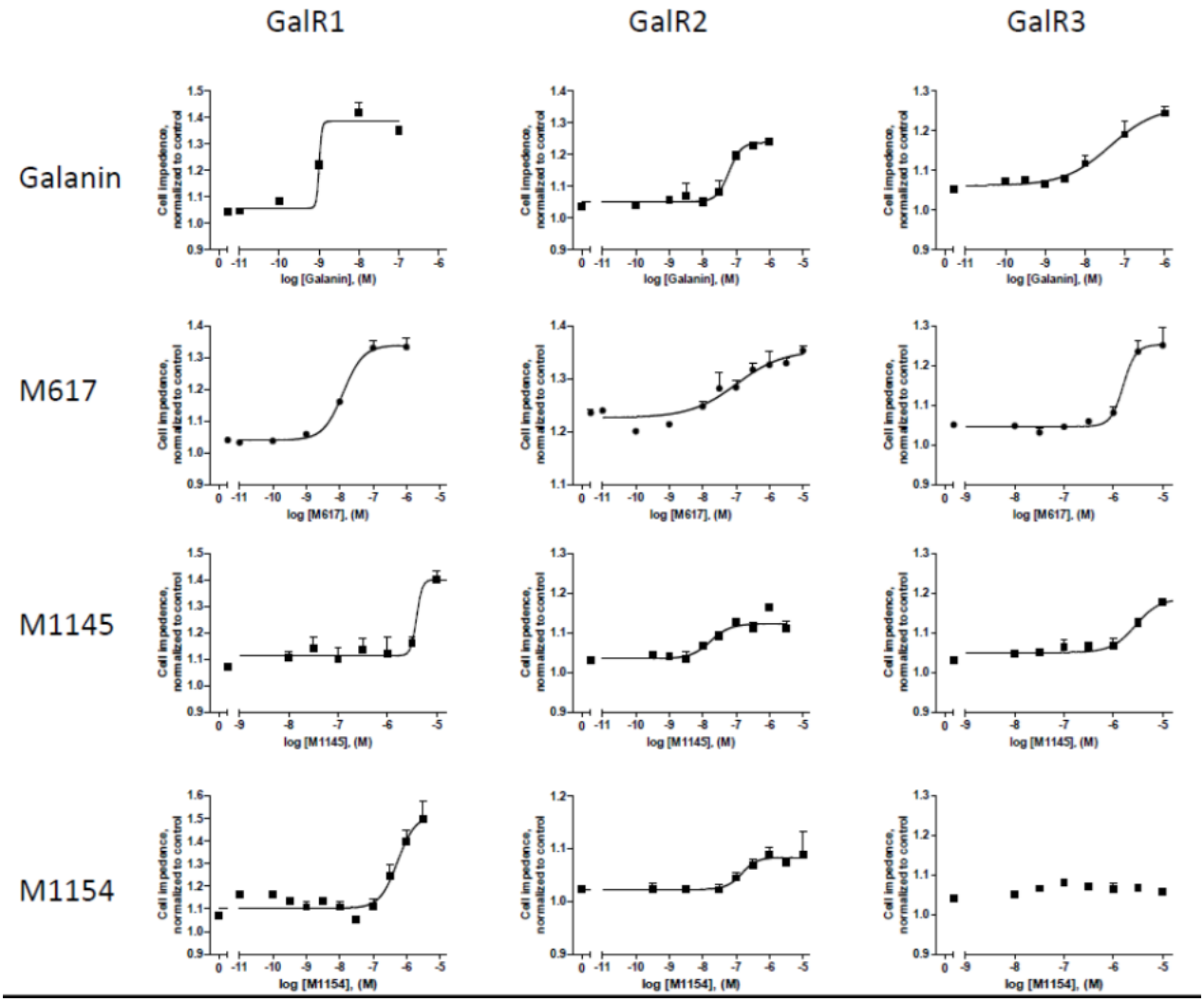

GalR1
GalR2
GalR3
Galanin
M617
M1145
M1154
Cell impedence, normalized to control
log [Galanin], (M)
log [M617], (M)
log [M1145], (M)
log [M1154], (M)

Figure 3.4 Galanin receptor signaling studies at HEK cells stably transfected with rGalR2.. Galanin dose response curve (closed circle) with an EC_{50} value of 8.2 nM was shifted to the right by 1 nM (open square) M1154 given an EC_{50} value of 6.0 nM, 10 nM (closed triangle) given an EC_{50} value of 5.8 nM, 100 nM (closed diamond) given an EC_{50} value of 0.56 nM and 1000 nM (closed square) M1154 given an EC_{50} value of 0.045 nM. Experiment was performed in duplicates and presented as mean ± SEM.

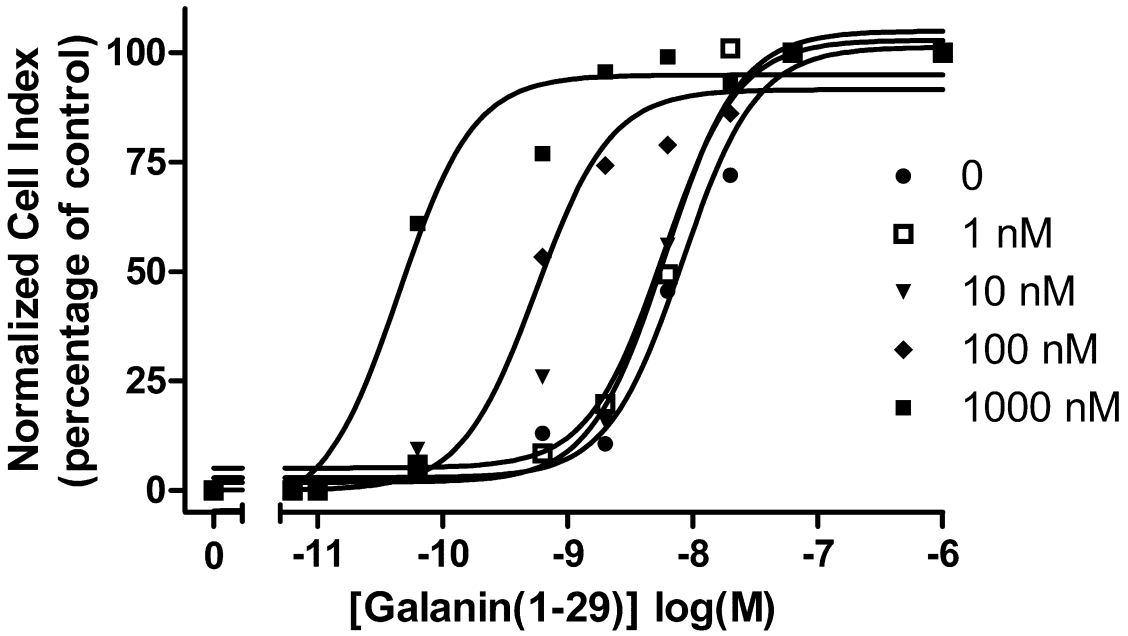

Normalized Cell Index
(percentage of control)
100
75
50
25
0
0
-11
-10
-9
-8
-7
-6
[Galanin(1-29)] log(M)
0
1 nM
10 nM
100 nM
1000 nM

Figure 3.5 Average seizure rating following ICV administration of either saline, M617, M1145 or M1154 followed by ICV injection of kainic acid. Data are presented as means ± SEM. No significant differences in seizure rating between treatments were found using a two-way contingency table analysis.

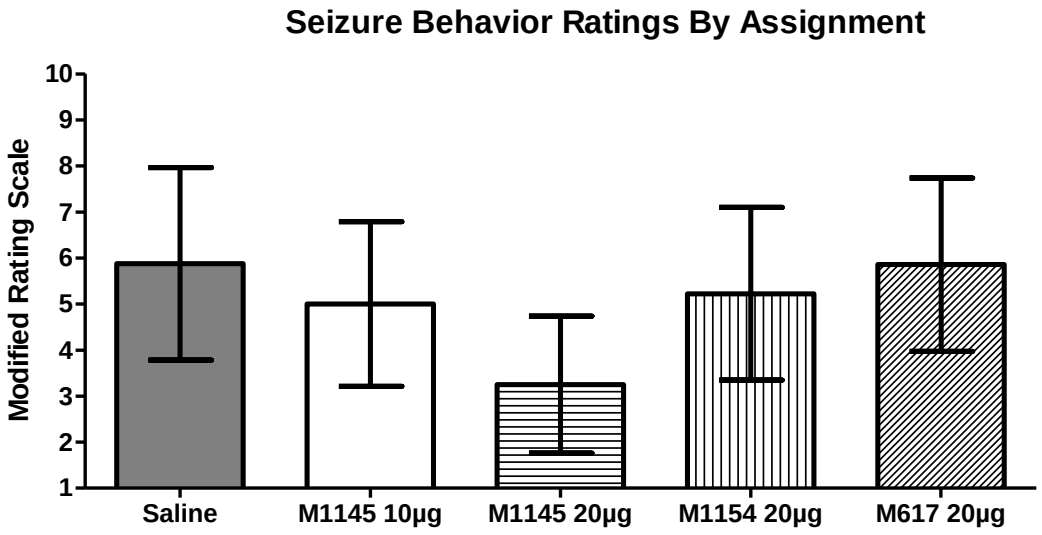

Seizure Behavior Ratings By Assignment
Modified Rating Scale
10
9
8
7
6
5
4
3
2
1
Saline
M1145 10µg
M1145 20µg
M1154 20µg
M617 20µg

Figure 3.6 Cell count from both dorsal (open bars) and ventral (closed bars) hippocampus (region CA3) after ICV administration of either saline, M617, M1145 or M1154 followed by ICV injection of kainic acid. Data are presented as means ± SEM. $^{\#}$ $p<0.05$ $^{\#\#\#}$ $p< 0.001$ as compared to ventral hippocampus in vehicle exposed animals, ** $p< 0.01$ as compared to dorsal hippocampus in vehicle exposed animals; Kruskal-Wallis pairwise comparisons test.

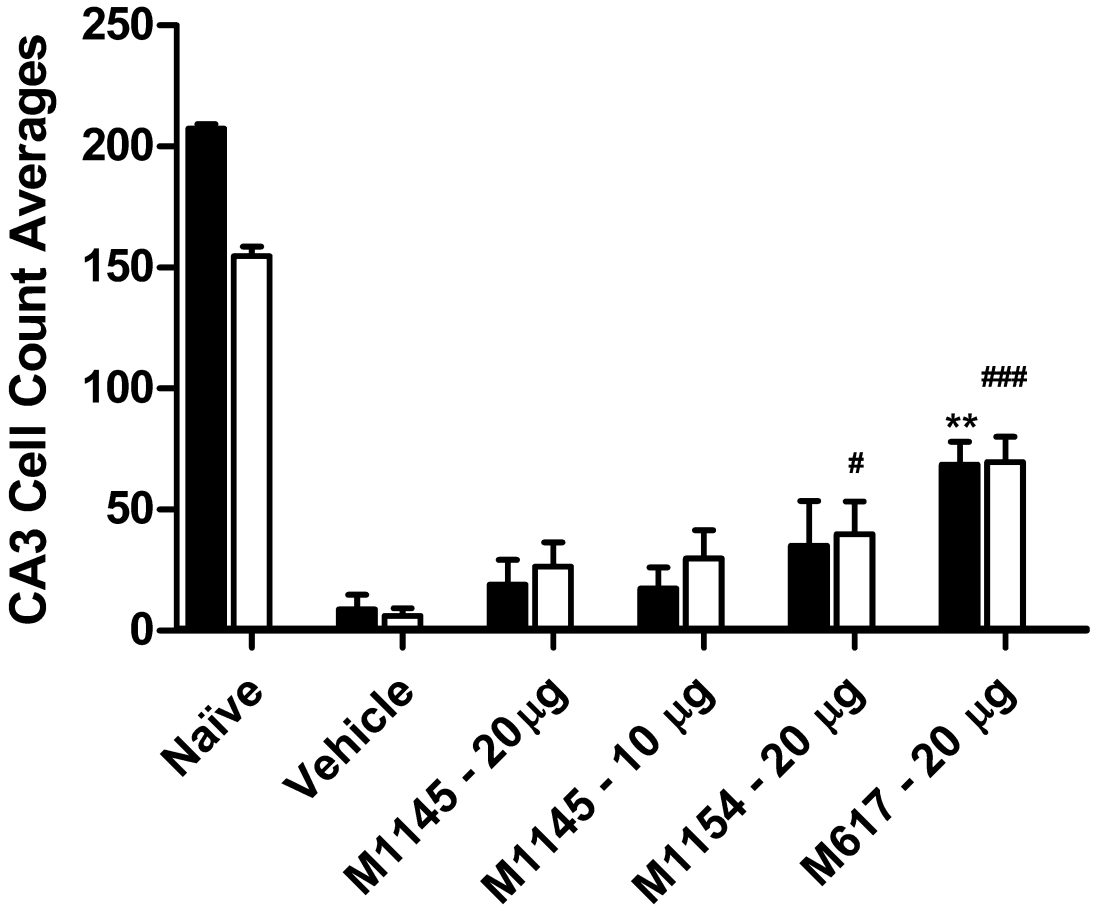
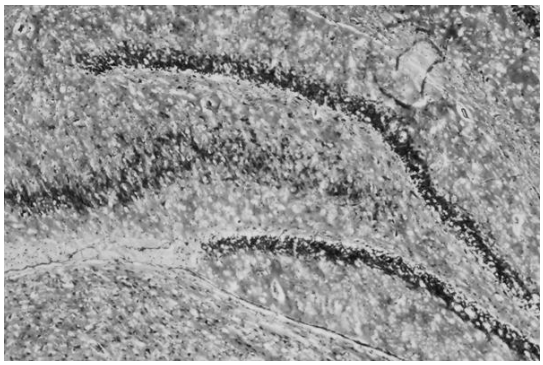
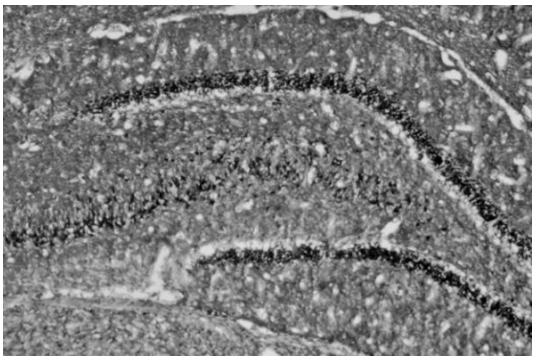

CA3 Cell Count Averages
250
200
150
100
50
0
**
###
#
Naïve
Vehicle
M1145 - 20μg
M1145 - 10 μg
M1154 - 20 μg
M617 - 20 μg

Figure 3.7 Nissl stain images of dorsal hippocampus (CA3 region): A. Naïve control, B. saline+KA, C. M1154+KA, D. M617+KA

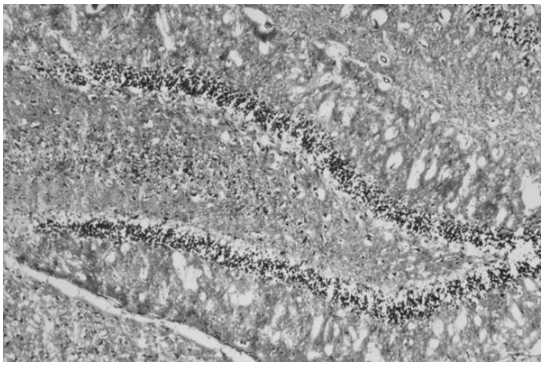
A.



C.



B.



D.

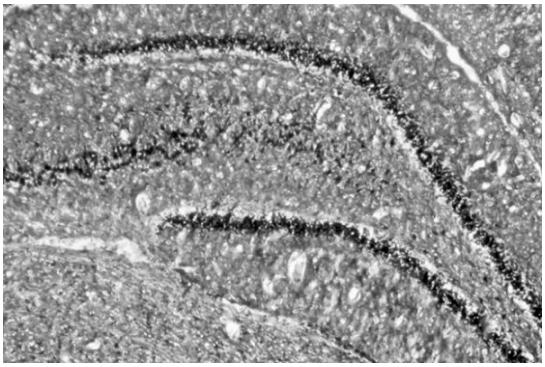
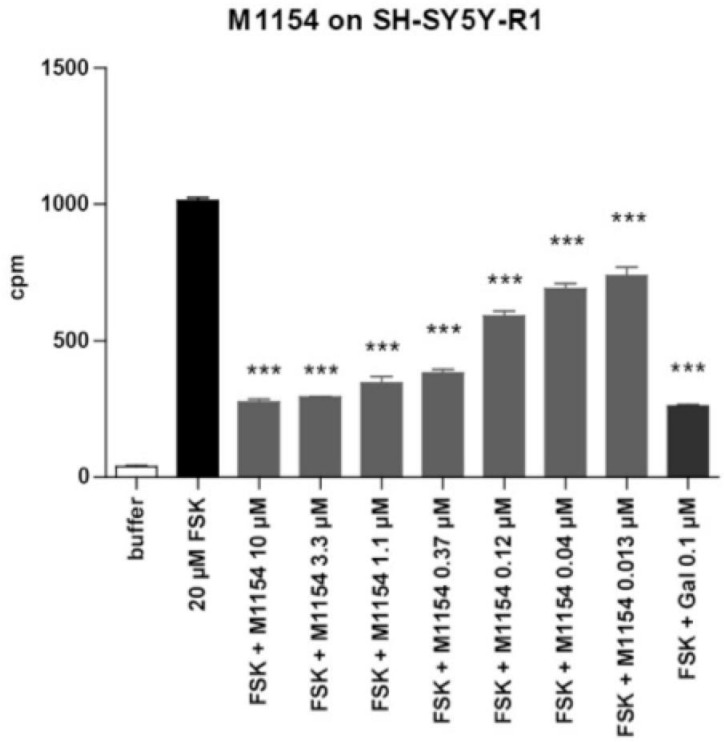
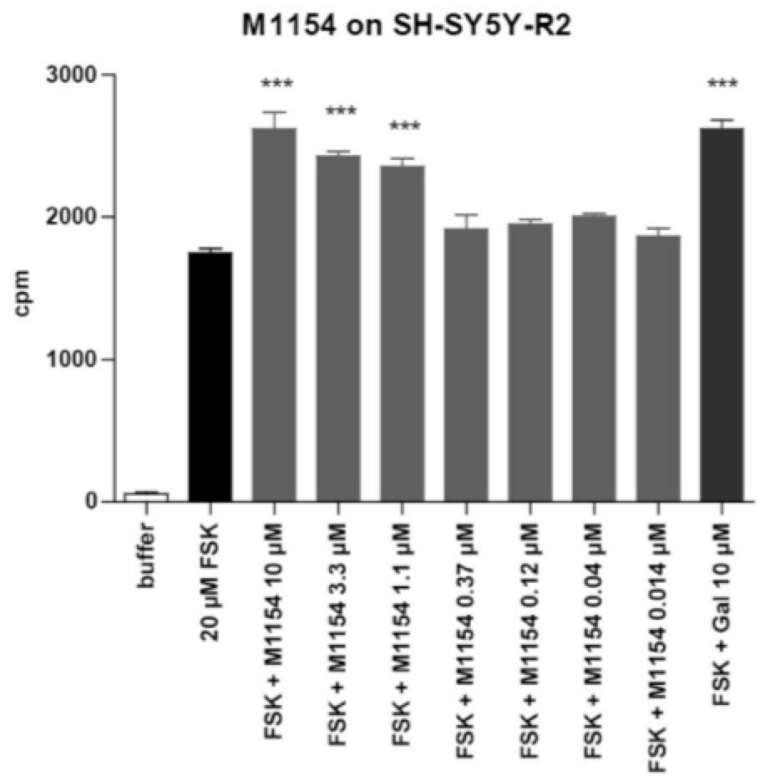


Figure 3.8 cAMP measurements of M1154 on SHSY5Y cells expressing GalR1 (A) and GalR2 (B), presented as means and SEM

A


M1154 on SH-SY5Y-R1
cpm
1500
1000
500
0
***
buffer
20 µM FSK
FSK + M1154 10 µM
FSK + M1154 3.3 µM
FSK + M1154 1.1 µM
FSK + M1154 0.37 µM
FSK + M1154 0.12 µM
FSK + M1154 0.04 µM
FSK + M1154 0.013 µM
FSK + Gal 0.1 µM

B


M1154 on SH-SY5Y-R2
cpm
3000
2000
1000
0
***
buffer
20 µM FSK
FSK + M1154 10 µM
FSK + M1154 3.3 µM
FSK + M1154 1.1 µM
FSK + M1154 0.37 µM
FSK + M1154 0.12 µM
FSK + M1154 0.04 µM
FSK + M1154 0.014 µM
FSK + Gal 10 µM

CHAPTER 4

THE EFFECTS OF VOLUNTARY WHEEL RUNNING ON COCAINE AND STRESS-PRIMED REINSTATEMENT IN A COCAINE-PRIMED CONDITION PLACE PREFERENCE MODEL[1]

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[1]Groves-Chapman, J.L., Cazares, D., & Holmes, P.V. To be submitted to *Psychopharmacology*.

Abstract

Regular exercise confers protective benefits in the brain, through neurotrophic and neuromodulatory effects. Exercise also protects against addiction-related behaviors, possibly through galaninergic innervation of the dopamine systems associated with these behaviors. Models involving three weeks of voluntary activity wheel running in rats have reported an exercise-induced upregulation of galanin mRNA and protein levels within the locus coeruleus (LC). Recent evidence suggests a role for galanin in the regulation of addiction-related behaviors. To evaluate the potential influence of running-induced galanin on modulation of the mesocorticolimbic dopamine system, as well as influences on addiction-related behaviors, a condition place preference (CPP) to cocaine paradigm was employed. Animals were assigned to three weeks of activity wheel or sedentary condition before undergoing training for CPP, followed by an extinction protocol. Upon achievement of extinction, all animals were exposed to a reinstatement test that involved a priming dose of cocaine or inescapable footshock. The results indicate that access to a running wheel differentially affects performance in the reinstatement models. Running animals showed greater reinstatement to drug seeking when they were primed with cocaine, compared to their sedentary counterparts. Interestingly, running animals showed less reinstatement to drug seeking when primed with footshock. These results, combined with the *in situ* hybridizations results, suggest that these two models are modulated by different pathways.

Keywords: galanin, dopamine, addiction, condition place preference, cocaine-primed reinstatement, stress-primed reinstatement, footshock

Introduction

Exercise and physical activity provide a non-pharmacological treatment modality for many neurological and psychological diseases and disorders including depression, anxiety, stress, and cognitive impairments (Duman, Schlesinger, Russell, & Duman, 2008; Dunn & Dishman, 1991; Garcia-Capdevila, Portell-Cortes, Torras-Garcia, Coll-Andreu, & Costa-Miserachs, 2009; Garza, Ha, Garcia, Chen, & Russo-Neustadt, 2004; Gomez-Pinilla, Vaynman, & Ying, 2008; Griffin, Bechara, Birch, & Kelly, 2009; Kramer & Erickson, 2007; Lee & Rhyu, 2009; Ma, 2008; Marais, Stein, & Daniels, 2009; Ploughman, 2008; Radak et al., 2010; Reiss, Dishman, Boyd, Robinson, & Holmes, 2009; Sciolino, Dishman, & Holmes, 2012). The mechanisms by which physical activity ameliorates symptoms of psychological disorders and brain diseases have been a focal point for recent research. The catecholamines, in particular, have been an integral component of understanding the neurochemistry of psychological well-being. More specifically, disruptions in both norepinephrine and dopamine are implicated in rodent models of anxiety, depression, and stress. Evidence from animal studies involving voluntary wheel or treadmill running reflects an increase in these neurotransmitters post-activity, further supporting a role for exercise in regulating mood and responses to stress (Buckworth et al., 2013; de Castro & Duncan, 1985; Dishman et al., 2006; Dunn & Dishman, 1991; Greenwood et al., 2011; MacRae, Spirduso, Walters, Farrar, & Wilcox, 1987).

Voluntary wheel running in rodent behavioral models has also been shown to upregulate galanin, a neuropeptide with widespread projections throughout the brain, which is also co-localized with neurotransmitters such as norepinephrine (Ash & Djouma, 2011; Melander et al., 1986; Robinson & Brewer, 2008; Runesson, Robinson, et al., 2009). Galanin has been directly associated with alterations in the dopaminergic systems, with evidence indicating galanin

decreases the firing rate of VTA neurons (Grenhoff et al., 1993; Weiss et al., 2005), and DA release from striatal slices (Tsuda et al., 1998). Galanin has also been associated with regulating stress responses, mood, and learning and memory (Runesson et al., 2009). Galanin's role in addiction is just now being evaluated, and ongoing work from this lab has determined that the mixed galanin receptor agonist galnon reduces cocaine-induced dopamine overflow within the prefrontal cortex (PFC), while enhancing dopamine release within the nucleus accumbens (NAc; (Ogbonmwan). Additionally, it was reported that galanin reduced cocaine-induced locomotor activity and cocaine-primed reinstatement in a self-administration model (Ogbonmwan). This suggests that galanin's role in modulating dopamine release following cocaine administration is complex, but galanin's influence on drug seeking behavior may depend specifically on modulation of mesocorticolimbic DA systems.

Conditioned place preference (CPP) is a model of learning that pairs a positive or negative cue with a particular context (Tzschentke, 2007). CPP can be established with food or drug administration, and represents an alternative to self-administration models for evaluating drug-seeking behaviors by focusing on the impact of contextual cues on appetitive motivation. Additionally, CPP allows for evaluations of extinction and reinstatement responses, which may provide further understanding of the different stages of the addiction process. In studies of addiction, specific emphasis is often placed upon the ability of a drug to enhance the preference of paired contexts as an indication of the rewarding or "approach" qualities of the drug, which are often associated with projections within the dopaminergic systems (Bardo & Bevins, 2000). It is hypothesized that cocaine-primed CPP requires activation of the mesocorticolimbic system, primarily relying on VTA projections to the PFC and back to the ventral striatum (VS); however,

understanding the dopamine projections directly involved in cocaine-primed CPP needs to be more accurately established (Tzschentke, 2007).

At present, no known efficacious treatment exists for cocaine addiction; however, evidence supporting exercise as a possible non-pharmacological intervention for drug addiction has become a new focal point of addiction research (Lynch et al., 2013). It has been established that both messenger ribonucleic acid (mRNA) and protein levels of galanin in the locus coeruleus (LC) are elevated following three weeks of free access to running wheels in Sprague Dawley rats (Groves-Chapman et al., 2011; Holmes et al., 2006; Murray et al., 2010; Sciolino et al., 2012; Van Hoomissen et al., 2004). The finding that galanin produces inhibitory effects on VTA signaling and dopaminergic tone (Grenhoff et al., 1993; Tsuda et al., 1998; Weiss et al., 2005), supports the hypothesis that enhancement of galanin activity in the LC by exercise may lead to a decrease in dopaminergic tone, thereby diminishing reward-based learning in the CPP

On the other hand, exercise enhances neurogenesis and synaptic plasticity in the hippocampus, a structure essential for learning and memory, through upregulation of trophic factors such as BDNF and enhanced synaptic plasticity (Cotman & Berchtold, 2002; Duman et al., 2008; Farmer et al., 2004; Garza et al., 2004; Fernando Gomez-Pinilla et al., 2008; Gomez-Pinilla et al., 2002; B. N. Greenwood et al., 2009; Griesbach et al., 2004; Griffin et al., 2009; Marais et al., 2009; Neeper et al., 1996; Russo-Neustadt & Chen, 2005; Sartori et al., 2009; Winter et al., 2007). The resulting cognitive enhancement is clearly evident in the exercise literature, which reveals that exercise enhances learning and memory in a variety of paradigms (Eisenstein & Holmes, 2007; Etnier & Chang, 2009; Etnier, Nowell, Landers, & Sibley, 2006; Garcia-Capdevila et al., 2009; Gobbo & O'Mara, 2005; Gomez-Pinilla et al., 2008; Griffin et al., 2009; Hillman, Erickson, & Kramer, 2008; Kramer & Erickson, 2007; Ma, 2008; Ploughman,

2008; Winter et al., 2007). It is this differential activation that may be responsible for the stronger learning patterns observed in exercising animals. Previous reports of facilitation or enhancement of acquisition of CPP following free access to running wheels is consistent with the literature on exercise and cognition (Lynch et al., 2013).

The existing literature on exercise and cocaine addiction; however, is contradictory, with some evidence reporting that forced exercise (treadmill running) reduces acquisition of place preference, while others indicate that exercise facilitates or enhances acquisition (Lynch et al., 2013). The question that arises from this is simple: Does exercise enhance learning in the CPP paradigm or not? The difficulty with answering this question is not just in how the data is interpreted between studies involving forced or voluntary exercise, but in how the CPP model is understood. As mentioned previously, it is known that exercise produces long term adaptations in the mesolimbic DA system. Following from these known effects of exercise, it is expected that a reduction in CPP acquisition would be observed in an exercise-CPP model. Yet exercise also induces plasticity in hippocampal and cortical circuits that promote learning, which would be expected to enhance CPP. These variant mechanisms may counteract each other in the CPP, and explain the conflicting reports on exercise and activity effects on CPP.

To further elucidate the neural systems involved in CPP, and specifically exercise effects on CPP acquisition and reinstatement, experiments were conducted to measure the behavioral and neurochemical effects of exercise on CPP. For the following experiments it was hypothesized that exercise would enhance acquisition of cocaine-primed conditioned place preference. Additionally, it was proposed that exercise would yield faster extinction and provide protection against reinstatement of place preference following a cocaine-priming injection or stress-priming with inescapable footshock. Stress strongly activates mesocortical dopamine

systems as indicated by dopamine overflow in the frontal cortex (Sciolino et al.). We therefore also hypothesized that exercise-induced upregulation of glalanin in the LC to VTA pathway would dampen stress-induced DA activity, which would attenuate stress-induced reinstatement in the CPP paradigm. Finally, we measured mRNA for the transcription factors cFos and zif268 in cortical areas to determine the impact of exercise on general markers of neural activity following cocaine-primed or stress-primed reinstatement. Differential expression at the level of the frontal cortex, LC or NAc post-reinstatement may provide evidence as to system activation during cocaine-and/or stress-primed recall between activity and sedentary animals. If different systems are involved in the recall component of this paradigm, it may provide support for the observed enhancements in conditioned place preference acquisition reported in exercising animals.

Materials and Methods

Animals and Experimental Design

Adult, male Sprague-Dawley rats (Harlan, Inc.) were housed individually in 42L×22W×20H cm polycarbonate cages (N=68) in a temperature and humidity-controlled environment on a 12-hour light/dark schedule. Food and water were provided *ad libitum* and animals were weighed weekly. All procedures were conducted in accordance with National Research Council Guide for Care and Use of Laboratory Animals.

Exercise Protocol

Rats were randomly assigned to either activity wheel (AW) or sedentary (SED) conditions. Stainless steel activity wheels (MiniMeter) with a circumference of 106 cm were placed in cages and attached to a magnetic distance counter. Home cages of sedentary rats did not contain an activity wheel. AW rats were given unlimited access to activity wheels for 21

days, with continued access throughout the training and testing procedures. Running distance was recorded every other day to avoid excessive interruptions of the animals' behavior.

Conditioned Place Preference

Apparatus

The CPP chambers were MedAssociates open field boxes that were fitted with a clear, acrylic neutral chamber with openings into two different contexts. One side had a black wall with white stripes and rod floors (dark chamber), the other side contained black squares on one wall and wire mesh floors (light chamber). A light (60 watts) was placed over the center of the entire chamber to allow equal light exposure to all chambers (zones).

Behavior Measurements

The protocol for conditioned place preference (CPP) is a modified version of protocols provided by Mueller and Stewart (2000). Animals underwent a 30 minute baseline preference test (Bias Test, Figure 4.1) for the conditioned place preference chambers at the cessation of the 21 day sedentary or activity wheel assignment (Day 1). Activity was monitored and recorded by Med Associates software, and time spent per chamber was analyzed to determine initial bias to one chamber over another. Animals were randomly assigned to a drug-paired chamber, dark or light; if their initial preference scores were determined to be approximately 50% for either chamber. Animals that presented with a biased preference of 70% + for one chamber over another were counter bias assigned to the non-preferred side for training. CPP training began 24 hours post-bias testing. Training consisted of an injection of saline in the morning and placement into one of the two chambers (as previously determined). The neutral area was blocked off with a clear piece of acrylic during training and the animal was confined to the

assigned chamber for a total of 15 minutes. Following the termination of the morning training session all animals were returned to their home cage assignments for 4 hours. At the end of the 4 hours animals received a cocaine injection (10 mg/kg) and placed in the corresponding chamber for another 15 minutes. This was repeated over 6 consecutive days (duration previously determined, data not shown). Animals were tested for preference (CPP test, Day 8, Figure 4.1) in the absence of drug or saline exposure 24 hours after the last training session. To evaluate learning, animals were placed into the neutral chamber and allowed free access to both chambers for a total of 30 minutes. Activity was monitored and recorded for analysis.

Extinction

Extinction procedures began on day 9, 24 hours post-CPP testing, and were identical to the training procedure with the exception that all injections contained only saline (Figure 4.1). Extinction procedures continued until chamber preference returned to 50%, previously determined to take 4 days (pilot data not shown). A 5 minute mini-extinction test was conducted after the two sessions of extinction training on day 11 (second day of training) to determine if some animals extinguish the learned behavior at a faster rate.

Reinstatement Tests

Cocaine-Primed

Twenty-four hours post extinction (Day 14, Figure 4.1), a reinstatement test was conducted. This test involved an injection of a smaller dose of cocaine (5 mg/kg) and placement into the open chamber. Activity was monitored for 30 minutes. Post-reinstatement testing, all animals were rapidly decapitated for brain extraction and brain tissue was stored at -80˚C until *in situ* hybridization procedures.

Stress-Primed

A subset of animals was tested in a stress-primed reinstatement protocol. This protocol was identical to the cocaine-primed methods used except for the priming injection. Animals in this group were administered inescapable footshock identical to that outlined elsewhere (McFarland, Davidge, Lapish, & Kalivas, 2004). Briefly, animals received intermittent shock (0.75 mAmps, 0.5 sec duration) at a variable interval schedule (10–70 sec range) for 10 min in a different room. Immediately post-footshock, animals were placed in the open CPP apparatus for reinstatement testing. Post-reinstatement testing, all animals were rapidly decapitated for brain extraction (euthanization time course indicated on Figure 4.1) and brain tissue was stored at -80˚C until *in situ* hybridization procedures.

In Situ Hybridization

Brains were sectioned into 12µm slices at the level of the locus coeruleus and nucleus accumbens using a Microm cryostat (Carl Zeiss, Waldorff, Germany) and thaw-mounted to gelatin coated microscope slides. In situ hybridization methods used are reported in detail elsewhere (Groves-Chapman et al., 2011; Murray et al., 2010; Van Hoomissen et al., 2004). Briefly, slides were fixed in 4% formaldehyde in 0.12M sodium phosphate-buffered saline, rinsed in PBS, and placed in 0.25% acetic anhydride. Sections were dehydrated through a series of ethanol washes, delipidated in chloroform, rinsed again in ethanol, and allowed to dry.

Oligonucleotide probes (Oligos Etc., Wilsonville, OR) for galanin, cFos, and zif268 were labeled at the 3′ end with [35S]-dATP (New England Nuclear, Boston, MA), terminal deoxynucleotidyl transferase (TdT, 25 units/ml; Roche, Indianapolis, IN), and tailing buffer. Column separation was used to remove unbound nucleotide. Sections were hybridized with radiolabeled probe in solution containing formamide, NaCL, Tris-HCl, EDTA, sodium

pyrophosphate, sodium dodecyl sulfate, and dextran sulfate. Sections were incubated in hybridization solution, followed by a series of washes to reduce nonspecific binding. Microscope slides were rinsed in deionized water and allowed to dry. Hybridized brain sections were exposed to autoradiographic film for a period of 3 weeks and then developed. Probes for immediate-early genes cFos and zif 268 were also applied to brain sections for the locus coeruleus and nucleus accumbens regions, respectively.

Film Analysis

Autoradiographic film analysis was conducted with NIH ImageJ (Rasband, 1997-2012), a computerized analysis system to determine optical density (OD) within the locus coeruleus, nucleus accumbens, piriform and frontal cortices.

Data Analysis

Behavioral analysis for CPP, Extinction, and Reinstatement tests were conducted for time spent per zone and zone entries. Preference scores calculated as time spent in drug paired zone/time spent in drug paired zone + time spent in unpaired zone. Preference scores were analyzed in ten minute bins over the course of the session. Repeated measures analyses of variance (RM-ANOVA) were used to evaluate differences between assignments in ambulatory distance travelled over time for each test, and for weights between assignments. Analyses for *in situ* hybridization data were conducted to determine group (exercise vs sedentary) and testing (cocaine-primed vs stress-primed reinstatement) effects. Bonferroni post-hoc analyses were included for all RM-ANOVAs. Running distances were assessed by week using descriptive statistics. All analyses were conducted using Graph Pad Prism version 6.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Conditioned Place Preference

Repeated measures analysis of variances (RM-ANOVA) were conducted for preference scores of total time spent in paired chamber during each behavioral test to assess differences between sedentary and activity animals. Sedentary and activity animals learned the paired association between drug and context, evident by a preference score above 0.50. No significant interaction or main effects of assignment or time were observed for the CPP test (Interaction: F (2,132) = 0.10, p = 0.91; Time: F (2,132) = 0.82, p = 0.44; Assignment: F (1, 66) = 0.00, p = 0.96; Figure 4.2). Comparisons were conducted using scores averaged into 10 minute time bins per subject. Additionally, both groups presented with extinction of association between drug and chamber during the mini-extinction test (Figure 4.3) and the extinction test (Figure 4.4) indicated by a preference score of less than 0.50. No difference was observed between assignments for the mini-extinction test, calculated using an unpaired t-test because this test was only 10 minutes long (p = 0.24). There was also no significant interaction or main effects observed for the full extinction test (Interaction: F (2,126) = 0.80, p = 0.45; Time: F (2,126) = 0.70, p = 0.50; Assignment: F (1, 63) = 0.36, p = 0.55). No animals were excluded from the behavioral analyses due to previous chamber bias, as this was addressed with counter balance of chamber assignment. No significant differences between assignments for total entries into the paired chambers were observed for any test (data reported as means and SEM in Table 4.1).

Cocaine-Primed Reinstatement

Both groups reinstated to time spent in the previously paired chamber following a smaller dose of cocaine administered IP as indicated by a preference score greater than 0.50. RM-ANOVA revealed no significant interaction or main effect for assignment (F (2, 98) = 1.75, p =

0.18; F (1, 49) = 0.11, p = 0.74; respectively). A significant main effect for time was observed for the cocaine-primed reinstatement test (F (2, 98) = 3.58, $p < 0.05$). To further evaluate this difference, planned comparisons were conducted between assignments for each 10 minute time bin. The difference noted on the graphs between activity and sedentary animals indicate that activity animals reinstated better than sedentary counterparts during the first 10 minute time bin (t(49) = -2.46, $p < 0.05$; Figure 4.5) only. No difference between sedentary and activity assignments was observed for the second or third 10 minute time bins of this model (p = 0.67 and p = 0.82, respectively).

Stress-Primed Reinstatement

RM-ANOVA revealed no interaction or assignment effects for the stress-primed reinstatement test (F (2, 26) = 2.62, p = 0.09; F (1, 13) = 2.25, p = 0.16; respectively). However, a significant effect of time was observed (F (2, 26) = 5.38, $p < 0.05$). Upon closer evaluation of this time effect, the graphs indicate that following a 20 minute priming with inescapable footshock, both sedentary and activity animals presented with an initial reinstatement to time spent in the previously paired chamber as indicated by a preference score greater than 0.50. However, activity animals preference scores quickly dropped below reinstatement levels during time bin 2 and 3 (preference score less than 0.50). To further evaluate this difference, planned comparisons were conducted between assignments for each 10 minute time bin. The difference noted on the graphs between activity and sedentary animals indicate that activity animals reinstated less than sedentary counterparts during the second 10 minute time bin (t(13) = 2.31, $p < 0.05$; Figure 4.6) only. No difference between sedentary and activity assignments was observed for the first or third 10 minute time bins of this model (p = 0.06 and p = 0.19, respectively).

Galanin mRNA Expression in the Locus Coeruleus

Galanin mRNA expression in the LC was analyzed using unpaired t-tests for assignment for both cocaine-primed reinstatement and stress-primed reinstatement tests (Table 4.5). There was no observed difference in galanin mRNA expression between sedentary and activity animals in the cocaine-primed reinstatement model ($p = 0.29$; Figure 4.9) or in the stress-primed reinstatement model ($p = 0.18$; Figure 4.10).

Zif268 and cFos mRNA Expression in the Nucleus Accumbens, Piriform and Frontal Cortices

mRNA expression for immediate-early genes zif268 and cFos were analyzed in the NAc, piriform (PC) and frontal cortices (FC) using unpaired t-tests for assignment for both cocaine-primed reinstatement and stress-primed reinstatement tests (Table 4.6 and 4.7, respectively). There was no observed difference in zif268 mRNA expression between sedentary and activity animals in the cocaine-primed reinstatement model (NAc: $p = 0.92$, PC: $p = 0.92$, FC: $p = 0.80$), or the stress-primed reinstatement model (NAc: $p = 0.21$, PC: $p = 0.29$, FC: $p = 0.76$) for any region. Additionally, no observed difference in cFos mRNA expression was observed for the stress-primed reinstatement model (Figure 4.12; NAc: $p = 0.66$, PC: $p = 0.41$, FC: $p = 0.16$). cFos mRNA expression in the cortical areas was determined to be significant between assignments, with sedentary animals exhibiting higher levels of cFos mRNA expression than activity animals (Figure 4.11; PC: $t(42) = 2.65$, $p = 0.01$; FC: $t(42) = 2.03$, $p < 0.05$). cFos mRNA expression was not observed to be different between sedentary and activity animals for the NAc ($p = 0.86$) cFos mRNA expression was not present in the LC.

General Activity Data: Ambulatory Distance over Time

RM-ANOVAs were conducted for each test to evaluate assignment differences in distance travelled over time. Means and standard errors for all tests by assignment are presented in Table 4.2. Significant main effects for time were observed for all tests, including baseline activity, with all animals showing a decrease in ambulatory distance travelled over time (Baseline: $F(29, 1856) = 41.30$, $p < 0.01$; CPP: $F(29, 1827) = 98.99$, $p < 0.01$; Mini-extinction: $F(9, 585) = 58.48$, $p < 0.01$) =, $p < 0.01$; Extinction: $F(29, 1798) = 104.69$, $p < 0.01$; Cocaine-primed reinstatement: $F(29, 1421) = 91.12$, $p < 0.01$; Stress-primed reinstatement: $F(29, 348) = 16.49$, $p < 0.01$). Additionally, a significant main effect for assignment was observed for the following tests: Baseline/Bias test ($F(1, 1856) = 15.01$, $p < 0.01$), CPP test ($F(1, 1827) = 8.43$, $p < 0.01$), Extinction test ($F(1, 1798) = 52.21$, $p < 0.01$). Interaction effects of assignment over time were also determined to be significant for the following tests: Baseline/bias ($F(29, 1856) = 2.14$, $p < 0.01$), cocaine-primed reinstatement test ($F(29, 1421) = 1.73$, $p = 0.01$), and stress-primed reinstatement ($F(29, 348) = 2.07$, $p < 0.01$).

Running Distances and Weights

Average weekly running distances are reported in Table 4.3 and Figure 4.7. Wheel running increased over the 3 weeks prior to testing, and a notable decrease was observed for the weeks during testing and training. Average weights by assignment are reported in Table 4.4 and Figure 4.8. All animals remained in a normal weight range throughout the experiments, with some between assignment differences observed during the initial three weeks, consistent with typical activity versus sedentary assignments (Groves-Chapman et al., 2011; Murray et al., 2010).

Discussion

The results indicate that three weeks of voluntary wheel running differentially affects reinstatement in a cocaine-cued and stress-cued condition place preference (CPP) model of addiction. We observed both behavioral and gene expression differences between assignments for both of the reinstatement models evaluated. While our results indicate that sedentary and activity animals both acquired CPP and extinguished the behavior similarly, reinstatement behaviors differed between exercising and sedentary rats depending on the paradigm. First, it was observed that activity animals reinstated significantly better than sedentary animals to their previously drug-paired chambers in the cocaine-primed reinstatement condition. However, in the stress-primed reinstatement condition, activity animals did not reinstate post-inescapable footshock. The difference observed between sedentary and activity animals in this condition was marked enough to suggest a possible aversion to the previously drug-paired chamber for activity animals. We also observed associated changes in cFos mRNA expression in cortical areas between assignments. The immediate early gene, cFos, was observed to be elevated in cortical areas in sedentary animals compared to activity animals for the cocaine-primed reinstatement model only.

Previous studies reveal that voluntary wheel running attenuates cocaine-primed reinstatement in a cocaine self-administration paradigm (Smith et al., 2012). The present findings suggest that the CPP model is not sensitive to exercise effects on cocaine-primed reinstatement. Interestingly, we also did not observe exercise-induced increases in the acquisition of CPP, as has previously been reported (Smith et al., 2008). However, both activity and sedentary animals clearly learned and extinguished the behavior. One explanation for the differences in behavior observed in the cocaine-primed reinstatement test may be that exercising

animals performance reflects a learning pattern, modulated by systems external to the ventral tegmental area (VTA) – ventral striatum (VS) – cortex loop typically associated with addictive-related behaviors (Morales & Pickel, 2012). The present study did not provide a direct measure of other potential circuitry involved in the acquisition, maintenance, and retention of place preference models (i.e. the hippocampus); however, it is possible that the observed behavioral effects of activity animals in the cocaine-primed model reflect the involvement of other brain systems. This would provide one explanation for the difference in cFos mRNA expression in cortical areas of activity animals compared to sedentary animals. cFos has previously been indicated to be activated in areas associated with addiction-related behaviors during drug use (Harlan & Garcia, 1998). Recently, models of learning have proposed that a VTA-hippocampus loop is involved in the transfer of new information into long-term memory (Lisman & Grace, 2005). Since ample evidence exists regarding exercise effects on neurogenesis and plasticity within the hippocampus (Buckworth et al., 2013; Dishman et al., 2006; Etnier & Chang, 2009; Farmer et al., 2004; Garcia-Capdevila et al., 2009; Garza et al., 2004; Gobbo & O'Mara, 2005; F. Gomez-Pinilla et al., 2002; B. Greenwood et al., 2003; Griffin et al., 2009; Lee & Rhyu, 2009; Neeper et al., 1996; Ploughman, 2008; Sartori et al., 2009), it seems conceivable that this is what is occurring in this model. Also, exercise is often associated with enhanced performance on learning tasks, including one report of enhanced CPP (Buckworth et al., 2013; Dishman et al., 2006; Eisenstein & Holmes, 2007; Etnier & Chang, 2009; Garcia-Capdevila et al., 2009; Gobbo & O'Mara, 2005; Griffin et al., 2009), lending credence to this hypothesis.

Contrary to previous reports from our lab, we did not find a difference in galanin mRNA expression within the LC between sedentary and activity conditions. This finding is remarkable in that our lab has consistently shown that three weeks of voluntary wheel running yields

elevations in both galanin mRNA expression and protein levels (Holmes et al., 2006; Murray et al., 2010; Reiss et al., 2009; Sciolino et al., 2012; Van Hoomissen et al., 2004). The most straight forward explanation for the lack of galanin expression differences observed between sedentary and activity animals is that the cocaine and stress-primed models utilized for this study provoke an acute response that diminishes the exercise-induced difference in galanin expression. Either cocaine treatment or footshock prior to brain harvesting may have influenced galanin gene expression, as we have not previously examined the effect of acute cocaine or footshock treatment on exercise-induced upregulation of galanin.

While these results indicate exercise may confer protective effects in stress and addiction-related behaviors, more research is needed to further elucidate the neurochemical and neurobiological systems involved in these models. More importantly, caution should be taken when utilizing models of association, acquisition, retention, and recall with exercise interventions. As was indicated here, enhanced performance on a task should not immediately be assumed to be an increased sensitivity to a drug, if the addiction model used to evaluate exercise effects involves learning and memory processes.

Table 4.1 Preference scores for average zone entries. Data are presented as means and SEM.

Average Zone Entries Preference Scores

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
CPP	0.5280	0.0112	0.5157	0.0144
Mini-Extinction	0.4468	0.0191	0.4617	0.0231
Extinction	0.4933	0.0156	0.5173	0.0159
Cocaine Reinstatement	0.4981	0.0159	0.5409	0.0166
Stress Reinstatement	0.5152	0.0367	0.4168	0.0462

Table 4.2 Average ambulatory distance (centimeters) by assignment (sedentary and activity) animals for all tests. Data are presented as means and SEM.

Average Ambulatory Distance By Test
*****indicates significant difference between assignments, $p < 0.05$

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
Baseline	118.09*	6.12	86.36	6.49
CPP	130.87*	10.60	110.27	10.68
Mini-Extinction	113.22	10.09	108.94	11.02
Extinction	120.62*	11.67	83.61	10.04
Cocaine Reinstatement	145.30	12.74	126.2	12.21
Stress Reinstatement	110.90	9.29	107.00	11.88

Table 4.3 Average wheel running distances (meters) over time (weeks). Data are presented as means and SEM.

Average Running Distance (m)

	Mean	SEM
Week 1	3096.85	203.58
Week 2	6504.69	309.37
Week 3	9093.01	489.54
Week 4	4004.73	126.04
Week 5	4156.46	148.70

Table 4.4 Average weights (grams) by assignment (sedentary vs. activity). Data are presented as means and SEM.

Average Weights

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
Day 1	234.85	1.87	236.61	1.92
Day 7	280.29	3.68	262.52	3.80
Day14	308.86	2.67	287.85	3.17
Day 21	326.21	3.82	304.82	4.39
Day 29	325.00	3.82	307.76	4.29
Day 33	335.91	4.23	323.48	4.47

Table 4.5 Average Galanin mRNA expression (grayscale units) in the LC by assignment (sedentary vs. activity). Data are presented as means and SEM.

Average Galanin mRNA Expression By Assignment and Test

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
Cocaine Reinstatement				
Locus Coeruleus	136.50	2.30	131.62	3.91
Stress Reinstatement				
Locus Coeruleus	137.13	2.25	141.31	1.97

Table 4.6 Average zif268 mRNA expression (grayscale units) by brain region (NAc, PC, FC) by assignment (sedentary vs. activity). Data are presented as means and SEM.

Average Zif268 mRNA Expression By Assignment, Test, and Region

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
Cocaine Reinstatement				
Nucleus Accumbens	83.44	1.32	84.07	1.19
Piriform Cortex	101.69	1.62	101.48	1.21
Frontal Cortex	101.86	1.56	101.32	1.39
Stress Reinstatement				
Nucleus Accumbens	79.63	1.53	83.26	2.42
Piriform Cortex	103.65	1.63	107.27	3.07
Frontal Cortex	100.192	2.55	101.32	2.41

Table 4.7 Average cFos mRNA expression (grayscale units) by brain region (NAc, PC, FC) by assignment (sedentary vs. activity). Data are presented as means and SEM.

Average cFos mRNA Expression By Assignment, Test, and Region
*indicates significant difference between assignments, $p < 0.05$

	Sedentary		Activity	
	Mean	**SEM**	**Mean**	**SEM**
Cocaine Reinstatement				
Nucleus Accumbens	78.94	0.80	78.73	0.95
Piriform Cortex	*****104.64	2.00	98.21	1.37
Frontal Cortex	*****89.43	1.44	85.66	1.18
Stress Reinstatement				
Nucleus Accumbens	76.68	0.73	75.68	0.74
Piriform Cortex	99.73	0.83	102.26	0.93
Frontal Cortex	85.15	1.53	82.38	0.95

Figure 4.1 Time line image for sedentary vs activity assignment and CPP experiments.

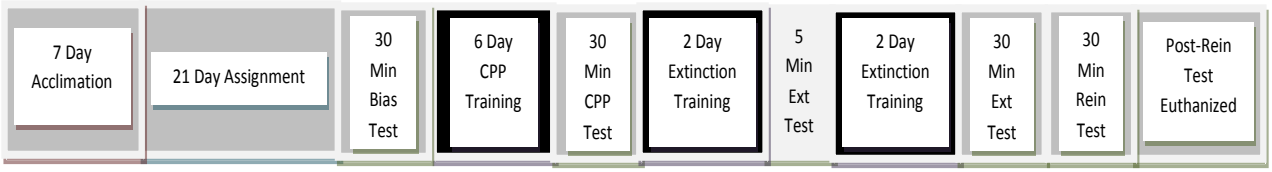

7 Day Acclimation
21 Day Assignment
30 Min Bias Test
6 Day CPP Training
30 Min CPP Test
2 Day Extinction Training
5 Min Ext Test
2 Day Extinction Training
30 Min Ext Test
30 Min Rein Test
Post-Rein Test Euthanized

Figure 4.2 Average preference scores for time in paired chamber: CPP test. Data are presented as means ± SEM. RM-ANOVA revealed no significant difference between sedentary and activity assignment.

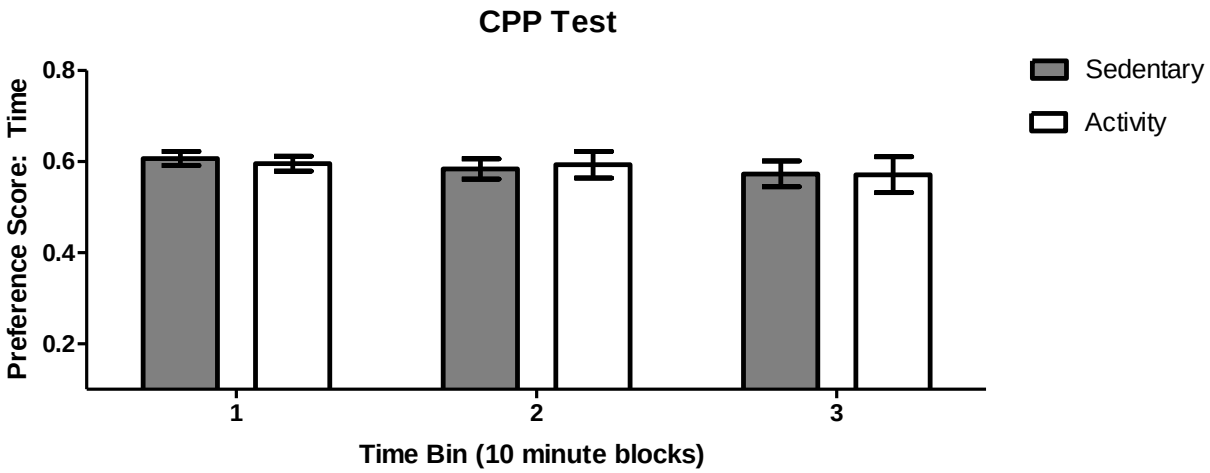

CPP Test
Preference Score: Time
0.8
0.6
0.4
0.2
1
2
3
Time Bin (10 minute blocks)
Sedentary
Activity

Figure 4.3 Average preference scores for time in paired chamber: Mini-extinction test. Data are presented as means ± SEM. RM-ANOVA revealed no significant difference between sedentary and activity assignment.

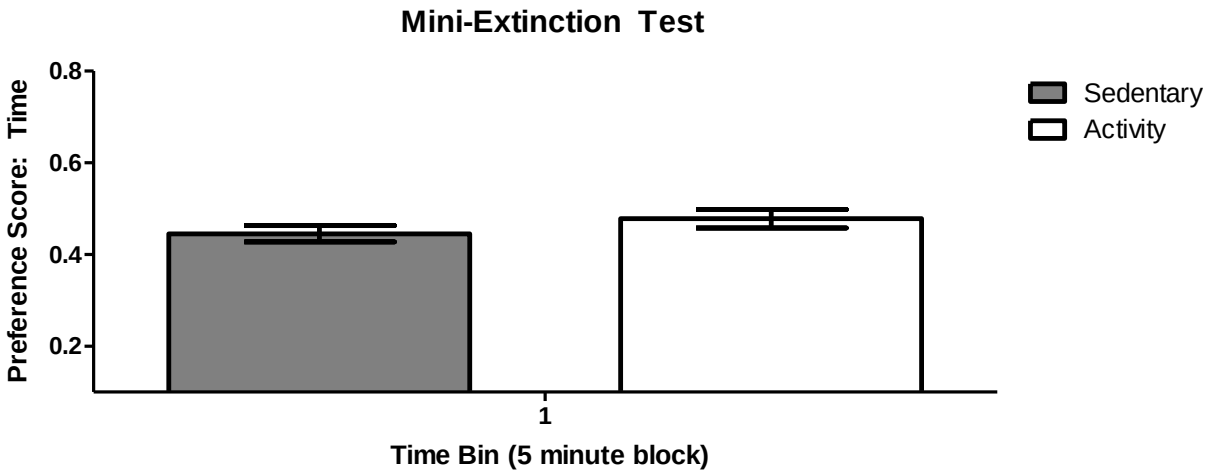

Mini-Extinction Test
Preference Score: Time
0.8
0.6
0.4
0.2
1
Time Bin (5 minute block)
Sedentary
Activity

Figure 4.4 Average preference scores for time in paired chamber: Extinction test. Data are presented as means ± SEM. RM-ANOVA revealed no significant difference between sedentary and activity assignment.

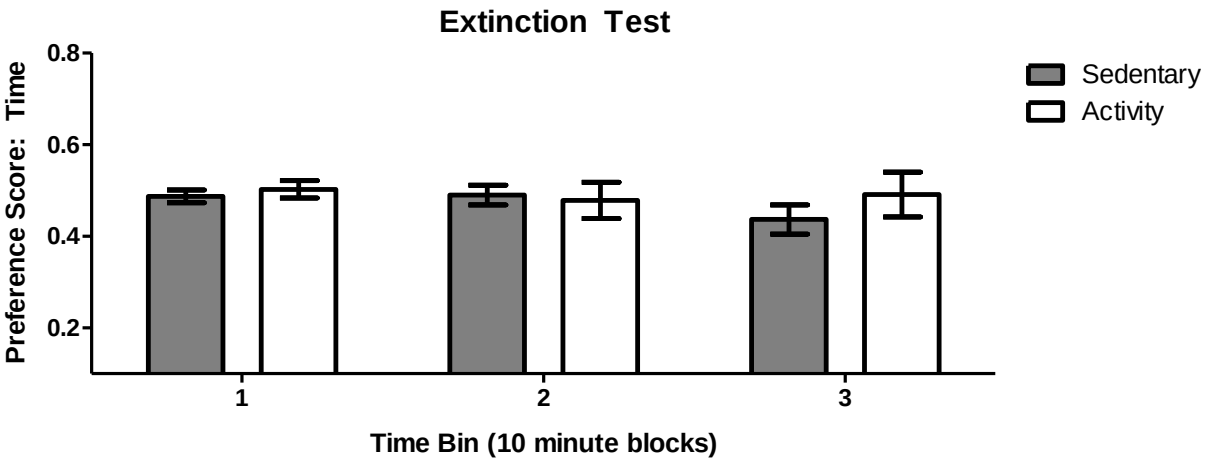

Extinction Test
Preference Score: Time
0.8
0.6
0.4
0.2
1
2
3
Time Bin (10 minute blocks)
Sedentary
Activity

Figure 4.5 Average preference scores for time in paired chamber: Cocaine reinstatement test. Data are presented as means ± SEM. Post-hoc analysis indicated a significant difference for time spent in paired chamber for time bin 1 only. Activity animals' preference scores were observed to be higher for this time bin than sedentary animals. *indicates significant difference between assignments, $p < 0.05$

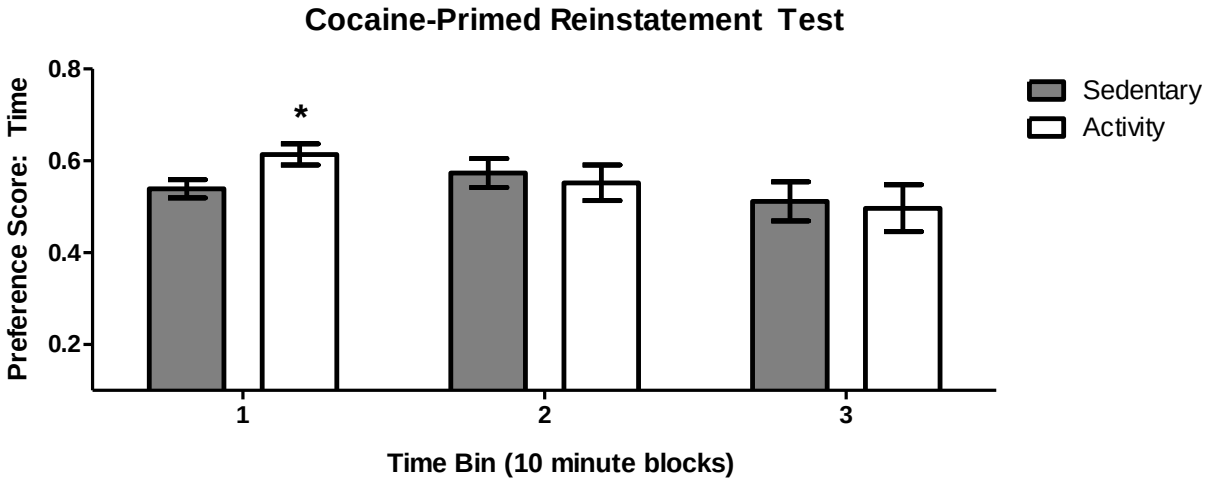

Cocaine-Primed Reinstatement Test
Preference Score: Time
0.8
0.6
0.4
0.2
*
1
2
3
Time Bin (10 minute blocks)
Sedentary
Activity

Figure 4.6 Average preference scores for time in paired chamber: Stress reinstatement test. Data are presented as means ± SEM. Post-hoc analysis resulted in a significant difference for time spent in paired chamber for time bin 2 only. Sedentary animals' preference scores were observed to be higher for this time bin than activity animals. *indicates significant difference between assignments, $p < 0.05$

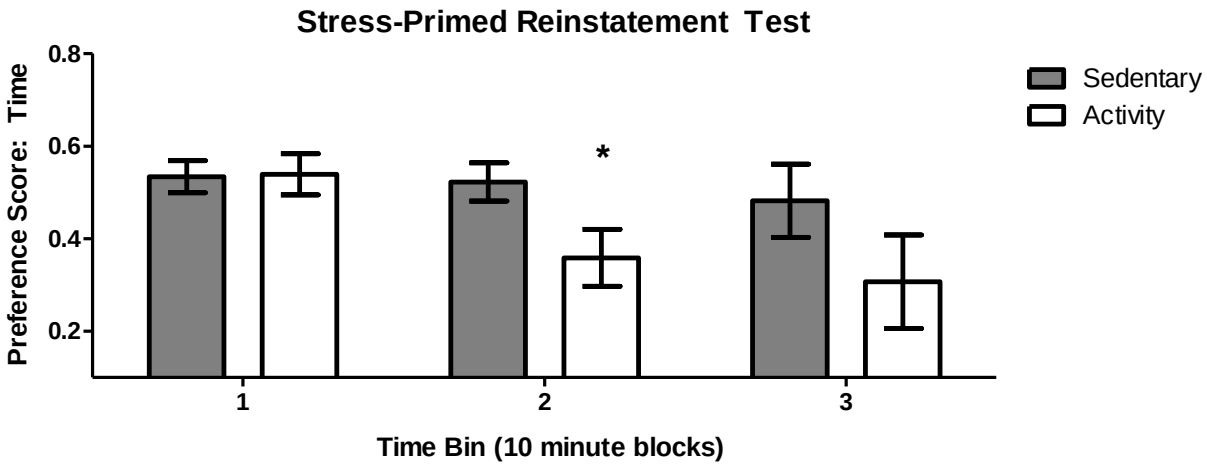

Stress-Primed Reinstatement Test
Preference Score: Time
0.8
0.6
0.4
0.2
*
1
2
3
Time Bin (10 minute blocks)
Sedentary
Activity

Figure 4.7 Average running distances (meters) by week. Data are presented as means ± SEM.

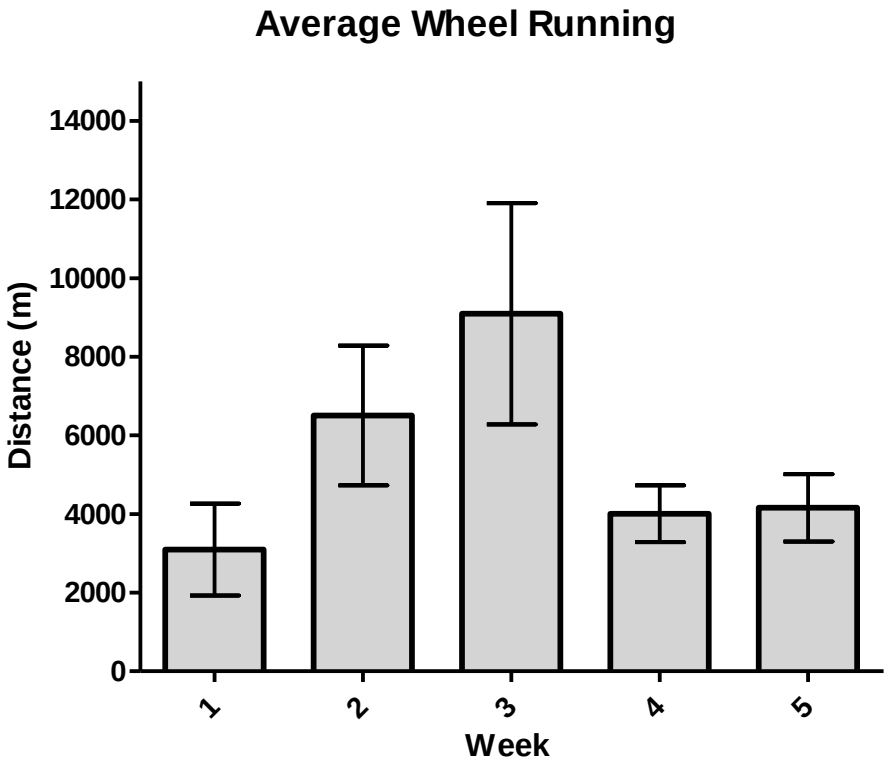

Average Wheel Running
Distance (m)
14000
12000
10000
8000
6000
4000
2000
0
1
2
3
4
5
Week

Figure 4.8 Average weights (grams) by assignment. Data are presented as means ± SEM.

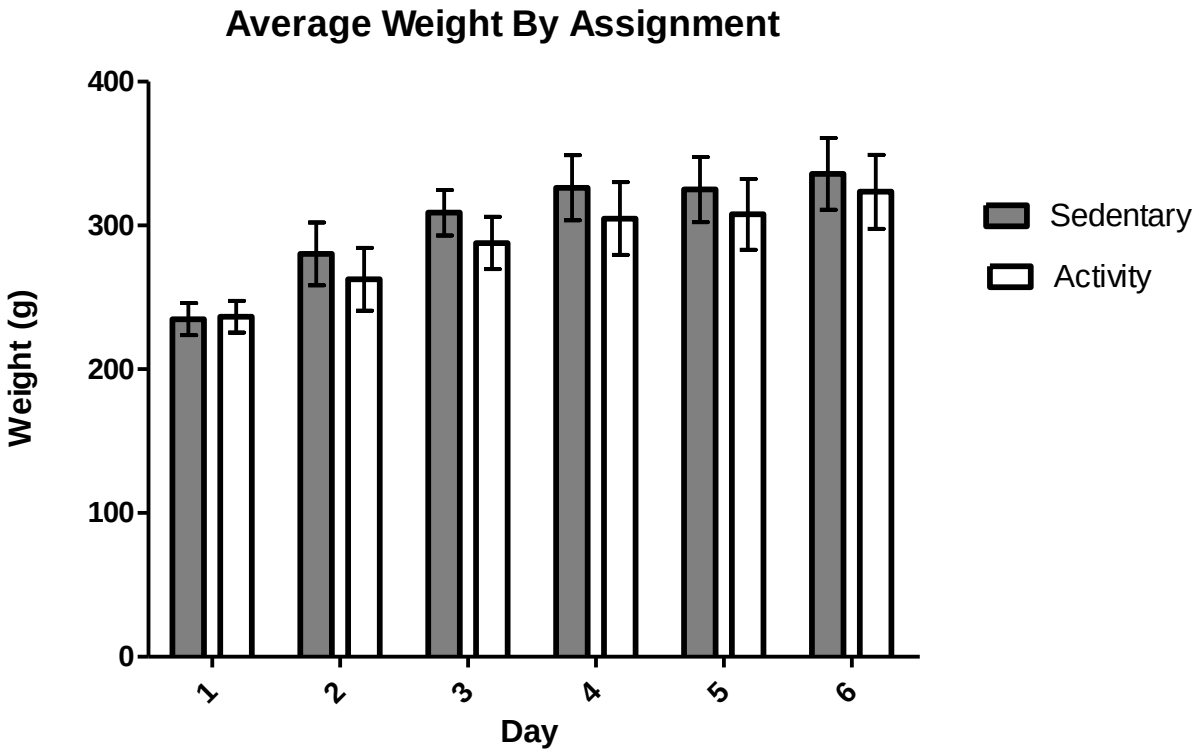

Average Weight By Assignment
400
300
200
100
0
Weight (g)
1
2
3
4
5
6
Day
Sedentary
Activity

Figure 4.9 Average Galanin mRNA expression (grayscale units) in the LC by assignment (sedentary vs. activity) for cocaine-primed reinstatement. Data are presented as means ± SEM. An unpaired t-test found no significant difference in galanin mRNA expression between sedentary and activity assignments.

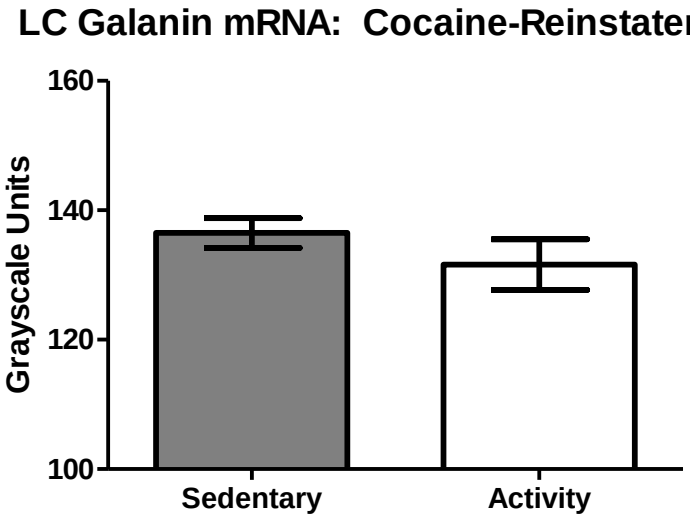

LC Galanin mRNA: Cocaine-Reinstatement
Grayscale Units
160
140
120
100
Sedentary
Activity

Figure 4.10 Average Galanin mRNA expression (grayscale units) in the LC by assignment (sedentary vs. activity) for stress-primed reinstatement. Data are presented as means ± SEM. An unpaired t-test found no significant difference in galanin mRNA expression between sedentary and activity assignments.

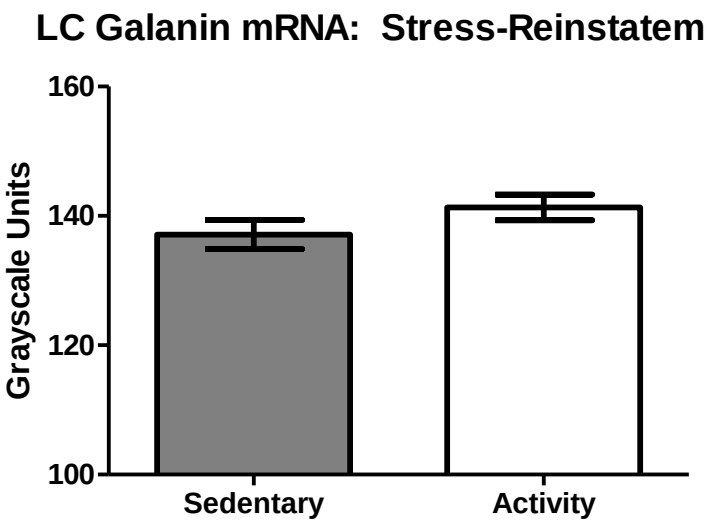

LC Galanin mRNA: Stress-Reinstatement
Grayscale Units
160
140
120
100
Sedentary
Activity

Figure 4.11 Average cFos mRNA expression (grayscale units) by brain region (NAc, PC, FC) and assignment (sedentary vs. activity) for cocaine-primed reinstatement. Data are presented as means ± SEM. An unpaired t-test revealed a significant difference in cFos mRNA expression between sedentary and activity animals for both the PC and FC regions, with sedentary animals exhibiting higher expression levels. No significant difference in cFos mRNA was observed in the NAc. *indicates significant difference between assignments, $p < 0.05$

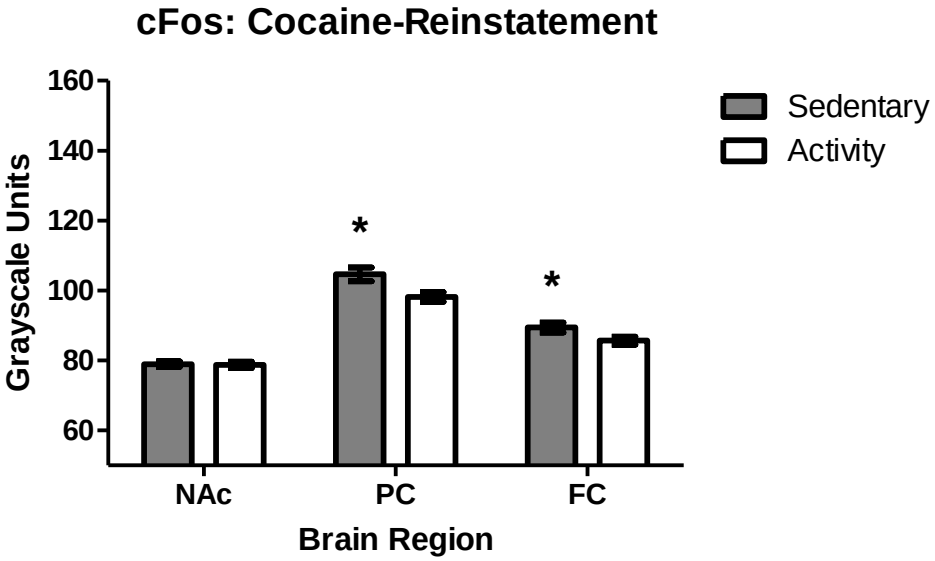
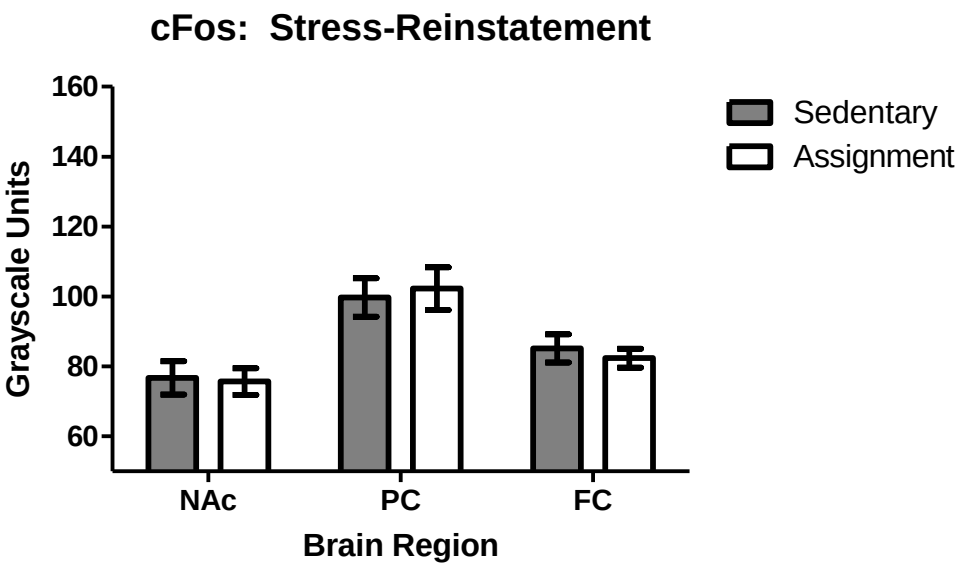

cFos: Cocaine-Reinstatement
Grayscale Units
160
140
120
100
80
60
*
*
NAc
PC
FC
Brain Region
Sedentary
Activity

Figure 4.12 Average cFos mRNA expression (grayscale units) by brain region (NAc, PC, FC) and assignment (sedentary vs. activity) for stress-primed reinstatement. Data are presented as means ± SEM. An unpaired t-test indicated no significant difference in cFos mRNA expression between sedentary and activity assignments.


cFos: Stress-Reinstatement
Grayscale Units
160
140
120
100
80
60
NAc
PC
FC
Brain Region
Sedentary
Assignment

CHAPTER 5

SUMMARY

The studies presented provide evidence for the influence of specific receptor subtypes of the neuropeptide galanin on neuroprotective effects within the hippocampus, and exercise-induced galanin effects on the modulation of addiction-related behaviors. In paper 1 (Chapter 3) we presented evidence that activation of GALR1 was essential to induce the neuroprotective effects observed in a kainic acid (KA)-excitotoxicity model; however, we failed to find similar effects utilizing a GALR2 specific agonist (M1145). It was also observed that the mixed GALR1/GALR2 agonist (M1154) conferred protective effects from cell loss in the CA3 region of the hippocampal formation to a lesser extent than the GALR1 specific agonist (M617). Contrary to our proposed effects of these agonists on reducing the seizure-typical behaviors associated with KA excitotoxicity, we found that none of the galanin receptor agonists, regardless of affinity for GALR1 or GALR2, produced anticonvulsant effects. One possible explanation of this finding may be in the inconsistency of seizures induced through ICV administrated KA. This particular model most likely yields seizure-typical behaviors through excitotoxicity in the limbic and motor cortex, and therefore any variances in observed seizure behavior may be due to variations in dispersal of the KA in the brain.

In paper 2 (Chapter 4) we examined the effects of three weeks of voluntary wheel running on performance in a cocaine-cued condition place preference (CPP) model of addiction. We evaluated ambulatory time spent in drug-paired zone in relation to time spent in unpaired zone for each subject, and calculated a preference score average for each testing procedure. Our

results indicate that sedentary and activity animals acquired CPP and extinguished the behavior similarly. However, we observed performance differences between assignments (sedentary vs. activity) for both the cocaine-primed and stress-primed reinstatement models. In the cocaine-primed reinstatement test, activity animals reinstated significantly better than sedentary animals to their previously drug-paired chambers. These findings are in direct contradiction to other reports indicating that access to wheel running decreases cocaine-primed reinstatement (Smith et al., 2012). Yet activity animals did reinstate comparably to sedentary animals for the first 10 minute bin in the stress-primed reinstatement model; however this shifted to a dramatic reduction in time spent in drug-paired chamber in the second 10 minute bin. In fact, during the second 10 minute time bin activity animals reflected preference scores below the scores they presented with during the extinction tests, indicating a possible aversion to the previously drug-paired chamber. This suggests that a stress-primed reinstatement model may be optimal in assessing protective exercise effects on addiction-related behaviors.

Interestingly, we also observed associated changes in cFos mRNA expression in cortical areas between assignments for the cocaine-primed reinstatement. cFos expression was observed to be elevated in sedentary animals compared to activity animals in both the piriform and frontal cortices, suggesting that cortical areas are not involved in the behavioral differences observed in activity animals for the cocaine-primed reinstatement model. Whether these values of expression would be different from animals injected with saline has yet to be determined. While this study did not provide a direct measure of other potential circuitry involved in the acquisition, maintenance, and retention of place preference models (i.e. the hippocampus), it is possible that the observed behavioral effects of activity animals in the cocaine-primed reinstatement model reflect the involvement of other brain systems not typically associated with addiction-related

behaviors. More importantly, it is plausible that activity animals reflect an enhancement in learning and retention of that learning in the cocaine-primed model. This would explain the lack of cFos, an immediate early gene associated with general activation (Harlan & Garcia, 1998), expression in cortical areas in the activity animals compared to sedentary animals.

The studies presented here reflect the complexities of galanin's role in neuroprotection and neuromodulation. The first study provides results supporting the specificity of galanin receptor activation in neuroprotection from excitotoxic toxins like KA. Furthermore, it offers supportive evidence for the necessary role of GALR1 in protection from excitotoxic insults. While study two does not provide evidence directly linking galanin with the neuroprotective effects of exercise in a CPP model of addiction, it does present evidence for exercise effects on addiction-related behaviors; particularly in protection against stress-primed reinstatement. Future studies of addiction-related behaviors, like those involving voluntary wheel running models, should take caution in the interpretation of results. Delineating between learning effects and drug sensitivities should be considered when assessing results from cocaine-primed reinstatement models.

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