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Gap Junction Communication in Memory Retrieval and Extinction of Cocaine Seeking

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GAP JUNCTION COMMUNICATION
IN MEMORY RETRIEVAL AND EXTINCTION OF COCAINE SEEKING

by

Michael K. Fitzgerald

A Dissertation Submitted in
Partial Fulfillment of the
Requirements for the Degree of

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ABSTRACT

GAP JUNCTION COMMUNICATION IN MEMORY RETRIEVAL AND EXTINCTION OF COCAINE SEEKING

by

Michael K. Fitzgerald

The University of Wisconsin-Milwaukee, 2016
Under the Supervision of Devin Mueller

Blocking drug-associated memory retrieval or enhancing extinction of drug-seeking behavior are two strategies that could limit relapse in drug addicts. The loci of retrieval and extinction memory processes include the prelimbic medial prefrontal cortex and the infralimbic medial prefrontal cortex, respectively. The neurochemical and synaptic mechanisms underlying drug-related behavior have received considerable attention, but extrasynaptic mechanisms are relatively unexplored. One form of cellular communication, gap junction communication, may play a role in drug-related learning and memory. Gap junction communication between neurons and astrocytes provide a cytoplasmic continuity between connected cells and both neuronal and astrocytic gap junction communication have been demonstrated to be involved with development and maintenance of the CNS, and also aversive and appetitive paradigms of learning. However, in retrieval or extinction of drug-seeking behavior, the role of gap junction communication is unknown. Here I describe a series of experiments that investigate astrocytic and neuronal gap junction communication in the retrieval and extinction of a cocaine conditioned place preference using microinfusions and confocal imaging.

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TABLE OF CONTENTS

Page 1: Introduction

Page 1: Overview

Page 2: Conditioned place preference

Page 2: PL-mPFC and drug-associated memory retrieval

Page 4: IL-mPFC and extinction of drug-seeking

Page 5: Gap junctions

Page 6: Neuronal gap junction communication

Page 8: Astrocytic gap junction communication

Page 13: Gap junction communication in reward-seeking and addictive behaviors

Page 16: Dissertation Goal and Aims

Page 17: Methods

Page 17: Methods: Aim 1a, retrieval of cocaine CPP

Page 17: Cannula surgery

Page 18: Drugs and microinfusions

Page 18: Place conditioning

Page 19: Experimental manipulations

Page 20: Alternative Strategies Implemented

Page 22: Methods: Aim 1b, extinction of cocaine CPP

Page 22: Cannula surgery

Page 22: Drugs and microinfusions

Page 22: Place conditioning

Page 22: Experimental manipulations

Page 24: Methods: Aim 2

Page 24: Tissue preparation and removal for astrocyte identification

Page 25: Loading of astrocytes with calcium indicator

Page 26: Experimental manipulations

Page 26: Confocal microscopy, recording astrocytic calcium activity

Page 29: Results

Page 29: Aim 1a

Page 39: Aim 1b

Page 41: Aim 2

Page 52: Discussion

Page 53: Aim 1

Page 55: Aim 2

Page 59: Future directions

Page 60: Clinical relevance

Page 61: Conclusion

Page 62: References

Page 76: Curriculum Vitae

LIST OF FIGURES

Figure 1, Page 31: PL-mPFC blockade of general or astrocyte-specific gap junctions prevent extinction of a cocaine-CPP.

Figure 2, Page 33: PL-mPFC infusion of both neuron-specific gap junction blocker quinine and GABA_A receptor agonist muscimol restores normal drug-seeking behavior and extinction of the CPP.

Figure 3, Page 36: PL-mPFC infusion of both unopposed hemichannel blocker Gap19 and astrocyte-specific gap junction blocker IRL-1620 persistently disrupts retrieval of CPP.

Figure 4, Page 38: PL-mPFC infusion of both unopposed hemichannel blocker Gap19 and astrocyte-specific gap junction blocker IRL-1620 disrupts retrieval of an established CPP.

Figure 5, Page 40: IL-mPFC blockade of general or astrocyte-specific gap junctions prevent extinction of a cocaine-CPP.

Figure 6, Page 43: Imaging of astrocytes using confocal microscopy.

Figure 7, Page 44: Pre-bath application of NE increases PL-mPFC astrocyte calcium levels.

Figure 8, Page 45: Pre-bath application of non-selective gap junction blocker CBX does not change PL-mPFC astrocyte calcium levels.

Figure 9, Page 46: Pre-bath application of astrocyte gap junction blocker IRL-1620 does not change PL-mPFC astrocyte calcium levels.

Figure 10, Page 47: Pre-bath application of neuron-specific gap junction blocker quinine increases PL-mPFC astrocyte calcium levels.

Figure 11, Page 48: Pre-bath application of CBX + NE does not significantly change PL-mPFC NE treated astrocyte calcium levels.

Figure 12, Page 49: Pre-bath application of IRL-1620 + NE prevents increase of NE Fluo-4-AM in PL-mPFC astrocytes.

Figure 13, Page 50: Pre-bath application of quinine + NE does not change PL-mPFC NE treated astrocyte calcium levels.

Figure 14, Page 51: Summary of area under curves levels for each group.

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Introduction

Drug-associated cues can trigger craving and relapse in drug addicts. Preventing retrieval of cue-associated memories or reducing cue reactivity through extinction has been demonstrated to reduce drug-seeking behavior in various drug-seeking models (LaLumiere et al., 2010; Groblewski et al., 2012). Retrieval of a cocaine-associated memory is dependent on activation of the prelimbic medial prefrontal cortex (PL-mPFC; Otis et al., 2013) whereas extinction of cocaine seeking is consolidated in the infralimbic medial prefrontal cortex (IL-mPFC; Otis et al., 2014). Although the neurochemical and synaptic mechanisms underlying drug-related behavior have been investigated intensively, little is known regarding the contribution of neural or astrocytic gap junction communication in the PL-mPFC and IL-mPFC.

Both neurons and astrocytes express gap junctions, which are specialized membrane structures built of connexin channels that allow cytoplasmic continuity between connected cells (Connors & Long, 2004; Pannasch & Rouach, 2013). Neuronal or astrocytic gap junction communication can alter neuronal activity and plasticity (Palacios-Prado et al., 2014; Pannasch et al., 2011). However, how gap junction communication blockade affects memory retrieval and extinction of drug-seeking behavior is not known. Here I describe how gap junction communication in the brain plays integral roles in neuronal and astrocytic development, maintenance, and ongoing plasticity. Next I describe a series of experiments that were performed to investigate gap junction communication in the retrieval and extinction of drug-seeking behavior using the conditioned place preference (CPP) paradigm and confocal microscopy.

The conditioned place preference procedure

The mechanisms of drug-associated memory retrieval can be investigated using the CPP paradigm. This paradigm is an animal model of drug seeking in which rats learn to associate environmental stimuli with cocaine. During training, rats are given experimenter-delivered cocaine or saline followed by exposure to one or another of two chambers. Following training, rats are given full access to both the cocaine- and saline-paired chambers, along with a neutral center chamber. Rats spend more time in the previously cocaine-paired chamber during this trial, thus expressing a cocaine-induced CPP (Mueller & Stewart, 2000). When a CPP is expressed, investigators can be certain that the rats acquired, consolidated, and retrieved this cocaine-associated memory. Additionally, rats that express a CPP on additional test trials demonstrate that they are subsequently able to retrieve the cocaine-associated memory.

The PL-mPFC and drug-associated memory retrieval

Recall or retrieval of memory refers to the subsequent re-accessing and behavioral expression of previously encoded and stored information. Studies have demonstrated that memory expression can be enhanced by pre-test treatment of nicotine (Faiman et al., 1992), cocaine (Rodriguez et al., 1993), or amphetamine (Sara & Deweer, 1982). These drugs are nonspecific, but all of them enhance noradrenergic signaling. Stimulation of the noradrenergic system can influence memory task performance. For example, blockade of α_2 -adrenergic autoreceptors, which normally prevent norepinephrine from being released, leads to an increase in rat performance in maze navigation after the task had been forgotten (Sara, 1985; Sara & Devauges, 1989). Moreover, stimulation of the locus coeruleus (LC), a major nucleus of

noradrenergic cell bodies (Dahlstroem & Fuxe, 1964), also enhances memory expression after forgetting. This enhancement is prevented by the β -adrenergic receptor (β -AR) antagonist propranolol (Devauges & Sara, 1991). Work from our lab has demonstrated that systemic β -AR blockade can prevent retrieval of a cocaine CPP (Otis & Mueller, 2011). Additionally, β -AR blockade prevents an increase in neuronal excitability in PL-mPFC neurons (Otis et al., 2013). Investigation of the loci of retrieval of a cocaine CPP revealed a role for the PL-mPFC. The PL-mPFC has previously shown to be necessary for expression of memories and decision making (Corcoran and Quirk, 2007; Kim et al., 2013; Euston et al., 2012). Additionally, glutamatergic driven neuronal activity within PL-mPFC has been shown to be necessary for expression of innate (Freitas et al., 2013) and learned (Davies et al., 2013; Gilmartin et al., 2013) behaviors. Infusions of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or the GluN2B-selective antagonist Ro 25-6981 into PL-mPFC reduced span capacity in rats, a measure of working memory. Furthermore, CNQX or Ro 25-6981 also induced a failure to retrieve the correct choice in an appetitive choice task for rats (Davies et al., 2013). Based on these studies demonstrating the necessity of PL-mPFC in memory retrieval across tasks, our lab examined the effects of microinfusions of non-selective β -AR antagonists into the PL-mPFC in retrieval of a cocaine CPP (Otis et al., 2013). We found that blockade of β -ARs in PL-mPFC impaired retrieval of the cocaine CPP. Thus, the PL-mPFC is necessary for cocaine-associated memory retrieval necessary for drug-seeking behavior.

The IL-mPFC and extinction of drug seeking

Extinction is the reduction in drug seeking across trials when the drug-predictive stimuli no longer predict the delivery of a drug reward. Importantly, extinction is an active learning process (Sutton et al., 2003; Knackstedt et al., 2010), is context specific (Bouton et al., 2000, 2004; Wells et al., 2011) and does not erase the original drug-related memory (Tobena et al., 1993; Di Ciano & Everitt, 2004). Rather, extinction learning results in the formation of an inhibitory memory that suppresses drug seeking. Extinction of ethanol seeking (Groblewski et al., 2012), morphine seeking (He et al., 2011), and cocaine seeking (Otis et al., 2014) is consolidated in the IL-mPFC. Retrieval of the extinction memory is dependent on the IL-mPFC, as lesioning of this structure does not disrupt acquisition of extinction within a session, but does impair retrieval of the extinction memory when tested in later trials (Quirk et al., 2000). For example, Do-Monte et al. (2015) optogenetically activated or silenced the IL-mPFC during extinction training and found that activation reduced the expression of freezing and facilitated extinction learning during both the acquisition and retrieval test days. Furthermore, silencing the IL-mPFC during extinction training yielded similar results to Quirk et al (2000) where IL-mPFC silencing did not disrupt acquisition of extinction within the initial session, but did impair retrieval of the extinction memory in later trials.

So far, I have described how the PL-mPFC and IL-mPFC are involved in the retrieval and extinction of drug seeking. Next, I will describe gap junctions and how they have been demonstrated to be involved in drug-seeking behavior. I will begin with a background of the function of gap junctions to show their involvement in development and in different forms of plasticity.

Gap junctions

Gap junctions were first detected by electron microscopy in the brains of primates and rats. For example, the CA1 and CA3 regions of the hippocampus (Kosaka, 1983), the sensorimotor cortex (Sloper and Powell, 1978), and the brainstem and cerebellum all contain gap junctions (Sotelo and Korn, 1978). Gap junctions allow intercellular electro- and metabolic-coupling between adjoining cells and are found in organs and tissues that rely on fast intercellular signaling, transfer and communication, including the brain, heart, skin, and inner ear. Gap junctions have critical roles in brain development and maturation, and contribute to neural precursor cell proliferation, as well as cell migration and differentiation (Bruzzone and Dermietzel, 2006).

Gap junctions are intercellular channels with a diameter of 1.2 nm, and form an aqueous pore that penetrates the lipid bilayer of two connected cells. These channels are formed by two hemichannels or connexons, with a connexon contributed by each cell. Connexons are composed of six protein subunits called connexins (Kumar and Gilula, 1996). Gap junctions permit the bidirectional diffusion of nutrients, ions, metabolites, and second messengers, including potassium, calcium, cyclic adenosine monophosphate (cAMP), inositol 1,4,5-triphosphate (IP₃), cyclic monophosphate, glucose, and other small molecules of about 1000 Da or less (Dobrowolski and Willecke, 2009; Zoidl and Dermietzel, 2010).

Connexin genes and their corresponding proteins are named according to the molecular weight of different connexin proteins (e.g., Cx43 has a mass of 43 kilodaltons). The molecular topography of connexin proteins include four alpha-helical transmembrane domains, intracellular N- and C- termini, two extracellular loops, and a cytoplasmic loop. There are two to

three cysteine residues located in the extracellular loops, which are needed for the proper alignment of two connexons to form a continuous gap junction channel (Yeager, 1998). The cytoplasmic C-terminus carries several serine, threonine, and tyrosine residues, which serve as targets for a number of protein kinases and phosphatases for posttranslational modifications (Solan and Lampe, 2005).

To date more than 20 different connexin genes have been identified and are expressed in many different cells and tissues in mammals (McCracken and Roberts, 2006). I will focus on two, Cx36 and Cx43, as these are the two brain connexins expressed in neurons and astrocytes, respectively.

Neuronal gap junction communication

The combination of *in situ* hybridization and immunocytochemistry for the neuron-specific DNA-binding protein NeuN demonstrated that, in rat and human cortices, approximately 10% of neurons express Cx36 (Belluardo et al., 1999, 2000; Condorelli et al., 2000). Co-localizing Cx36 mRNA with parvalbumin, a GABAergic interneuron marker, confirmed that GABAergic inhibitory interneurons express Cx36 (Belluardo et al., 2000). Furthermore, simultaneous recordings revealed that cortical GABAergic interneurons, and not pyramidal cells, are interconnected via electrical synapses (Galarreta and Hestrin, 1999; Gibson et al., 1999). The gap junctions of inhibitory neurons were shown by ultrastructural studies to be located between dendrites, or between dendrites and somata (Szabadics et al., 2001; Tamas et al., 2000). Cx36 has been thoroughly investigated and has been shown to be selectively expressed in neurons and to participate in neuronal gap junctions found in the hippocampus, neocortex,

basal ganglia, cerebellum, inferior olive, suprachiasmatic nucleus, pedunculo-pontine nucleus, and the thalamic reticular nucleus (Garcia-Rill et al., 2007; Landisman et al., 2002; McCracken and Roberts, 2006; Sohl et al., 2005, 2006).

The generation of mice with disruption of the Cx36 gene (Gueldenagel et al., 2001; De Zeeuw et al., 2003) provided insight and confirmation of the inclusion of Cx36 in the formation of gap junctions between inhibitory neurons. In the Cx36-deficient mice, a gap junction-like structure was still observed, but the interneuronal space was reduced to 9 nm. Dye-coupling experiments demonstrated a failure of direct electrical coupling in the Cx36 knockout mice (De Zeeuw et al., 2003).

The availability of Cx36 knockout mice has also allowed for the study of the role of gap junctions in learning and memory (Frisch et al., 2005). Allen et al (2011) demonstrated that Cx36 knockout mice displayed impaired spatial memory in the open field and in zigzag maze running. Additionally, slower hippocampal theta oscillations were observed in the Cx36 knockout mice. The authors theorized that Cx36-coupled interneurons play a functional role in spatial coding and cognition (Allen et al., 2011). A possible explanation for the failure of these Cx36 knockout mice to learn could be deficiencies in the mechanisms of long-term potentiation (LTP). The induction of LTP, which is considered a cellular model of learning and memory, is impaired in Cx36-deficient animals in the visual cortex (Postma et al., 2011) and the hippocampus (Wang and Belousov, 2011). Interestingly, Wang and Belousov performed a western blot analysis of NMDA receptor subunits and found a higher GluN2A/GluN2B ratio in Cx36 knockout mice and theorized that there is shift in the threshold for LTP induction in knockout animals. Postma et al (2011), however, found equal levels of GluN2A and GluN2B

mRNAs in Cx36 knockout animals and controls. One could argue that deletion of Cx36 would have an impact on an animal's healthy development and subsequent normal function. Although Cx36 is expressed early in the development of the embryonic brain (Gulisano et al., 2000), no developmental or morphological abnormalities were observed by Sohl et al (2004) in the neocortex of Cx36-deficient mice. However, it has also been reported that knockdown of Cx36 significantly reduces the number of differentiated neurons and increases the number of differentiated astrocytes (Hartfield et al., 2011). Thus, Cx36 may regulate early neurogenesis during development.

In addition to being able to genetically alter Cx36, pharmaceutical treatment can block to block Cx36-containing gap junctions (Juszczak & Swiergiel, 2009). One compound is the antimalarial drug mefloquine (Cruikshank et al., 2004). Mefloquine has been utilized for local microinfusions and has shown to be effective at impairing context-dependent fear learning when infused into the dorsal hippocampus in rats prior to or following training (Bissiere et al., 2011). Furthermore, unilateral infusion of mefloquine into the ventral hippocampus combined with a contralateral infusion into the mPFC decreases anxiety-like behavior on the elevated plus maze in mice (Schoenfeld et al., 2014). However, it should be noted that the dose of mefloquine used in these experiments (100 mM) can block astrocyte Cx43 junctions as well (Cruikshank et al., 2004).

Astrocytic gap junction communication

In the adult brain, Cx43 is the main connexin spanning the astrocytic gap junction network (Yamamoto et al., 1992). The term astrocyte or “star-like cell” was first used by Michael von Lenhossek in 1891 (Lenhossek, 1891). Astrocytes can have a star-like appearance, and express glial fibrillary acidic protein (GFAP), a protein used to identify astrocytes (Tong et al., 2014). The list of astrocyte functions is extensive, and is generally focused on maintaining CNS homeostasis. For example, astrocytes form and maintain the blood brain barrier (Abbott, 2005), and deletion of Cx43 can weaken the integrity of the blood brain barrier (Ezan et al., 2012). Astrocytes have also been shown to be involved in neurogenesis, both pre- and post-natally (Alvarez-Buylla et al., 2001). Astrocytes also contribute to morphological homeostasis by defining the migration of neural cells during development (Nedergaard et al., 2003), and also help regulate synaptogenesis and synaptic pruning to shape the microarchitectural framework of gray matter (Pfrieger, 2009). Astrocytes also help regulate molecular homeostasis by regulating the concentrations of ions, neurotransmitters, and neurohormones in the CNS (Danbolt, 2001; Newman, 1995) and also detect systemic fluctuations of carbon dioxide, pH, sodium, and potassium (Gourine and Kasparov, 2011; Gourine et al., 2010; Huckstepp et al., 2010; Shimizu et al., 2007). Astrocytes are also involved with metabolic homeostasis by accumulating energy substrates and supplying neurons with lactate (Magistretti, 2006). Another role of astrocytes is their defensive protection and structural maintenance of neural tissue following trauma or the introduction of pathogens (Pekny and Nilsson, 2005).

From the aforementioned functions of astrocytes, it is easy to see how involved astrocytes are in supporting the cellular and molecular components of neural activity. However,

the last fifteen years has seen an explosion of research showing how astrocytes are not only directly involved with supporting neural network integrity, but also are directly involved in cellular and molecular mechanisms through interactions with neurons to build higher cognitive functioning in the brain. An integral part of this ability is gap junction communication of calcium between astrocytes. In 1992, Steven Finkbeiner loaded astrocytes with a calcium-attaching dye and was able to visualize diffusing calcium moving from one cell to the next in a curvilinear pattern (Finkbeiner, 1992). Astrocytes each contain an endoplasmic reticulum (ER), an internal complex that stores calcium concentrations as high as 0.2-1 mM, whereas the calcium concentration inside the cytoplasm at baseline is set at about 50-100 nM (Alonso et al., 1999; Mogami et al., 1998; Solovyova and Verkhratsky, 2002). ERs each contain ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate (IP₃)-gated receptors. Activation of either RyR or IP₃ receptors triggers an ER to release its calcium. RyRs can become activated by calcium whereas IP₃ receptors can be activated by either calcium or by the second messenger IP₃ (IP₃ is produced by activation of metabotropic receptors linked with phospholipase C) (Bezprozvanny, 2005). Hence, astrocytes are capable of calcium-induced calcium release (Verkhratsky and Kettenmann, 1996). Finkbeiner (1992) demonstrated that this calcium-induced calcium release is possible following glutamatergic activation of metabotropic G-protein coupled receptors that are linked to phospholipase C and the second messenger IP₃. Roughly 230 gap junctions connect a pair of astrocytes and 50-100 neighboring astrocytes can be influenced by a single calcium wave (Innocenti et al., 2000). Probably the most intriguing aspect of calcium sharing between astrocytes is the fact that astrocytes, like neurons, use calcium as a catalyst to release transmitters into the extracellular space. In 1994, Parpura and colleagues discovered that

astrocytes are capable of releasing glutamate into the extracellular space after a calcium wave spreads between astrocytes (Parpura et al., 1994). Additionally, astrocytes release transmitters in a calcium-dependent manner involving SNARE protein mediated fusion of vesicles with the cell's plasma membrane (Araque et al., 2000; Zhang et al., 2004). Furthermore, astrocytes have vesicles located in close proximity to synapses that contain different neuroactive molecules/transmitters like glutamate, d-serine, ATP, adenosine, GABA, tumor necrosis factor α , and prostaglandins (Bezzi et al., 2004, Volterra & Bezzi, 2002).

The body of work by Finkbeiner and others during the 90's has stimulated investigation of the relationship between neurons and astrocytes in structures throughout the brain. Pirrtimaki and colleagues (2011) showed that neuronal corticothalamic glutamate release can trigger astrocytic calcium waves, and astrocytes can subsequently release glutamate primarily targeting NMDA receptors on neurons. Furthermore, Jourdain and colleagues (2007) studied communication between astrocytes and neurons in the hippocampus in the dentate molecular layer using a combination of patch-clamp electrophysiology and calcium imaging, and demonstrated that glutamate release from astrocytes enhances synaptic strength within the surrounding neurons. By measuring the flow of positively charged ions into the postsynaptic neuron, they observed that when astrocytes were stimulated to release a calcium wave, there was an increase in neuronal electrical activity as compared to the activity of neurons stimulated independently of astrocyte stimulation (Jordain et al., 2007).

Astrocytes have also been implicated in LTP and long-term depression (LTD). Henneberger and his colleagues (2010) investigated the role of astrocytes in LTP at Schaffer collateral – CA1 pyramidal cell synapses. Henneberger and colleagues (2010) observed that

holding and preventing elevations of internal calcium levels in astrocytes in the CA1 region blocked LTP at excitatory synapses. Furthermore, they determined that performing a calcium clamp on the astrocytes prevented astrocytes from releasing D-serine since LTP blockade could be reversed by exogenous application of D-serine (Henneberger et al., 2010). Regarding LTD, Chen's group (2013) demonstrated that LTD is dependent on calcium elevation in astrocytes leading to release of ATP that then acts on P2Y receptors on hippocampal neurons. Blocking P2Y receptors or buffering astrocyte calcium at a low level prevented LTD (Chen et al., 2013).

So far, I have discussed how neuronal and astrocytic gap junction communication is capable of altering neural activity and plasticity, and have provided some examples of how gap junction communication is involved in non-addictive behavior. Next, I will focus on data that has been collected relating gap junction communication to addictive behaviors.

Gap junction communication in reward-seeking and addictive behaviors

A growing body of literature has emerged investigating gap junction communication in relation to addictive behaviors. Here I describe gap junction involvement in drug-seeking behavior within different brain regions during different time points of drug-seeking.

The ventral tegmental area (VTA) is the beginning of the mesolimbic pathway, which is also known as the reward pathway (Kauer, 2004). The VTA projects to areas such as the nucleus accumbens, prefrontal cortex, and amygdala, which interpret environmental stimuli and internal states that can affect drug-seeking behavior (Cohen et al., 2012; Schultz, 2006). Gap junction communication can alter behavior associated with activity in the mesolimbic pathway. For example, Lassen and colleagues (2007) systemically injected either the non-selective gap junction blocker quinidine or the Cx36-specific blocker mefloquine and found that general and Cx36 blockade significantly increased the threshold within the VTA for responding to rewarding self-stimulation. They theorized that VTA gap junction communication blockade reduces the rewarding impact of self-stimulation. It is interesting to note that the animals were still able to search out pleasure/rewarding stimuli, but required greater stimulation. Notably, this study involved systemic administration, and the assessment of this data should be taken carefully because all regions of the brain were accessed by gap junction antagonists.

A reduction of pleasure-seeking behavior by gap junction communication blockade also occurred in an experimental design reminiscent of Olds and Milner (1954) performed by Kokarovtseva and colleagues (2009), Kokarovtseva et al. trained rats to press a lever to receive an electrical current in the hypothalamus. The hypothalamus, particularly the lateral region, when stimulated causes pleasure and feelings of reward through glutamatergic projections to

the VTA. After training, the authors microinfused the non-selective gap junction blocker carbenoxolone (CBX) into the nucleus accumbens to investigate drug-seeking behavior. Intra-accumbens CBX reduced lever-pressing activity. Infusions of tetrodotoxin (TTX) also produced a similar decrease in lever-pressing behavior. Kokarovtseva et al. theorized that blocking gap junctions reduces the level of neuronal excitability. Additionally, as a control, CBX or TTX were infused into the motor cortex to examine the function of gap junctions in forelimb motor ability. No effect was found, indicating that the effects of CBX and TTX were reward-specific.

The previous experiments demonstrated that blocking gap junction communication following training can decrease drug-seeking behavior. In animals that have gone through a prolonged withdrawal from a drug, an opposite effect is observed. Bull and colleagues (2014) investigated gap junction communication in the nucleus accumbens of rats following three weeks of withdrawal from ethanol. They demonstrated that after withdrawal, non-selective or astrocytic gap junction blockade caused an increase in lever pressing in ethanol-experienced rats compared to controls. Neuronal gap junction blockade had no effect on lever pressing. Additionally, they demonstrated that selective $G\alpha_q$ -DREADDs activation of astrocytic intracellular calcium in the nucleus accumbens, decreased lever pressing after three weeks of abstinence. Therefore, astrocytic, but not neuronal, gap junction communication is necessary for prevention of drug-seeking behavior following withdrawal. Astrocytic calcium gap junction communication may be needed for decreasing drug-seeking behavior during periods of withdrawal within the nucleus accumbens to counter dysregulation of glutamate exchange between astrocytes and neurons (Kalivas 2009). During withdrawal, insertion of calcium-permeable AMPA receptors into the presynaptic membrane of nucleus accumbens neurons

occurs (Wolf & Tseng, 2012), as well as the addition of structural proteins such as actin and myosin to form new synapses. These structural changes increase output from the nucleus accumbens and strengthen the drive to drug-seek.

Dissertation Goal and Aims

The PL-mPFC and IL-mPFC underlie drug-associated memory retrieval and extinction of drug-seeking behavior, respectively. Above, I have described how blockade of activity within these structures prevents the behavioral expression of drug seeking. Additionally, I have described how blockade of gap junction communication prevents behavioral expression of both addictive and non-addictive behaviors. Thus, gap junction communication plays an important mechanistic role in memory processing.

Although gap junction blockade disrupts addictive memories, the specific neuronal or astrocytic gap junction communication necessary for retrieval and extinction within the PL-mPFC and IL-mPFC has remained unexplored. Therefore, the overall goal of this dissertation was to investigate general, neuronal, or astrocytic gap junction communication in the retrieval and extinction of a cocaine CPP. This was addressed in the following two aims.

Aim 1: Determine whether gap junction communication is a mechanism for memory retrieval and extinction of a cocaine CPP. Here I evaluated the behavioral effect of blocking general, neuronal, or astrocytic gap junction communications within PL-mPFC and IL-mPFC on retrieval and extinction of a cocaine CPP. We hypothesized that general or astrocyte specific gap junction blockade would prevent a net increase in available transmitter shared at the synapse thereby

Aim 2: Evaluate PL-mPFC astrocytic calcium levels following general, neuronal, or astrocytic gap junction communication blockade. Next, using confocal microscopy I determined the underlying effects of the gap junction blockers used in Aim 1 on calcium levels in PL-mPFC astrocytes.

Methods

Subjects

Adult male Long-Evans rats weighing 275-300 grams were housed individually in clear plastic cages with access to standard laboratory rat chow (Harlan Laboratories) and water *ad libitum* unless otherwise noted. Rats were maintained on a 14 hour light/10 hour dark cycle (lights on at 7am), and were weighed and handled daily. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Milwaukee in accordance with National Institutes of Health guidelines.

Methods: Aim 1

Aim 1 was completed to determine the behavioral effect of general, neuronal, or astrocytic gap junction communication disruption within the PL-mPFC for retrieval (Aim 1a) and IL-mPFC for extinction (Aim 1b) of a cocaine CPP.

Aim 1a: Retrieval of cocaine CPP

Cannula surgery

PL-mPFC cannula surgeries were performed to allow PL-mPFC gap junction blockade for retrieval of a cocaine CPP. Rats were anesthetized with ketamine/xylazine (90 mg/kg, 10.5 mg/kg, i.p.). Following anesthetization, double-barrel guide cannula (26 gauge; Plastics One, Roanoke, VA) were implanted within PL-mPFC (AP, +2.9; ML, ± 0.6 ; DV, -2.9 mm relative to bregma). Cannula were fixed into place with 3 stainless steel skull screws and grip cement.

Following surgery, rats were treated with an antibiotic (penicillin g procaine, 75,000 units in 0.25 ml, s.c.) and an analgesic (carprofen, 5.0 mg in 0.1 ml, s.c.). Rats were given a minimum of 7 days for recovery following surgeries, during which behavioral experiments were not conducted. Stylets remained within the guide cannula following surgery to maintain patency until microinfusions were performed.

Drugs and microinfusions

For conditioning, cocaine HCl (National Institute on Drug Abuse) was dissolved in sterile 0.9% saline at a concentration of 10 mg/mL, and administered i.p. at a dose of 10 mg/kg. PL-mPFC infusions of vehicle (0.9% saline), the non-selective gap junction blocker carbenoxolone (CBX; 50 mM, Tocris), the neuron specific gap junction blocker quinine (100 uM, Tocris), or the astrocyte specific gap junction blocker IRL-1620 (0.1 uM, Tocris) were administered at 0.3 µl / side over a 2 minute time period. All drugs were dissolved into sterile 0.9% saline. Microinfusion injectors were then left in place for a minimum of 1 minute following microinfusions. Doses of each gap junction blocker were based on previous studies (Blomstrand et al., 1999; Cruikshank et al., 2004; Sun et al., 2012).

Place conditioning

Place conditioning and testing was conducted in a 3-chamber apparatus that contained 2 distinguishable conditioning chambers (13" x 9" x 11.5") which were separated by a smaller center chamber (6" x 7" x 11.5"). One of the conditioning chambers had wire mesh flooring with white walls, whereas the other conditioning chamber had gold- grated

flooring with a black wall. The smaller center chamber had aluminum sheeting as flooring and all white walls. Each of the larger chambers contained two infrared photobeams separated by 3". If the beam furthest from the center chamber was broken, then the rat was determined to be in the larger chamber. If only the beam closest to the center chamber was broken, then the rat was determined to be in the center chamber. In addition, the total numbers of photobeam breaks were recorded during microinfusion trials to quantify locomotor activity.

Baseline preferences were assessed by placing the rats into the center chamber with free access to the entire CPP apparatus for 15 minutes. We previously demonstrated that rats spend equivalent time within the larger chambers before conditioning (Otis and Mueller, 2011). Thus, following baseline testing, rats were conditioned to associate one chamber, but not another, with cocaine in a counterbalanced fashion over 8 days. Injections of saline or cocaine were administered immediately before each 20 minute conditioning session, during which rats were confined to the appropriate chamber. Following conditioning, microinfusion adaptation was performed by lowering injector tips 1.0 mm past the guide cannula for 2 minutes. The following day, the same procedure took place, except that saline was infused (0.3 μ l / side / 2 min). These procedures allowed for the rats to adapt to mechanical stimulation and changes in cranial pressure that occurred during microinfusions.

Experimental manipulations

For retrieval testing, rats were separated into one of four groups. The vehicle group contained control animals that received vehicle (0.9% saline). Group CBX received the non-selective gap junction blocker carbenoxolone. Group IRL-1620 received the astrocyte-specific

gap junction blocker IRL-1620. Group Quinine received the neuron-specific gap junction blocker quinine. Following conditioning and 10 minutes prior to the first CPP test, all groups received their respective drug manipulation. During the CPP test, all animals had full access to all chambers for 15 minutes (Otis et al., 2013). Additional drug-free daily CPP tests followed to determine if gap-junction blockade had any persistent effect on memory retrieval. Animals continued to receive daily drug-free CPP tests until they reached extinction criterion of two consecutive days of no significant CPP.

Alternative Strategies Implemented: Aim 1

As described in the Alternative Strategies portion of the original proposal, different possibilities were described as to why the experiments proposed in Aim 1a would result in trends, but not significant differences in retrieval disruption. Significant differences were observed within the original experiments proposed in Aim 1a, however additional retrieval experiments were performed following completion of the original experiments proposed in Aim 1a.

Additional Experiment 1: Restoration of GABAergic tone following neuronal gap junction blockade via quinine rescues normal CPP behavior

Cannula surgery, drugs and microinfusions, place conditioning, and behavioral testing were all performed as described above with the exception of quinine (100 μ M) and the potent GABA_A receptor agonist muscimol (0.0001 μ M, dose determined by Sajdyk et al., 2008) being co-infused prior to the first test trial.

Additional Experiment 2: Preventing retrieval of CPP via blockade of astrocyte gap junctions with IRL-1620 in addition to blockade of astrocyte hemichannels with Gap19

Gap junctions as described earlier are formed by two hemichannels contributed by each adjacent cell. However, hemichannels do not always form gap junction channels and have been reported to be present in noncontacting membranes of astrocytes to allow intracellular-extracellular communication (Ye et al., 2003). These functional hemichannels have been shown to release ATP and glutamate (Orellana et al., 2011). To decrease the net transmitter released by astrocytes during retrieval in the PL-mPFC we used the same cannula surgery, drugs, microinfusions, and place conditioning as described above with the exception of both Gap19 (142 uM) and IRL-1620 (0.1uM) being co-infused prior to the first test trial.

Additional Experiment 3: Preventing retrieval of established CPP via blockade of astrocyte gap junctions with IRL-1620 and blockade of astrocyte hemichannels with Gap19

To test if retrieval of a CPP could be disrupted by Gap19 + IRL-1620 after a CPP had already been expressed we used the same cannula surgery, drugs, microinfusions, and place conditioning as described above with the exception of both Gap19 (142 uM) and IRL-1620 (0.1uM) being co-infused prior to the second test trial after a CPP had been established in the first drug-free test trial.

Aim 1b: Extinction of cocaine CPP

Cannula surgery

Cannula surgeries were performed as described above, but double-barrel guide cannula (26 gauge; Plastics One, Roanoke, VA) were implanted within IL-mPFC (AP, +2.9; ML, \pm 0.6; DV, -4.4 mm relative to bregma).

Drugs and microinfusions

For conditioning, cocaine HCl (National Institute on Drug Abuse) was dissolved in sterile 0.9% saline at a concentration of 10 mg/mL, and administered i.p. at a dose of 10 mg/kg. IL-mPFC infusions of vehicle (0.9% saline), the non-selective gap junction blocker CBX (50 mM, Tocris), the neuron specific gap junction blocker quinine (100 uM, Tocris), or the astrocyte specific gap junction blocker IRL-1620 (0.1 uM, Tocris) were administered at 0.3 μ l / side over a 2 minute time period. All drugs were dissolved in sterile 0.9% saline. Microinfusion injectors were left in place for a minimum of 1 minute following microinfusions.

Place conditioning

Place conditioning and testing were conducted as described in Aim 1a.

Experimental manipulations

For extinction testing, rats was separated into one of four groups. The vehicle group contained control animals that received vehicle (0.9% saline). Group CBX received the non-selective gap junction blocker CBX. Group IRL-1620 received the astrocyte specific gap junction blocker IRL-1620. Group Quinine received the neuron specific gap junction blocker quinine. Following conditioning and 10 minutes prior to the first extinction test, all groups received their respective drug manipulation. During the CPP test, all animals had full access to all chambers for

30 minutes to accelerate extinction learning (Otis et al., 2014). Additional drug-free daily extinction tests followed to determine if gap-junction blockade had any persistent effect on extinction learning. Animals continued to receive daily drug-free CPP tests until they reached an extinction criterion of two consecutive days of no significant CPP.

Behavior data analysis

Cocaine seeking was analyzed by comparing time spent between the cocaine, saline, and center chambers across trials and between groups using one-way ANOVAs for each individual day of testing. If a significant effect of chamber was found, *post-hoc* Tukey's Honestly Significant Difference (HSD) tests were used to compare the amount of time spent in the cocaine-paired versus the saline-paired chambers during the CPP trial.

Methods: Aim 2

Aim 2 was completed using confocal microscopy to determine the effects of the gap junction blockers used in Aim 1 on PL-mPFC astrocyte cytoplasmic calcium levels.

Tissue preparation and removal for astrocyte identification

To image astrocyte morphology *in vivo*, a single intraperitoneal injection of the fluorescent dye sulforhodamine 101 (SR101; 100 mg/kg) was dissolved in saline and administered to the animal 40 minutes prior to sacrifice. Astrocytes have been shown to selectively take up SR101 *in vivo* (Nimmerjahn et al., 2004; Appaix et al., 2012; Perez-Alvarez et al., 2013). Although the mechanism of uptake is still unknown, there is evidence showing that metabolites such as glucose and sulforhodamine spread efficiently across astroglial networks through gap junctions present in the astrocytic membrane (Rouach et al., 2008). Astrocyte staining were visualized 40–60 min after injection.

For tissue removal rats were anesthetized with isoflurane, and brains were quickly removed and put into ice-cold (0-2°C) oxygenated (95% O₂ / 5% CO₂) artificial cerebral spinal fluid (aCSF) composed of the following (in mM): 124 NaCl, 2.8 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 20 dextrose. Coronal slices 300 μm thick containing prefrontal cortex were taken using a vibratome (Leica VT1200). Slices were then allowed recover in continuously oxygenated warm aCSF (32°C) for approximately 30 minutes, followed by incubation in continuously oxygenated room temperature aCSF for 0.5-8 hours when not in use.

Loading of astrocytes with calcium indicator

To prepare the tissue for calcium visualization within astrocytes, brain slices labeled with SR101 were loaded with the fluorescent calcium indicator Fluo-4-AM. Fluo-4-AM was used in conjunction with SR101 for calcium imaging of astrocytes (Appaix et al., 2012; Perez-Alvarez et al, 2013).

50 μg of Fluo-4-AM was added to 4.5 μl of fresh dimethyl sulfoxide (DMSO) and 4.5 μl of 70000 MW of Dextran (to aid in locating the Fluo-4-AM injection site) and was then vortexed thoroughly. Next we loaded a glass pipette with dye solution and lowered it to the surface of the slice above this field using a standard microelectrode holder. We then slowly lowered the pipette to approximately 40 μm below the slice surface using a standard micromanipulator (Narishige) and applied back pressure to the pipette to eject 2 nl of Fluo-4-AM per injection into the PL-mPFC. To ensure that a large number of astrocytes take up Fluo-4-AM, we injected a second and third Fluo-4-AM bolus short distances away, approximately 80-100 μm away from the first injection site, and repeated bolus injections at this site. We then slowly retracted the pipette tip from the slice by raising the pipette back to the surface of the slice, made sure that the pipette was not clogged, and removed the pipette. We then allowed 30 min before imaging for the astrocytes to take up the dye and for the background signal to diminish.

Experimental manipulations

Tissue slices loaded with SR101 and Fluo-4-AM were then separated into one of eight groups:

1. Control (no gap junction blockade treatment)
2. Treatment with 100 μ M CBX for 5 min prior to scan
3. Treatment with 0.1 μ M IRL-1620 for 5 min prior to scan
4. Treatment with 100 μ M Quinine for 5 min prior to scan
5. Treatment with 100 μ M Norepinephrine (NE) for 2 min prior to scan
6. Treatment with 100 μ M CBX for 5 min then with 100 μ M NE for 2 min prior to scan
7. Treatment with 0.1 μ M IRL-1620 for 5 min then with 100 μ M NE for 2 min prior to scan
8. Treatment with 100 μ M Quinine for 5 min then with 100 μ M NE for 2 min prior to scan

NE can evoke calcium-induced calcium sharing between astrocytes (Salm & McCarthy, 1990; Duffy & MacVica, 1995). All drugs were mixed into aCSF.

Confocal microscopy, recording astrocytic calcium activity

PL-mPFC Fluo-4-AM injection sites were visualized with differential interference contrast using a 60X water-immersion lens mounted on an Nikon C2 Scanning Confocal microscope (Nikon Instruments). To set up the confocal microscope for imaging, we set the default values for each laser to a high photomultiplier setting, 1x gain and 0.5% laser output power. We also applied a 1.5x zoom for better visualization of astrocytes. Next we set the field resolution to 512 x 512 pixels. SR101-fluorescence was detected through a bandpass filter of 503-548 nm for

the 488 nm laser. Fluo-4-AM-fluorescence was detected through a bandpass filter of 624-724 nm for the 559 nm laser (see Figure 6).

To record astrocyte calcium activity we drew boxes using the image acquisition NIS Elements Imaging Software over regions of interest (ROIs) within the cell, in this case over astrocyte cell bodies. We then recorded fluorescence over time from the ROI. Any increases in fluorescence over baseline indicated increases in cytoplasmic calcium concentration (Takahashi et al., 1999). Tissue was scanned 200 times with each scan lasting 4.5 seconds over the course of 15 minutes. Each individual scan measured and averaged the highest and lowest intensity of Fluo-4-AM intensity (mean intensity). We also subtracted away background fluorescence from our ROI fluorescent value for each scan. The mean intensity value of each scan was taken for analysis.

Analysis of astrocyte calcium activity

Since Fluo-4-AM naturally decays during exposure to the confocal laser, the mean intensity of each scan was compared to the initial scan. To normalize the data the initial scan mean was assigned the starting value of 100 and each subsequent scans value was calculated relative to the initial scan's value of 100. For example, if during the initial scan the mean intensity was 851.36 and received the starting value of 100, the second scan with a mean intensity of 848.85 was receive a value of 99.71. Use of these calculations allowed all the different cells in the same treatment groups to be collectively analyzed and plotted over the course of the 200 scans. Plotting data this way allowed a polynomial curve to be established for each tissue slice. Next, using the Riemann sum formula $(B1+B2)/2*(A2-A1)$ we were able to calculate the total area under the curve to provide a measure of the total amount of calcium

fluoresced over the entire 200 scans. Using Riemann sum formula for example, if B1 represents scan number 1, B2 is scan number 2, A2 is mean intensity value of scan 2, and A1 is the mean intensity value scan 1. You then continue to reapply this equation and subsequently B1 then represents the x-axis value of scan number 2, B2 is the x-axis value of scan number 3, A2 is mean intensity value of scan 3, and A1 is the mean intensity value scan 2. We repeated this area under the curve equation for all 200 scans to establish a relative overall intensity value for each scan. Finally, using ANOVA we were able to compare total areas under each curve for each treatment group. If a significant effect was found, *post-hoc* Tukey's Honestly Significant Difference (HSD) tests will be used to identify which individual treatment group significantly differed from another.

Results

Aim 1

Memory retrieval of conditioned place preference

Following conditioning, rats were given 15 minute CPP trials across days to examine both retrieval and retention of a cocaine-associated memory. We hypothesized that general gap junction or astrocytic gap junction blockade would prevent retrieval whereas neuronal gap junction blockade would not. PL-mPFC infusions (Figure 1) of vehicle, CBX (50 mM), IRL-1620 (.1 μ M), or quinine (100 μ M) were administered prior to the first trial. PL-mPFC CBX or IRL-1620 prevented CPP expression on the first and subsequent infusion-free trials whereas quinine prolonged CPP expression trials (Figure 1). Repeated measures ANOVA revealed a significant effect of chamber for vehicle ($F_{2,22}=30.14$, $p<0.05$), carbenoxolone ($F_{2,20}=20.70$, $p<0.05$), IRL-1620 ($F_{2,18}=29.37$, $p<0.05$), and quinine treatment ($F_{2,18}=23.06$, $p<0.05$). *Post hoc* analyses confirmed that vehicle, IRL-1620-treated, and quinine-treated groups expressed a CPP during the first trial ($p < 0.05$), whereas the CBX-treated group did not ($p > 0.05$). On subsequent test trials 2 and 3, vehicle and quinine-treated groups continued to express a CPP ($p < 0.05$), whereas the CBX-treated group continued to express a CPP deficit ($p > 0.05$). Moreover, the IRL-1620-treated group began to demonstrate a CPP deficit across trials ($p > 0.05$). Overall, these data demonstrate that general and astrocyte-specific gap junction blockade prevents retrieval of a CPP, an effect that persists even in the absence of additional treatment.

On trial 4, the vehicle, CBX-treated, and IRL-1620-treated groups expressed a CPP deficit ($p > 0.05$) whereas the quinine-treated group continued to express a CPP ($p < 0.05$). Thus,

neuron-specific gap junction blockade in the PL-mPFC prolongs CPP expression. Additionally, during test trials 5 and 6, the quinine-treated group continued to express a CPP ($p < 0.05$), whereas the vehicle-treated group did not, thus further demonstrating that neuron-specific gap junction blockade in the PL-mPFC prolongs CPP expression. For the CBX-treated and IRL-1620 treated groups in test trials 5 and 6, a weak but significant CPP re-emerged ($p < 0.05$), suggesting that the CPP deficit in previous trials was somewhat transient.

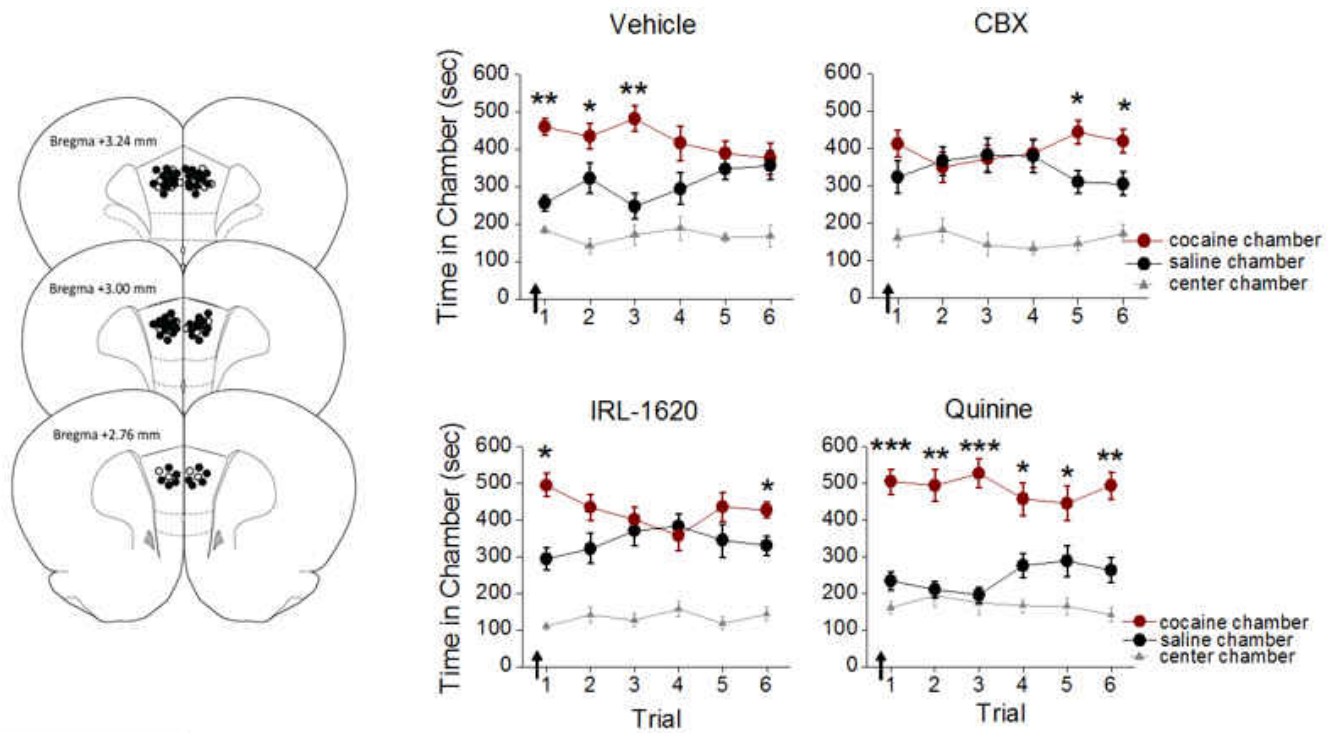


Figure 1. PL-mPFC blockade of general or astrocyte-specific gap junctions prevent extinction of a cocaine-CPP. **Left**, Coronal drawings showing injector tip placements for vehicle ($n = 11$), CBX ($n = 10$), IRL-1620 ($n = 9$), and quinine ($n = 10$). White circle represents Vehicle, black circles represent experimental groups. **Right**, IL-mPFC infusions (arrows) of CBX or IRL-1620 but not quinine before the first CPP trial prevented extinction of the CPP. $**p < 0.01$, $*p < 0.05$. Error bars indicate SEM.

Following the observation that neuron-specific gap junction blockade with quinine in the PL-mPFC prolonged CPP expression across days, we hypothesized that a reintroduction of GABAergic tone would oppose this effect and promote extinction. Following conditioning, rats were given 15 minute trials to test for CPP expression across days. As predicted, animals that received a PL-mPFC infusion of both the neuron-specific gap junction blocker quinine (100 μ M) and the potent GABA_A receptor agonist muscimol (.0001 μ M) prior to the first trial showed similar drug-seeking and extinction behavior as control animals (Figure 3). Repeated measures ANOVA revealed a significant effect of chamber for vehicle ($F_{2,20}=34.71, p<0.05$) and quinine + muscimol groups ($F_{2,18}=29.95.06, p<0.05$). *Post hoc* analyses confirmed that vehicle and quinine + muscimol-treated groups expressed a CPP during the first, second, and third trial ($p < 0.05$), but not during the fourth, fifth, and sixth trial ($p > 0.05$). Thus, restoring GABAergic tone in the presence of neuron-specific gap junction blockade in the PL-mPFC restores normal drug-seeking behavior and extinction of the CPP.

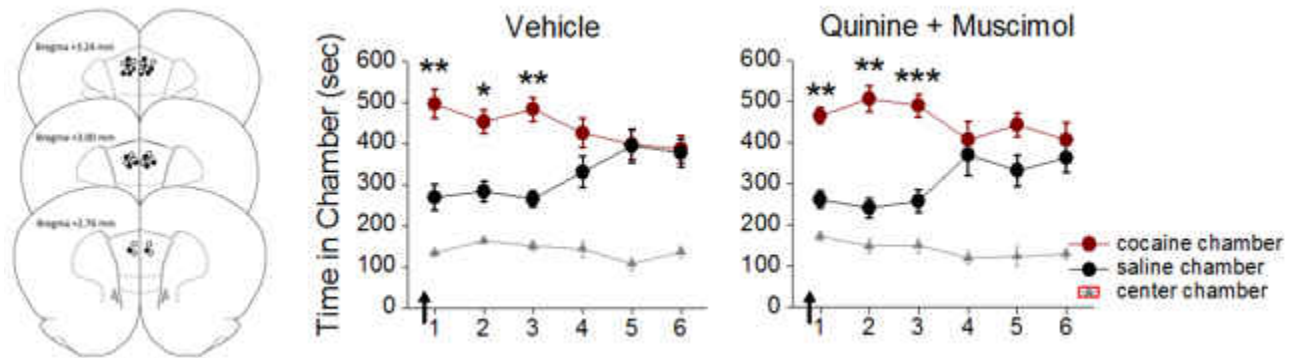


Figure 2. PL-mPFC infusion of both neuron-specific gap junction blocker quinine and GABA_A receptor agonist muscimol restores normal drug-seeking behavior and extinction of the CPP. **Left,** Coronal drawings showing injector tip placements for vehicle ($n = 10$) and quinine + muscimol ($n = 9$). White circle represents Vehicle, black circles represent experimental groups. **Right,** PL-mPFC infusions (arrows) of vehicle or quinine + muscimol before the first CPP trial resulted in similar retrieval and extinction of the CPP. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$. Error bars indicate SEM.

Gap junctions are formed by two hemichannels contributed by each adjacent cell. However, hemichannels do not always form gap junction channels and have been reported to be present in noncontacting membranes of astrocytes to allow intracellular-extracellular communication (Ye et al., 2003). These functional unopposed hemichannels have been shown to release ATP and glutamate (Orellana et al., 2011). Thus, we investigated if unopposed hemichannels affected drug-seeking behavior either independently or in conjunction with astrocyte-specific gap junctions. We hypothesized that animals receiving the unopposed hemichannel blocker Gap19 would show similar retrieval blockade as the IRL-1620 animals from the second experiment (see Figure 2) and animals receiving both Gap19 and IRL-1620 would demonstrate a more persistent retrieval blockade than either group of animals independently treated with Gap19 or IRL-1620.

Following conditioning, rats were given 15 minute trials to test for CPP expression across days. Animals that received a PL-mPFC infusion of the unopposed hemichannel blocker Gap19 (142 μ M) alone prior to the first trial showed weaker but longer-lasting CPP expression across days. Animals that received a PL-mPFC infusion of both Gap19 (142 μ M) and IRL-1620 (0.1 μ M) showed a lack of CPP expression across days (Figure 4). Repeated measures ANOVA revealed a significant effect of chamber for the vehicle ($F_{2,20}=34.22, p<0.05$), Gap19 ($F_{2,22}=43.31, p<0.05$), and Gap19 + IRL-1620 groups ($F_{2,22}=43.31, p<0.05$). *Post hoc* analyses confirmed that both the vehicle and Gap19 groups expressed a CPP during the first trial ($p < 0.05$). The vehicle group then continued to express a CPP during the second and third trial ($p < 0.05$), but not during the fourth, fifth, and sixth trial ($p > 0.05$). The Gap19 group continued to show a CPP during trial three and trial five ($p < 0.05$), but during trial two and trial four did not ($p > 0.05$). The Gap19 +

IRL-1620 group did not express CPP on any trial, and *post hoc* analyses confirmed no significance in time spent between the cocaine-paired and saline-paired chamber ($p > 0.05$). Thus, blockade of either astrocyte-specific gap junctions (Figure 2) or astrocyte unopposed hemichannels in the PL-mPFC does not prevent initial retrieval of a CPP, but impairs subsequent CPP expression across days. Joint blockade of both astrocyte unopposed hemichannels and astrocyte-specific gap junctions in the PL-mPFC persistently blocks CPP expression.

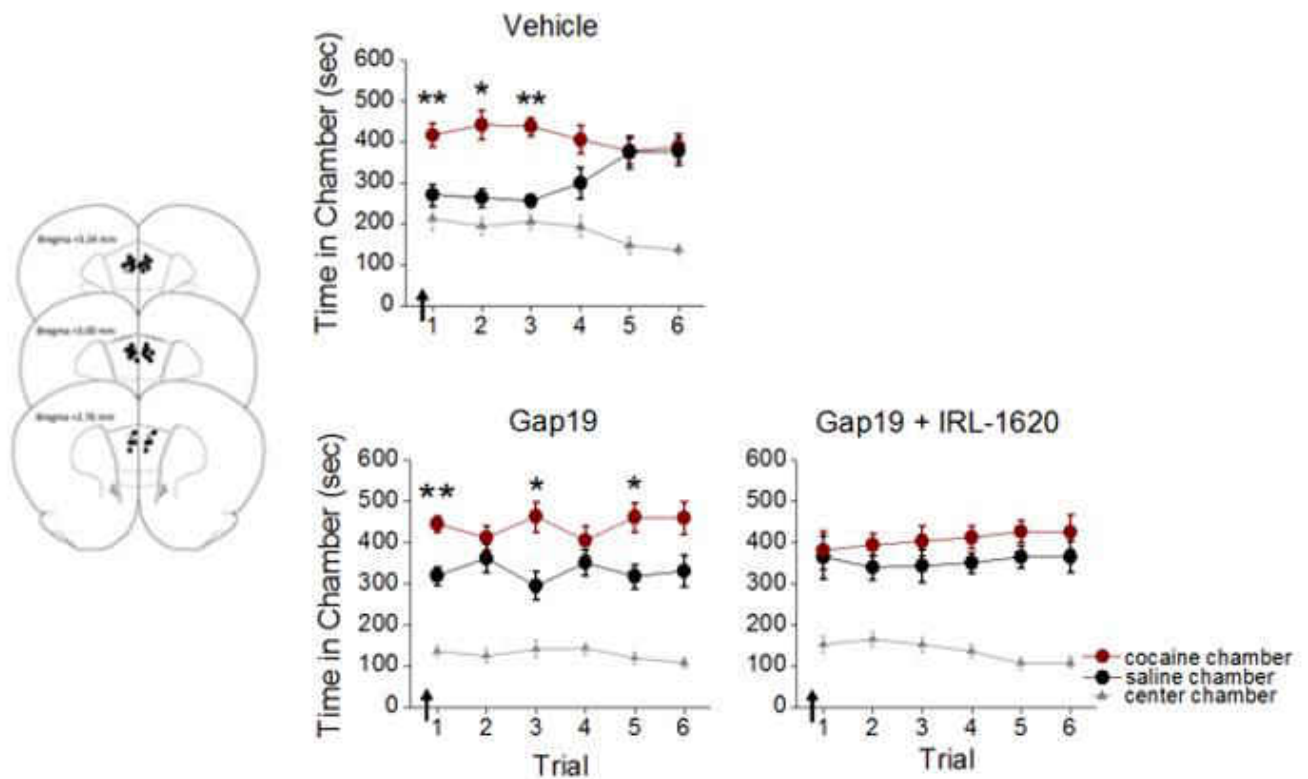


Figure 3. PL-mPFC infusion of both unopposed hemichannel blocker Gap19 and astrocyte-specific gap junction blocker IRL-1620 persistently disrupts retrieval of CPP. **Left**, Coronal drawings showing injector tip placements for vehicle ($n = 10$), Gap19 ($n = 11$), and Gap19 + Irl-1620 treated animals ($n = 11$). White circle represents Vehicle, black circles represent experimental groups. **Right**, PL-mPFC infusions (arrows) of Gap19 + Irl-1620 before the first CPP trial persistently disrupts retrieval of CPP whereas infusions of vehicle or Gap19 does not. $**p < 0.01$, $*p < 0.05$. Error bars indicate SEM.

To test if retrieval of a CPP could be disrupted by Gap19 + IRL-1620 after a CPP had already been expressed, we next examined the effect of Gap19 + IRL-1620 on retrieval by microinfusion prior to the second trial after a CPP had already been expressed. Following conditioning, all rats expressed a CPP for the previously cocaine-paired chamber during the first trial. PL-mPFC microinfusions of Gap19 + IRL-1620 ($n=11$), but not vehicle ($n=9$) before the second trial impaired expression of the CPP on that trial and all subsequent trials (Figure 5). Repeated measures ANOVA revealed an effect of chamber for both groups during the first trial (vehicle $F_{2,22}=24.07$, $p<0.05$; Gap19 + IRL-1620, $F_{2,18}=19.27$, $p<0.05$), and *post hoc* analyses confirmed that both groups spent significantly more time in the cocaine-paired chamber than the saline-paired chamber ($p<0.05$). Following microinfusion of Gap19 + IRL-1620 or vehicle prior to the second trial, only the vehicle-treated rats spent significantly more time in the previously cocaine-paired chamber (effect of chamber: $F_{2,22}=16.44$, $p<0.05$, *post hoc* $p<0.05$), whereas Gap19 + IRL-1620-treated rats spent an equivalent amount of time in all chambers ($F_{2,18}=4.11$, $p>0.05$). On subsequent infusion-free trials, vehicle-treated rats continued to spend significantly more time in the cocaine-paired chamber overall (effect of chamber: $F_{2,22}=7.61$, $p<0.05$, *post hoc* $p<0.05$), whereas Gap19 + IRL-1620-treated rats spent an equivalent amount of time in all chambers ($F_{2,18}=1.18$, $p>0.05$). Therefore, Gap19 + IRL-1620 infusions into PL-mPFC disrupted the retrieval of a previously expressed CPP.

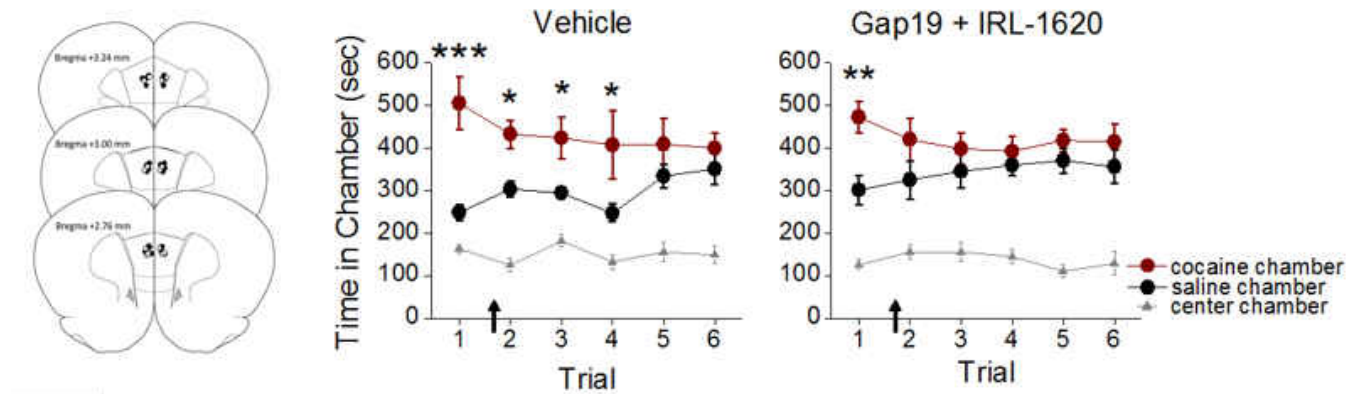


Figure 4. PL-mPFC infusion of both unopposed hemichannel blocker Gap19 and astrocyte-specific gap junction blocker IRL-1620 disrupts retrieval of an established CPP. **Left**, Coronal drawings showing injector tip placements for vehicle ($n = 9$), Gap19 + IRL-1620 treated animals ($n = 11$). White circle represents Vehicle, black circles represent experimental groups. **Right**, all rats expressed a CPP for the previously cocaine-paired chamber over the previously saline-paired chamber during the first test trial. PL-mPFC infusions (arrows) of Gap19 + IRL-1620 but not vehicle before the second CPP trial prevented rats from expressing a CPP during the second trial and subsequent trials. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$. Error bars indicate SEM.

Extinction of conditioned place preference

Following conditioning, rats were given longer 30 minute CPP trials that has been shown to promote extinction learning (Otis et al., 2014). We hypothesized that general gap junction or astrocytic gap junction blockade would prevent extinction whereas neuronal gap junction blockade would not. IL-mPFC infusions (Figure 1) of vehicle, CBX (50 mM), IRL-1620 (.1 uM), or quinine (100 uM) were administered prior to the first trial. IL-mPFC carbenoxolone or IRL-1620 impaired extinction across days (Figure 5). Repeated measures ANOVA revealed a significant effect of chamber for the vehicle ($F_{2,22}=18.45, p<0.05$), carbenoxolone ($F_{2,20}=14.635, p<0.05$), IRL-1620 ($F_{2,20}=11.78, p<0.05$), and quinine groups ($F_{2,20}=17.39, p<0.05$). *Post hoc* analyses confirmed that all groups expressed a CPP during the first trial ($p < 0.05$). However, only carbenoxolone-treated and IRL-1620-treated rats expressed a CPP during subsequent trials (i.e., trials 2-4; $p < 0.05$), whereas vehicle-treated and quinine-treated rats did not ($p > 0.05$). Thus, IL-mPFC general or astrocyte specific gap junction blockade impaired extinction of the CPP.

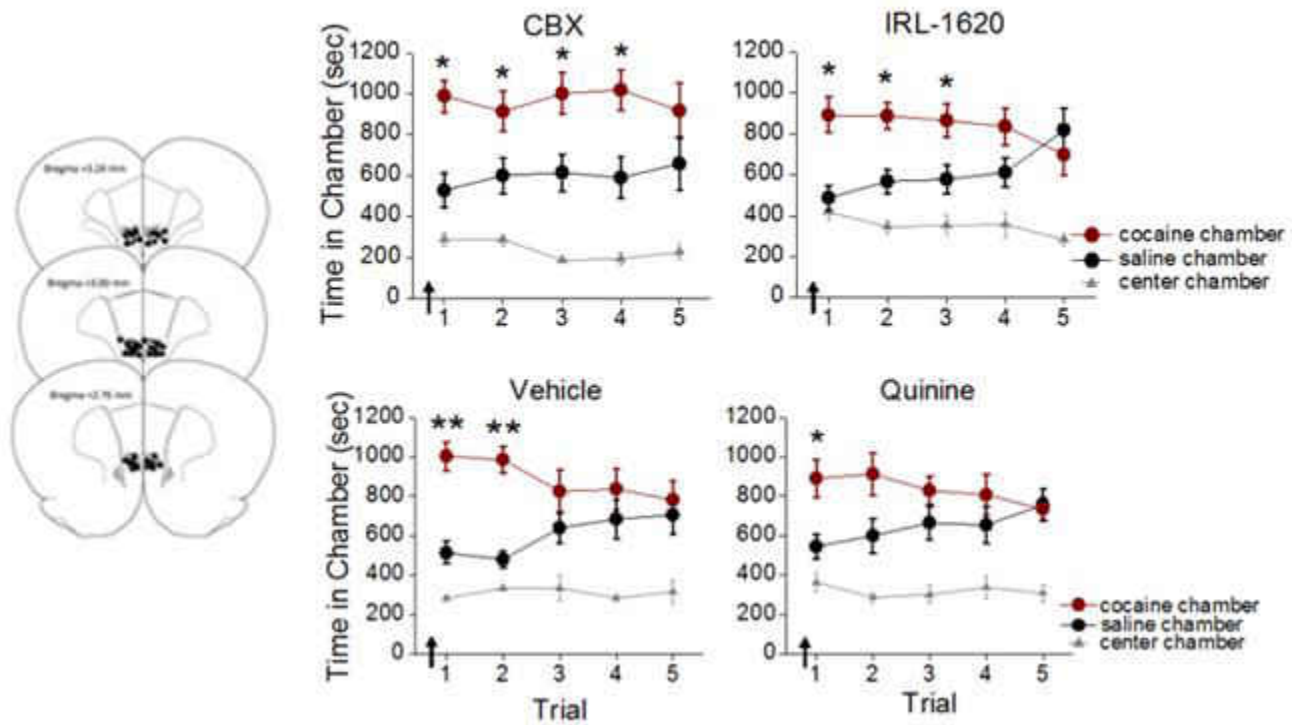


Figure 5. IL-mPFC blockade of general or astrocyte-specific gap junctions prevent extinction of a cocaine-CPP. **Left**, Coronal drawings showing injector tip placements for vehicle ($n = 11$), CBX ($n = 10$), IRL-1620 ($n = 9$), and quinine ($n = 10$). White circle represents Vehicle, black circles represent experimental groups. **Right**, IL-mPFC infusions (arrows) of CBX or IRL-1620 but not quinine before the first CPP trial prevented extinction of the CPP. ** $p < 0.01$, * $p < 0.05$. Error bars indicate SEM.

Aim 2

PL-mPFC astrocytic calcium levels following general, neuronal, or astrocytic gap junction communication blockade was next investigated. Following tissue removal and preparation of SR-101 and Fluo-4-AM (Figure 6), PL-mPFC tissue was treated with one of the following treatments:

1. Control (no gap junction blockade treatment) - 6 animals, 7 slices, 26 cells.
2. Treatment with 100 μ M for 5 min prior to scan - 6 animals, 6 slices, 21 cells.
3. Treatment with 0.1 μ M IRL-1620 for 5 min prior to scan - 6 animals, 6 slices, 22 cells.
4. Treatment with 100 μ M Quinine for 5 min prior to scan - 6 animals, 6 slices, 20 cells.

NE has previously been shown to evoke calcium-induced calcium sharing between astrocytes (Salm & McCarthy, 1990; Duffy & MacVica, 1995). So we also tested to see if various gap junction blockade treatments would prevent NE evoked calcium-induced calcium rise in astrocytes.

5. Treatment with 100 μ M NE for 2 min prior to scan - 6 animals, 6 slices, 25 cells.
6. Treatment with 100 μ M CBX for 5 min then with 100 μ M NE for 2 min prior to scan - 6 animals, 6 slices, 18 cells.
7. Treatment with 0.1 μ M IRL-1620 for 5 min then with 100 μ M NE for 2 min prior to scan - 6 animals, 6 slices, 18 cells.
8. Treatment with 100 μ M Quinine for 5 min then with 100 μ M NE for 2 min prior to scan - 6 animals, 6 slices, 16 cells.

Following tissue removal, slices containing the PL-mPFC were prepared as described above. We hypothesized neuronal gap junction blockade or NE treatment would increase PL-mPFC basal astrocyte cytoplasmic calcium levels whereas general gap junction or astrocytic gap junction blockade would not. Additionally, we hypothesized that tissue receiving NE treatment after neuronal gap junction blockade would increase PL-mPFC astrocyte cytoplasmic calcium levels whereas NE treatment after general gap junction blockade or astrocytic gap junction blockade would not. We observed that bath application of NE (Figure 7) or quinine (Figure 10) increased PL-mPFC basal astrocyte cytoplasmic calcium levels whereas bath application of CBX (Figure 8), or IRL-1620 (Figure 9) had no effect on PL-mPFC basal astrocyte cytoplasmic calcium levels. Additionally, CBX (Figure 11) or IRL-1620 (Figure 12) treatment prevented NE from increasing PL-mPFC astrocyte cytoplasmic calcium levels, whereas quinine (Figure 13) did not. ANOVA revealed an overall effect of group ($F_{7,164}=3.46$, $p<0.05$) and *post hoc* analyses confirmed that the area under the curve (AUC) for the control group was significantly lower than the NE group AUC ($ps<0.05$), the quinine group AUC ($ps<0.05$), and the quinine + NE group AUC ($ps<0.05$). Thus, NE or neuronal gap junction blockade via quinine increases cytoplasmic calcium levels in astrocytes. Additionally, the IRL 1620 + NE group AUC was significantly lower than the NE group AUC ($ps<0.05$). Thus, astrocyte-specific gap junction blockade via IRL-1620 prevented the NE-induced increase of calcium levels. Overall, NE or neuronal gap junction blockade increases basal cytoplasmic calcium levels in PL-mPFC astrocytes and blocking astrocyte gap junctions can prevent NE-induced calcium level increases.

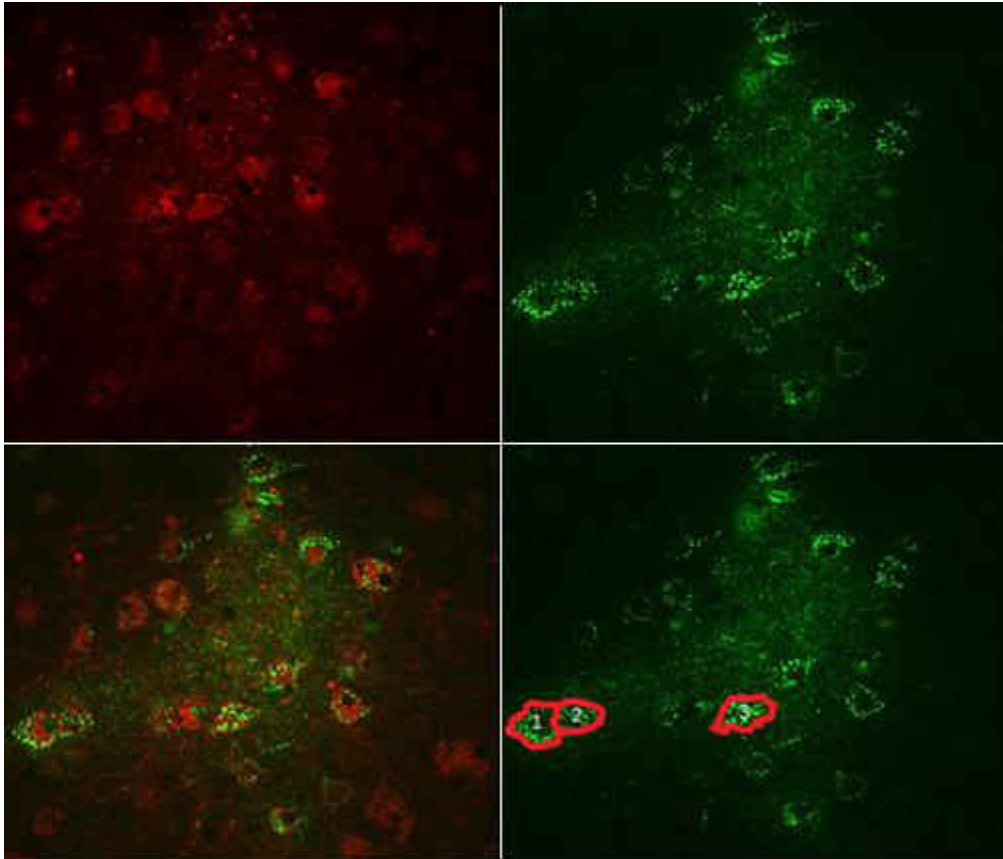


Figure 6. Imaging of PL-mPFC astrocytes using confocal microscopy.

Top left, At 40x magnification, astrocyte cell bodies were identified in red using SR-101 staining.

Top right, Fluo-4-AM staining in green identified calcium.

Bottom left, Merged images show that Fluo-4-AM staining of calcium is co-localized within astrocyte cell bodies.

Bottom right, Example ROI collection.

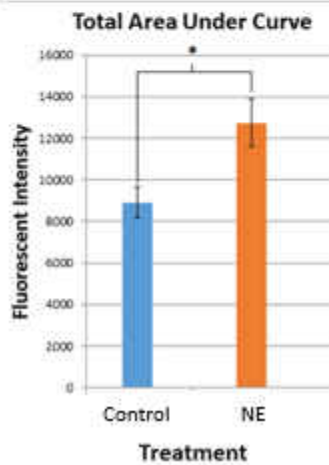
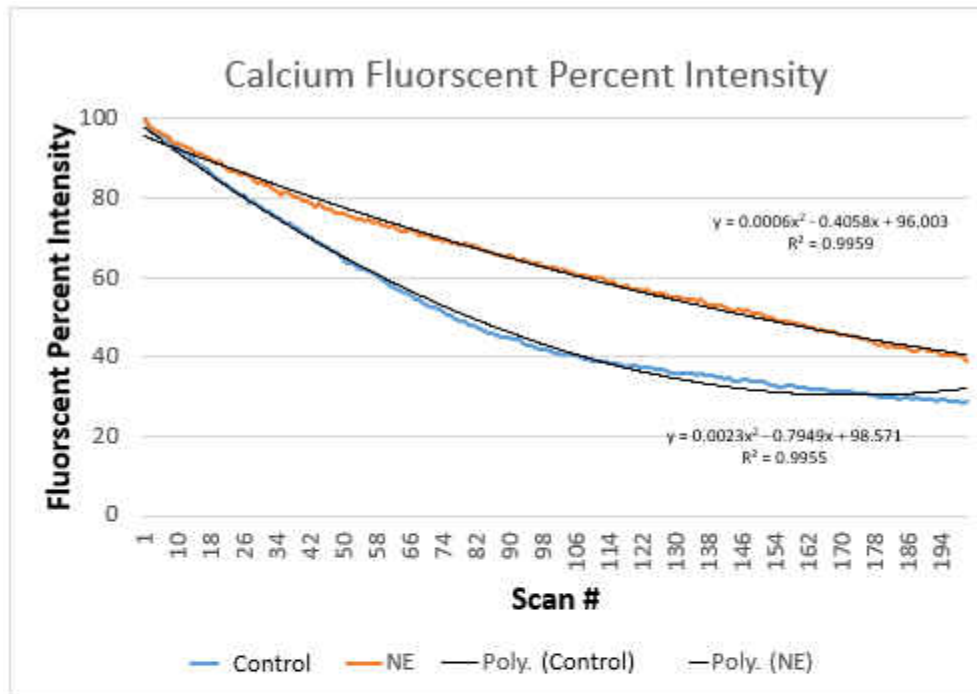


Figure 7. Pre-bath application of NE increases PL-mPFC astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of controls/baseline (light blue, $n = 26$ cells) and NE (orange, $n = 25$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, NE (orange) treated PL-mPFC tissue increases overall Fluo-4-AM intensity as measured by the area under curve as compared to controls/baseline (blue) treated tissue. * $p < 0.05$. Error bars indicate SEM

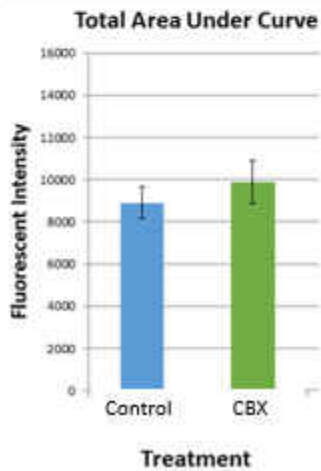
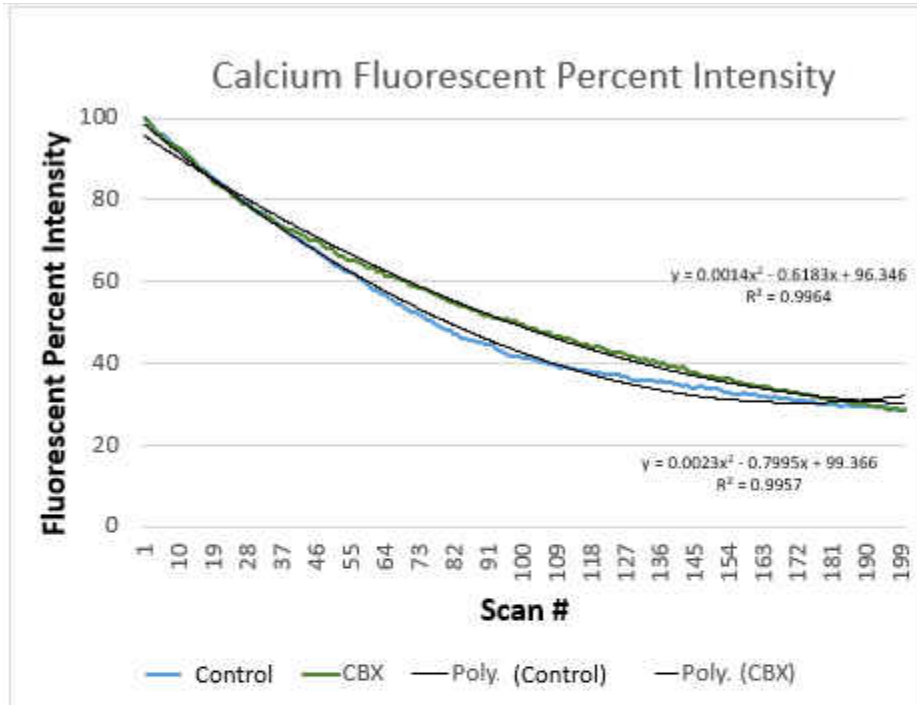


Figure 8. Pre-bath application of non-selective gap junction blocker CBX does not change PL-mPFC astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of controls/baseline (light blue, $n = 26$ cells) and CBX (green, $n = 21$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, CBX (green) treated PL-mPFC tissue and controls/baseline (light blue) treated tissue show similar overall Fluo-4-AM intensity as measured by the area under the curve. Error bars indicate SEM.

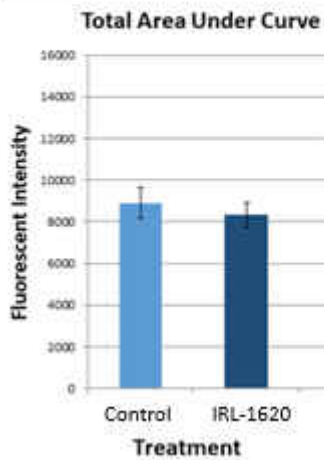
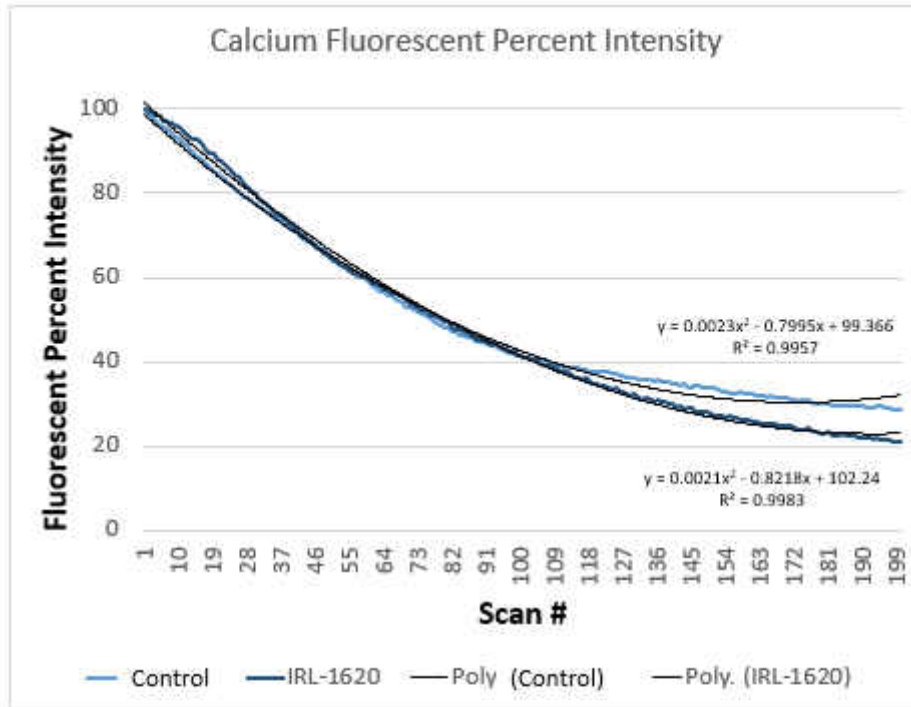


Figure 9. Pre-bath application of astrocyte gap junction blocker IRL-1620 does not change PL-mPFC astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of controls/baseline (light blue, $n = 26$ cells) and IRL-1620 (dark blue, $n = 22$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, IRL-1620 (dark blue) treated PL-mPFC tissue and controls/baseline (light blue) treated tissue show similar overall Fluo-4-AM intensity as measured by the area under curve. Error bars indicate SEM.

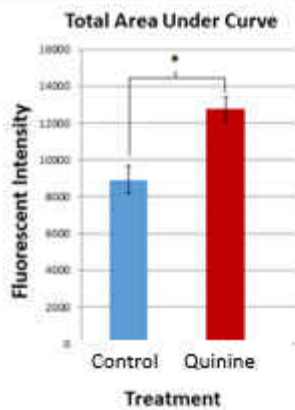
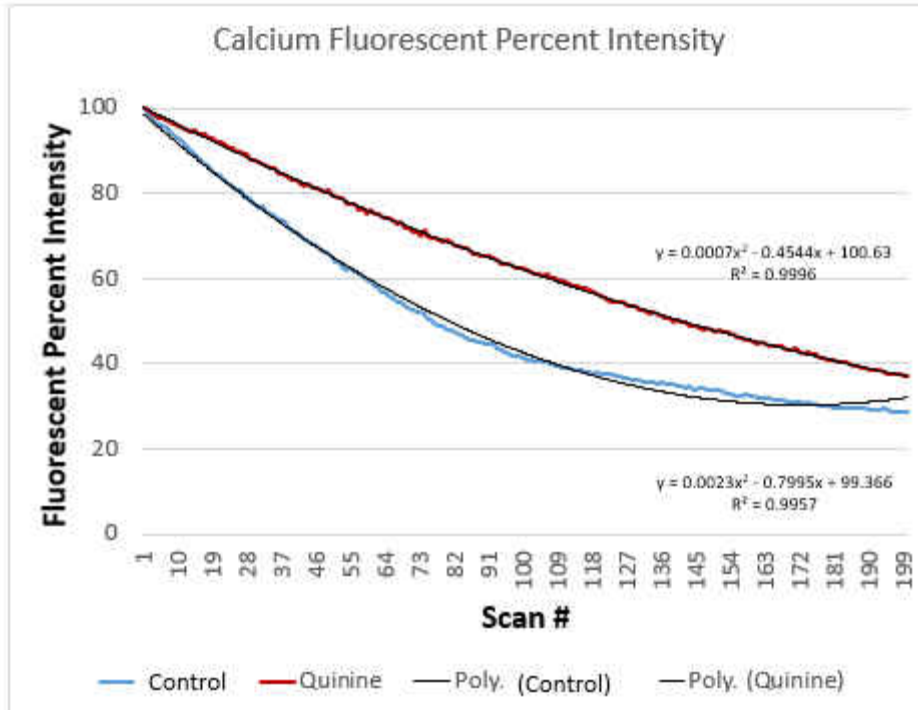


Figure 10. Pre-bath application of neuron-specific gap junction blocker quinine increases PL-mPFC astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of controls/baseline (light blue, $n = 26$ cells) and quinine (red, $n = 20$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, Quinine (red) treated PL-mPFC tissue increases overall Fluo-4-AM intensity as measured by the area under curve compared to controls/baseline (blue) treated tissue. $*p < 0.05$. Error bars indicate SEM.

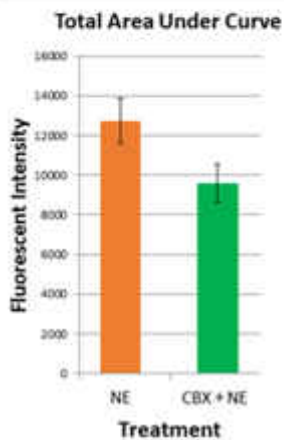
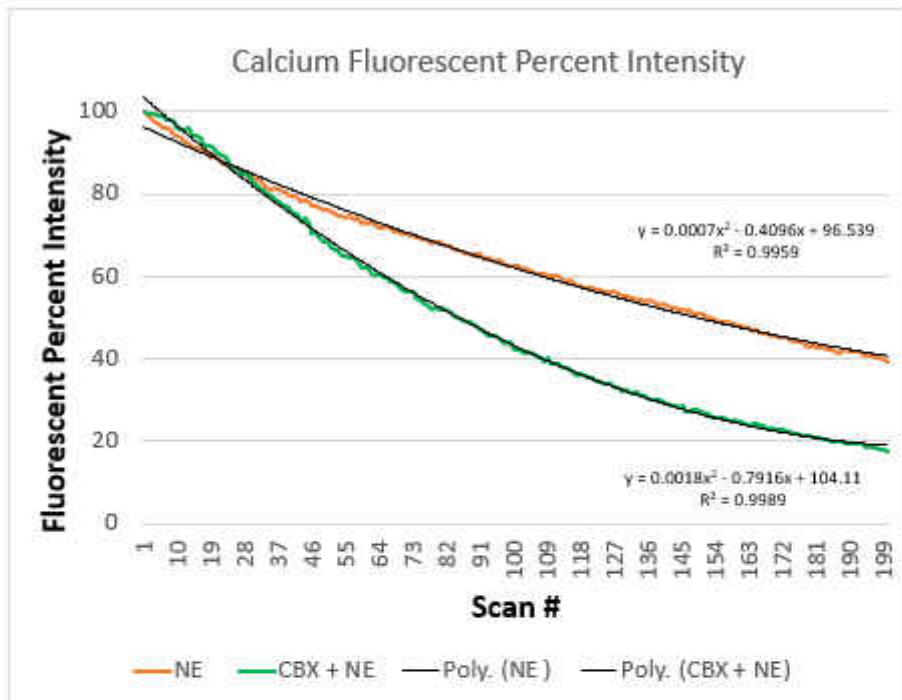


Figure 11. Pre-bath application of CBX + NE does not significantly change PL-mPFC NE treated astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of NE (orange, $n = 25$ cells) and CBX + NE (bright green, $n = 18$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, NE (orange) treated PL-mPFC tissue non-significantly increases overall Fluo-4-AM intensity as measured by the area under curve compared to CBX + NE (bright green) treated tissue. Error bars indicate SEM

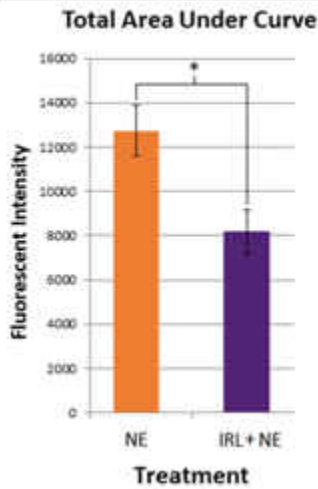
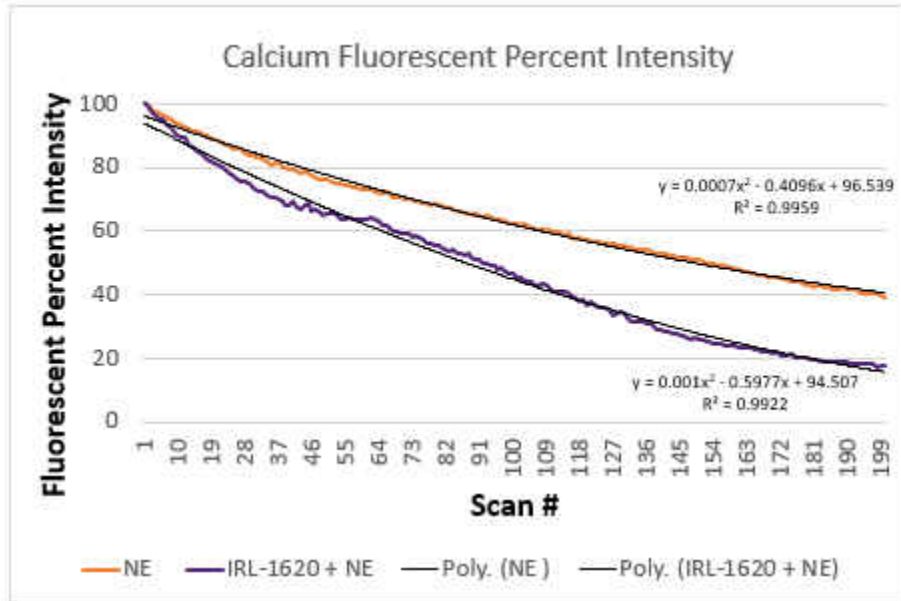


Figure 12. Pre-bath application of IRL-1620 + NE prevents increase of NE Fluo-4-AM in PL-mPFC astrocytes. *Top*, Fluo-4-AM intensity measured of NE (orange, $n = 25$ cells) and IRL-1620 + NE (purple, $n = 18$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, PL-mPFC IRL-1620 + NE (purple) treated tissue show significantly less overall Fluo-4-AM intensity as measured by the area under curve compared to NE (orange) treated tissue. * $p < 0.05$. Error bars indicate SEM.

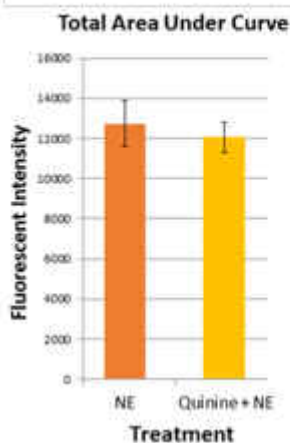
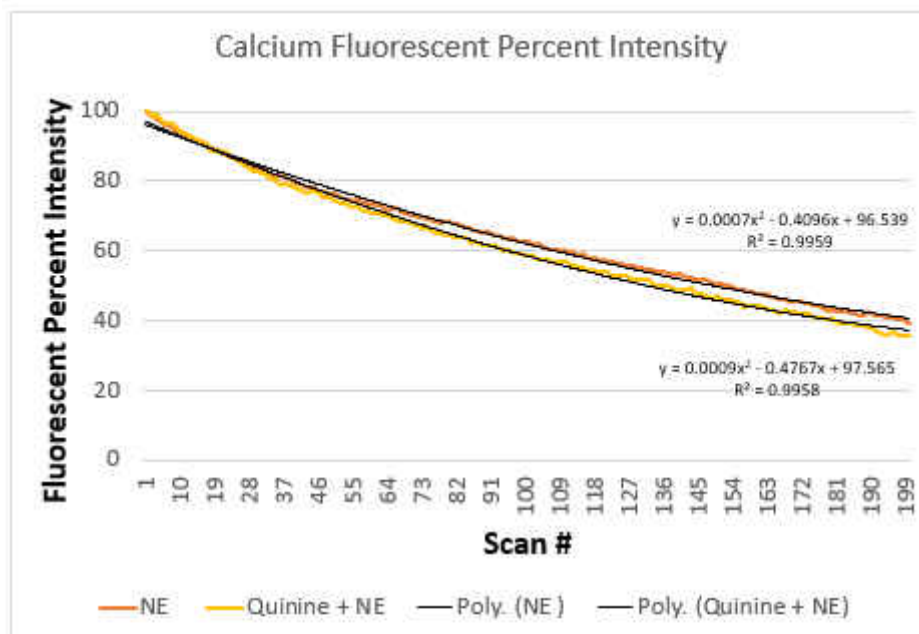


Figure 13 Pre-bath application of quinine + NE does not change PL-mPFC NE treated astrocyte calcium levels. *Top*, Fluo-4-AM intensity measured of NE (orange, $n = 25$ cells) and quinine + NE (yellow, $n = 18$ cells) over the course of 200 scans. Polynomial equation with R^2 was also calculated. *Left*, NE (orange) and quinine + NE (yellow) treated PL-mPFC tissue show similar overall Fluo-4-AM intensity as measured by the area under curve. Error bars indicate SEM.

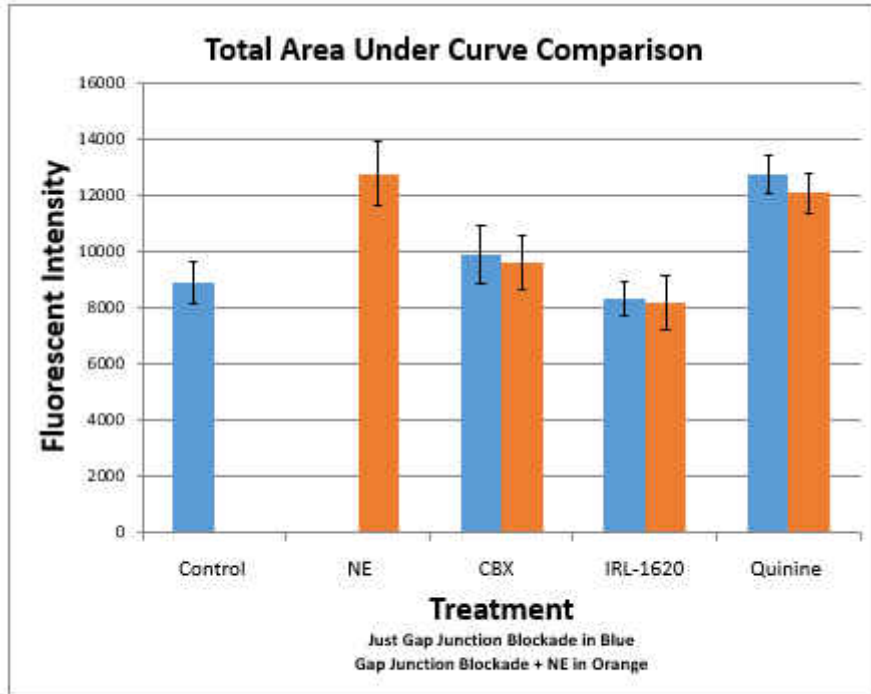


Figure 14. Summary of area under curves levels for each group. Error bars indicate SEM.

Discussion

These studies aimed (1) to determine whether gap junction communication is a mechanism for memory retrieval and extinction of a cocaine CPP and (2) to evaluate PL-mPFC astrocytic calcium levels following general, neuronal, or astrocytic gap junction communication blockade.

Results revealed that, within the PL-mPFC, general and astrocyte-specific gap junction blockade prevent retrieval of a CPP whereas neuronal-specific gap junction blockade prolonged drug-seeking behavior. Additional behavioral experiments within the PL-mPFC revealed that restoring GABAergic tone in conjunction with blockade of neuronal-specific gap junctions restored normal memory retrieval and extinction of a CPP. Furthermore, blocking unopposed astrocyte hemichannels in conjunction with astrocyte-specific gap junctions prevented initial and subsequent memory retrieval of a CPP, whereas blockade of just astrocyte gap junction or just unopposed astrocyte hemichannels did not. Additionally, blocking PL-mPFC unopposed astrocyte hemichannels in conjunction with astrocyte-specific gap junctions prevented memory retrieval of a CPP after a CPP had been established. Finally within aim 1, blockade of IL-mPFC general and astrocyte-specific, gap junctions prevented extinction of a CPP.

In Aim 2, I examined the effects of the various gap junction blockers on calcium dynamics in PL-mPFC. Neuronal-specific gap junction blockade increased cytoplasmic calcium levels in astrocytes, whereas astrocyte-specific gap junction blockade did not. Furthermore, NE exposure induced an increase in cytoplasmic calcium levels in astrocytes, but pre-application of an astrocyte-specific gap junction blocker prevented the NE-induced rise. In contrast, neuron-specific gap junction blockade did not prevent the NE-induced rise in calcium levels in

astrocytes. Taken together, astrocyte and neuronal gap junctions play differential roles in memory retrieval and extinction of a CPP and have opposing effects on basal and NE-induced calcium levels in PL-mPFC astrocytes.

Aim 1

Data revealed that blocking PL-mPFC astrocyte specific gap junctions and unopposed hemichannels prevented memory retrieval of a CPP both during the initial and subsequent test trials. Furthermore, data revealed that blocking PL-mPFC astrocyte specific gap junctions and unopposed hemichannels prevented memory retrieval of a CPP after a CPP had been established. Previous data has shown that astrocyte gap junction communication influences neural activity (Parri et al., 2001). Additionally, blocking unopposed hemichannels in astrocytes decreases the release of glutamate and ATP (Ye et al., 2003) and decreases CA1 pyramidal neurons EPSC amplitudes in mice (Chever et al., 2014). Our data further revealed that blockade of astrocyte gap junctions alone or unopposed astrocyte hemichannels alone did not prevent initial memory retrieval, but did prevent subsequent retrieval of a CPP. Additionally, a return of a CPP was observed on test trial 6 for the astrocyte gap junction blockade animals even though during test trials 2-5, a CPP was not expressed. Overall, our data support that astrocytes can communicate to other astrocytes and neurons to mediate cocaine-associated memory retrieval in PL-MFC. In fact, these data are the first to demonstrate that blocking both PL-mPFC astrocyte gap junction communication and unopposed astrocyte hemichannels can persistently prevent memory retrieval of a drug-associated memory. Further investigation is necessary to determine the specific transmitters being released by astrocytes to neurons within the PL-mPFC to allow

memory retrieval to occur and what short and long term plasticity events can occur to cause a return of CPP expression following gap junction blockade.

PL-mPFC neuron-specific gap junction blockade prolonged drug-seeking behavior as compared to vehicle control. Restoring PL-mPFC GABAergic tone in conjunction with blocking neuron-specific gap junctions restored animals to normal drug-seeking behavior comparable to controls. Previous work has shown that GABAergic inhibitory interneurons selectively express Cx36 (Belluardo et al., 2000). The application of quinine selectively blocks Cx36 (Cruikshank et al., 2004) and has been shown to increase spike frequency in CA3 recordings of guinea pig tissue slices (Yoshida et al., 1986). The authors proposed that quinine's mechanism of action on neural activity was through blockade of an after-hyperpolarizing potassium channel (Yoshida et al., 1986). Overall, our data support the conclusion that blocking Cx36 in inhibitory interneurons leads to a decrease of inhibitory signal in local circuitry and a net increase in activity within a structural region of the brain.

PL-mPFC non-specific gap junction blockade prevented memory retrieval of a CPP on test trials 1-4, but caused a return of preference on test trials 5-6. These data may be anomalous, or they may reflect the combined effects of astrocyte and neuron specific gap junction blockade. The initial trials appear similar to those from the IRL-1620 group, whereas later trials appear similar to those from the quinine group during which the preference returns even after the control animals have extinguished their drug-seeking behavior. CBX has been one of the most widely used non-selective gap junction blockers (Moradi et al., 2013; Chepkova et al., 2008; Schoenfeld et al., 2014) and has been shown to reduce neural activity (Tover et al., 2009) impair LTP (Chepkova et al., 2008), and decrease diffusion of gap junction channel-

permeable dye between astrocytes (Sun et al., 2012). No other data are currently available showing the long-lasting effect of CBX on neuron or astrocyte activity, but CBX can washout during electrophysiological recordings after 30 min (Alvarex-Maubecin et al., 2000).

Interestingly, it has been observed that CBX blocks unopposed astrocyte hemichannels in addition to being a non-selective gap junction blocker (Ye et al., 2003). Thus, further study of CBX's dynamics will be needed to determine the extent of effects on both neurons and astrocytes.

Results revealed that general or astrocyte-specific gap junction blockade in IL-mPFC prevented extinction learning whereas neuron-specific gap junction blockade accelerated extinction learning. These results are similar to those found in the PL-mPFC behavioral studies, where general and astrocyte-specific gap junction blockade prevented the function of the PL-mPFC whereas neuron specific gap junction blockade accelerated and had the opposite effect on the PL-mPFC. Taken together, drug-associated retrieval and extinction memories can be impaired or strengthened by blocking specific gap junction activities, with astrocyte and neuronal gap junctions serving opposing roles in regions of the brain involved in memory retrieval and extinction of drug-seeking behavior.

Aim 2

Our data revealed that astrocyte gap junction blockade prevented a NE-induced increase in cytoplasmic calcium levels in astrocytes. NE can evoke a calcium increase within astrocytes (Salm & McCarthy, 1990). In the absence of provoking conditions, spontaneous calcium activity in individual astrocytes does not spread among astrocytes as an intercellular calcium wave (Nett

et al.2002). In baseline conditions, the magnitude of correlated activity in nearby and distant astrocytes is quite similar (Hirase et al., 2004).

Gap junction blockade does not render an astrocyte unable to release transmitter, which could explain why PL-mPFC IRL-1620 treated animals in aim 1 showed a significant preference on day 1 of testing. Calcium and cAMP are active in SNARE-dependent merger of vesicles (Vardjan & Zorec, 2015) and calcium increases in a single astrocyte can lead to the release of transmitters like glutamate and ATP (Parpura et al., 1994). Thus, for a single astrocyte to release its own transmitter, gap junction communication is not always necessary. However, when gap junctions between astrocytes are open, calcium waves between astrocytes can lead to a much more unified release of transmitter from an ensemble of astrocytes that can then communicate to other nearby astrocytes and neurons. For example, glutamate can induce astrocytic calcium waves (Innocenti et al., 2000) in addition to causing a calcium rise inside a single astrocyte. Once calcium waves begin, they can exhibit oscillatory behavior that can persist for periods of 5–30 min at variable frequencies (10–110 mHz). A direct correlation between glutamate concentration and the frequency of oscillations has also been observed. At low concentrations (below 1 μ M), intracellular calcium transients are asynchronous and localized. In contrast, at higher concentrations of glutamate (10–100 μ M), calcium waves propagate over longer distances (Cornell-Bell et al., 1990). Additionally, rhythmic oscillatory calcium waves can release transmitters in a unified manner that affect the local field potentials of neurons (Tewari & Parpura, 2015). IRL-1620 decreases calcium wave propagation by roughly 80-90 percent in hippocampal and striatal astrocytes (Blomstrand et al., 1999). Taken together our data suggests that blocking astrocyte gap junction communication with IRL-1620 prevents NE-induced

oscillatory actions of calcium waves (Paukert et al., 2014; Salm & McCarthy, 1990).

Because the astrocytic release of transmitters is slower than neuron communication, some theorize that the astrocytic system regulates homeostatic processes. Astrocytic transmission thus models the endocrine system in that it is slower than the neuronal transmission but more widespread. For example, when astrocytes connected to arterioles elicit calcium waves, there is an increase in the release of vasoactive compounds (Bezzi et al. 1998). This work supports the idea that astrocytes function to regulate local circulation according to the metabolic needs of neurons.

PL-mPFC tissue exposed to the neuron-specific gap junction blocker quinine significantly increased cytosolic calcium in astrocytes. Our data from aim 1 revealed that increasing GABAergic tone within the PL-mPFC counteracts quinine's effect of prolonging drug-seeking behavior. These data offer evidence that a decrease in GABAergic tone on pyramidal neurons cause an increase in neuronal transmitter release that can trigger astrocytic calcium waves. Furthermore, co-localizing Cx36 mRNA with parvalbumin, a GABAergic interneuron marker, shows that GABAergic inhibitory interneurons express Cx36 (Belluardo et al., 2000) and quinine specifically blocks channel conductance of Cx36 (Cruikshank et al., 2004). Other data suggest that quinine may have another mechanism to increase neuronal excitability. Quinine increases the excitability of CA3 pyramidal neurons, possibly through blockade of an AHP – K⁺ channel (Yoshida et al., 1986). This proposed mechanism is activated by protein kinase A (PKA; Liu et al. 1999) and PKA is able to regulate AMPA receptor recruitment to the plasma membrane (Gomes et al., 2004) to further increase neuronal excitability.

PL-mPFC tissue exposed to the general gap junction blocker CBX did not significantly

decrease cytosolic calcium in astrocytes, as compared to tissue treated with NE. We originally hypothesized that CBX would prevent a NE-induced increase in astrocyte cytoplasmic calcium similar to the IRL-1620 treated tissue based on previous findings that CBX reduces neural activity (Tover et al., 2009) impairs LTP (Chepkova et al., 2008), and decreases diffusion of gap junction channel-permeable dye between astrocytes (Sun et al., 2012). Our data offers a possible explanation as to why CBX was unable to significantly decrease the cytoplasmic calcium levels in astrocytes. CBX blocks both astrocyte and neuron gap junctions, and our data reveal that quinine is able to significantly increase the cytoplasmic calcium levels in astrocytes compared to baseline/control readings. A future experiment of applying both IRL-1620 and quinine could clarify if quinine is able to increase the cytoplasmic calcium levels in astrocytes while astrocyte gap junctions are closed.

Another intriguing aspect of our data is that the animals in aim 1 who received CBX microinfusions into the PL-mPFC showed a retrieval deficit that was initially very prevalent, but turned out to not persist. Since CBX can block both gap junctions and unopposed hemichannels in astrocytes, a possible explanation as to why CBX treated animals showed a strong initial retrieval deficit is the CBX treated animals were under similar conditions as the PL-mPFC IRL-1920 + Gap19 group. However, since neuron-specific gap junctions are blocked as well with CBX, this could explain why the PL-mPFC CBX animals in later trials displayed CPP after controls had extinguished, similar to the PL-mPFC quinine-treated animals. Future experiments exploring CBXs long term effects on astrocytes and neurons would help clarify the length of time CBX is active and what plasticity triggering events follow.

Future Directions

Gap junctions play a role in learning and memory, emotions and reward-related behavior, and even psychiatric and behavioral disorders, but the mechanisms are poorly understood. However, investigation of the behavioral impact of gap junctions has revealed that pharmacological blockade of gap junctions can impair the acquisition and expression of cued fear (Bissiere et al., 2011), bilateral hippocampus blockade of gap junctions with CBX can impair rats performance on a water maze (Hosseinzadeh et al., 2005), and post-acquisition infusions of the non-selective gap junction blocker 18-alpha-glycyrrhetic acid can impair retention performance in a one-trial discrimination avoidance task in chicks (Verwey and Edwards, 2010).

I have demonstrated that within the cocaine-associated memory paradigm of a conditioned place preference that general or astrocyte gap junction blockade decreases drug-seeking behavior whereas neuronal gap junction blockade prolongs drug-seeking behavior. Gap junction blockade has been theorized to reduce the level of neuronal excitability (Kokarovtseva et al., 2013), however whether this is due to astrocytic and neuronal gap junction closure or just one specific type remains unclear. Whole cell patch clamp electrophysiology using the either IRL-1620 or quinine could determine which specific gap junction type mediates the effects observed with a general gap junction blocker such as CBX. Based on my results, I would predict that the application of IRL-1620 would decrease neuronal excitability whereas quinine would increase neuronal excitability.

Evidence described here indicates that blocking astrocyte gap junctions prevents an increase in calcium levels within astrocytes. Because astrocytes release transmitters in a calcium-dependent manner, it would be interesting to see what short and long-term plasticity

events can occur within both astrocytes and neurons following prolonged gap junction blockade. The blockade of glutamate exchange between astrocytes and neurons increases and decreases vesicular glutamate release in pre-synaptic neurons. It also promotes AMPA receptor insertion in post-synaptic neurons during withdrawal (Kalivas 2009) but it not known if this is dependent on gap junction communication of astrocytes. Electrode recordings of extracellular levels of glutamate (Hu et al., 1994) and protein analysis of AMPA receptors (Brown et al., 2002) at various time points following IRL-1620 application could determine if astrocyte gap junctions influence neuronal plasticity. From the results gathered here, I predict that blocking astrocyte gap junctions will decrease extracellular glutamate levels and decrease AMPA receptor levels in neurons.

Clinical Relevance

Previous work has demonstrated that gap junction dysfunction in the prefrontal cortex can induce depressive-like behaviors in rats (Sun et al., 2012). Following chronic unpredictable stress, animals exhibited significant decreases in diffusion of gap junction channel-permeable dye and expression of Cx43. However, the cellular and behavioral alterations induced by the chronic unpredictable stress were reversed and/or blocked by treatment with the antidepressants fluoxetine and duloxetine. Beyond fluoxetine's SSRI effect on depression, fluoxetine also has an anti-depressant effect by increasing expression of the vesicular glutamate transporter-1 (VGLUT1) in the PFC (Farley et al., 2012; Chen et al., 2014). However, it is not known if VGLUT1 expression is elevated in neurons and/or astrocytes. Therapeutically, targeting astrocytes or neurons can create a "chicken or egg" effect concerning which cell influences the

other, but further investigation could reveal a greater holistic view of antidepressants on both healthy gap junction and synaptic communication. Lastly, previous work from our lab has shown that blocking β -adrenergic receptors with specific and non-specific β -adrenergic blockers like propranolol or betaxolol can prevent retrieval of a CPP (Otis and Mueller, 2011; Otis et al., 2013, 2014; Fitzgerald et al., 2016). It would be interesting to investigate how β -adrenergic blockade also influences the communication between astrocyte and neuronal networks because astrocytes express a variety of Gq-coupled alpha adrenergic receptors (Cahoy et al., 2008), and exogenous norepinephrine triggers a rise in calcium in astrocytes (Salm and McCarthy, 1990) that can lead to SNARE-dependent merger of a vesicle membrane with the plasma membrane (Vardjan & Zorec, 2015).

Conclusion

These studies aimed (1) to determine whether gap junction communication is a mechanism for memory retrieval and extinction of a cocaine CPP and (2) to evaluate PL-mPFC astrocytic calcium levels following general, neuronal, or astrocytic gap junction communication blockade. Overall this dissertation provides evidence that both gap junction communication and the communication between astrocytes and neurons are both actively involved with memory.

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Publications

Fitzgerald MK, Otis JM, Mueller D. (2016). *Dissociation of β 1- and β 2-adrenergic receptor subtypes in the retrieval of cocaine-associated memory*. Behavioural Brain Research. 296:94-9

Otis JM, Fitzgerald MK, Mueller D. (2014). *Infralimbic BDNF/TrkB enhancement of GluN2B currents facilitates extinction of a cocaine conditioned place preference*. Journal of Neuroscience. 34(17):6057-64.

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Holden JM, Fitzgerald M, Bussell G, Ehlers V. (2011) *Triadimefon supports conditioned cue preference*. Behavioural Brain Research. 221,1,1,307–310

Posters

Fitzgerald MK, Otis JM, Drake MA, Burkard JL, Mueller D. (2014) Inhibition of PKA signaling in the prelimbic cortex persistently disrupts retrieval of a cocaine-associated memory and prevents subsequent reinstatement. Poster session presented at Society for Neuroscience, Washington DC.

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Otis JM, Fitzgerald MK, Mueller D. (2013) Hippocampal β -adrenergic receptor blockade impairs retrieval but not reconsolidation of a cocaine-associated memory. Poster session presented at Society for Neuroscience, San Diego CA.

Lenz A, Fitzgerald M, and Holden JM (2013) Effects of triadimefon on mouse light-dark performance in Swiss Webster mice. Poster session presented at Seven Rivers Conference, La Crosse, WI.

Otis JM, Fitzgerald MK, Dashew, KB, Mueller, D. (2012) *Infralimbic BDNF and TrkB signaling enhances extinction of cocaine seeking*. Poster session presented at SfN, New Orleans

Doncheck EM, Fitzgerald MK, Ruder SA, Mueller D. (2012) *Acquisition and extinction of cocaine-seeking behavior regulate brain expression of basic fibroblast growth factor*. Poster session presented at SfN, New Orleans

Holden JM, Fitzgerald M, Bussell G, Ehlers V. (2010) *Triadimefon supports conditioned cue preference*. Poster session presented at Seven Rivers Conference, La Crosse, WI.

Fitzgerald M, Glavinovich N, Howes N, Kryzyzanowski B. (2010) *Drug Survey: Is a Synthetic Cannabinoid (JWH018) Creating a New Trend?* Poster session presented at Tristate Conference, Cedar Rapids, IA

Presentations

M. Fitzgerald (2014) *Involvement of β -adrenergic receptor signaling in retrieval of a cocaine-associated memory*. Oral session presented at UW – Milwaukee Neuroseminar Series, Milwaukee WI.

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Teaching experience:

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