

Curriculum Vita

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EDUCATION

University of Texas at El Paso (El Paso, TX)

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AFFILIATIONS

Society for Neuroscience

National Hispanic Science Network on Drug Abuse

HONORS AND AWARDS

- Ford Diversity Predoctoral Fellowship 2008-2012

- Meyerhoff Graduate Fellowship 2008-Present

- Gordon Research Conference on Catecholamines Carl Storm Underrepresented Minority Fellowship Travel Award 2009 National

- Science Foundation Graduate Research Fellowship Program 2010-2013

- NIH Graduate Student Research Travel Award 2011 (poster award)

- Kappa-opioid therapeutics conference 2013 travel award

- Society for Neuroscience Neuroscience Scholars Program 2013-Present

UNDERGRADUATE HONORS AND AWARDS

- Career Opportunities in Research (NIMH) Fellowship 2005-2007
- MARC/AIM Program at Purdue University, Summer 2006
- Dilliard's Scholarship 2007-2008
- Society for Neuroscience 2007 Faculty for Undergraduate Neuroscience Annual Meeting Travel Award
- College of Liberal Arts Dean's list: Spring 2004, Fall 2004, Spring 2005, Fall 2005, Spring 2006, Fall 2006, Spring 2007, Fall 2007, Spring 2008
- University of Texas at El Paso Outstanding Academic and Research Achievement in Psychology 2008

RESEARCH EXPERIENCE

8/2008-8/2012

Graduate Student to Dr. Toni S. Shippenberg

National Institute on Drug Abuse

Integrative Neuroscience Section

Examining modulation of prefrontal cortex neurotransmission and affect by endogenous opioid systems using in-vivo microdialysis and behavioral measures. Analysis of monoamine neurotransmitters utilizing high performance liquid chromatography with electrochemical detection and amino acid neurotransmitters utilizing capillary electrophoresis with laser-induced fluorescence.

8/2008-Present

Graduate to Dr. Patricio O'Donnell

University of Maryland, Baltimore

Department of Anatomy and Neurobiology

Examination of the interaction of cortical and limbic afferents in the prefrontal cortex and opioid receptor modulation of limbic-cortico pathways using *in-vivo* juxtacellular and intracellular

electrophysiological recordings, *in-vivo* electrophysiology in freely-moving animals, and optogenetic techniques.

6/2005- 8/2008

Research Assistant to Dr. Laura E. O'Dell

University of Texas at El Paso

Department of Psychology

Used various behavioral tools such as the conditioned place paradigm, the elevated plus maze, conditioned taste aversion, two-bottle choice drinking paradigm, catalepsy test, intravenous self-administration, and the tail flick test. Employed several rodent surgeries including subcutaneous osmotic mini-pump implantations, intravenous catheter implantations, ovariectomies, and stereotaxic surgeries. I have assayed extracellular dopamine levels using *in-vivo* microdialysis with high performance liquid chromatography coupled with electrochemical detection.

6/2006-8/2006

Research Assistant to Dr. Julia A. Chester

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Department of Psychological Sciences

Utilized a genetic animal model of alcoholism to examine developmental differences in ethanol self administration. Utilized the handling-induced convulsions technique to examine the role of the endogenous kappa-opioid receptor in mediating alcohol withdrawal-induced convulsions and seizures.

PUBLICATIONS

1. **Tejeda H.A.**, Mejias-Apontes C., O'Donnell P. Regulation of limbic inputs to the mPFC by kappa-opioid receptors. Manuscript in preparation.
2. **Tejeda H.A.**, O'Donnell P. Amygdala-mediated prefrontal cortex deactivation disrupts information flow in the hippocampus to prefrontal cortex pathway. Manuscript in preparation.
3. Cabungcal, H.J., Counotte D.S., **Tejeda H.A.**, Piantidosi P.T., Lewis E.M., Calhoon G.G., Sullivan E., Presgraves E.Y., Cuenod M., Do K.Q., O'Donnell P. Juvenile antioxidant treatment prevents adult deficits in a developmental model of schizophrenia. *Neuron*. Under revision.

4. Dilgen J.*, **Tejeda H.A.***, O'Donnell P. (2013). Amygdala inputs drive feedforward inhibition in the medial prefrontal cortex. *J Neurophysiology*. 110(1):221-9. * *Co-first author*
5. **Tejeda H.A.**, Counotte D.S., Oh E., Rammamoorthy S., Schultz-Kuszk K.N., Bäckman C.M., Chefer V., O'Donnell P., Shippenberg T.S. (2013). Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion. *Neuropsychopharmacology*. 38(9):1770-9.
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7. Loewinger G.C., Beckert M.V., **Tejeda H.A.**, Cheer J.F. (2012). Methamphetamine-induced dopamine terminal deficits in the nucleus accumbens are exacerbated by reward-associated cues and attenuated by CB1 receptor antagonism. *Neuropharmacology*. 62(7):2191-200.
8. **Tejeda H.A.**, Shippenberg T.S., Henriksson R. (2010). The dynorphin/ κ -opioid receptor system and its role in psychiatric disorders. *Cell Mol Life Sci*. 69(6):857-96.
9. **Tejeda H.A.**, Chefer VI., Zapata A., Shippenberg T.S. (2010). The effects of kappa-opioid receptor ligands on prepulse inhibition and CRF-induced prepulse inhibition deficits. *Psychopharmacology (Berl)*. 210(2):231-40.
10. Natividad L.A., **Tejeda H.A.**, Torres O.V., O'Dell L.E. (2009). Nicotine withdrawal produces a decrease in extracellular levels of dopamine in the nucleus accumbens that is lower in adolescent versus adult male rats. *Synapse*. 64 (2): 136-145.
11. Torres O.V., Natividad L.A., **Tejeda H.A.**, Van Weelden S.A., O'Dell L.E. (2009). Female rats display dose-dependent differences to the rewarding and aversive effects of nicotine in an age-, hormone-, and sex-dependent manner. *Psychopharmacology (Berl.)*. 206 (2): 303-312.

12. Torres O.V., **Tejeda H.A.**, Natividad L.A., O'Dell L.E. (2008). Enhanced vulnerability to the rewarding effects of nicotine during the adolescent period of development. *Pharmacol Biochem Behav.* 90(4):658-663.
13. O'Dell L.E., Torres O.V., Natividad L.A. and **Tejeda H.A.** (2007). Adolescent nicotine exposure produces less affective measures of withdrawal relative to adult nicotine exposure in male rats. *Neurotoxicology and Teratology.* 29(1):17-22.

ORAL PRESENTATIONS

1. **Tejeda H.A.**, Shippenberg T.S., O'Donnell P. (October, 2013)
Prefrontal cortical kappa opioid receptors modulate mesocortical and limbic inputs to the prefrontal cortex. National Hispanic Science Network on Drug Abuse.
2. **Tejeda H.A.**, Counotte DS, Chefer V, Backman C, Shippenberg T, O'Donnell P. (April, 2013)
Kappa opioid receptor regulation of mesocortical and limbic inputs to the prefrontal cortex. *Kappa-opioid Therapeutics Conference.*
3. **Tejeda H.A.** (January, 2013)
Modulation of limbic and mesocortical prefrontal cortex afferents by kappa-opioid receptors. *Winter Conference on Brain Research.*
4. **Tejeda, H.A.** (July, 2011).
Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and affect. *University of Maryland, Baltimore Program in Neuroscience Annual Retreat.*
5. **Tejeda, H.A.** (September, 2008).
The role of kappa-opioid receptors in nicotine withdrawal. *National Academy of Science Ford Foundation Fellows Conference.*
6. **Tejeda, H.A.** (March, 2008).
Kappa opioid receptor modulation of nicotine withdrawal in adolescent and adult rats. *National Institute on Drug Abuse Seminar.*
7. **Tejeda, H.A.** & Chester J.A. (November, 2006).
Effects of alcohol drinking during adolescence on adult alcohol drinking behavior in a genetic animal model of alcoholism. *Career Opportunities in Research Meeting.*
8. **Tejeda, H.A.** & Chester J.A. (July, 2006).

Effects of adolescent alcohol exposure on alcohol drinking behavior in adulthood in a genetic animal model of alcoholism. *Annual MARC/AIM Conference at Purdue University*.

POSTER PRESENTATIONS

1. **Tejeda, H.A.**, O'Donnell P. Basolateral amygdala-evoked heterosynaptic suppression of hippocampal inputs to the prefrontal cortex. *Winter Conference on Brain Research, 2012*.
2. **Tejeda, H.A.**, Shippenberg T.S., O'Donnell P. Basolateral amygdala-evoked heterosynaptic suppression of hippocampal inputs to the prefrontal cortex. *American College of Neuropsychopharmacology, 2012*.
3. Counotte D.S., Cabungal J.H., Lewis E.M., Piantadosi P.T., Sullivan E.M., Calhoon G.G., **Tejeda H.A.**, Cuenod M., Do K.Q., O'Donnell P. Antioxidants as a treatment to prevent prefrontal cortical and behavioral deficits in a developmental model of schizophrenia. *American College of Neuropsychopharmacology, 2012*.
4. **Tejeda, H.A.**, Shippenberg T.S., O'Donnell P. Basolateral amygdala-evoked heterosynaptic suppression of hippocampal inputs to the prefrontal cortex. *Society for Neuroscience, 2012*.
5. **Tejeda, H.A.**, Shippenberg T.S., O'Donnell P. Basolateral amygdala inputs attenuate responses to other temporal cortical structures in the prefrontal cortex. *Society for Neuroscience, 2011*.
6. **Tejeda, H.A.**, Counette D.S., Schultz K.N., Oh E., Chefer V.I., O'Donnell P., Shippenberg T.S. Prefrontal cortical kappa-opioid regulation of local neurotransmission and affect. *Therapeutic Potential of Kappa Opioids in Pain and Addiction, 2011*.
7. Natividad, L. N., Torres, O. V., **Tejeda, H. A** and O'Dell, L. E. Adolescent nicotine exposure enhances the rewarding properties of nicotine in a graded manner during adulthood. *Society for Neuroscience, 2010*.
8. Beckert M. V., Loewinger G., **Tejeda H. A.**, Bernstein D. L., Cheer J. F. Endocannabinoid modulation of methamphetamine neurotoxicity. *Society for Neuroscience, 2010*.
9. **Tejeda, H.A.**, Schultz K.N., Chefer V.I., Shippenberg T.S. Regulation of dopamine and glutamate overflow in the prefrontal cortex by local mu- and kappa-opioid receptors. *Society for Neuroscience, 2010*.
10. Natividad, L.A., Roman F., Torres, O.V. **Tejeda, H.A.**, and O'Dell, L.E. Exposure to nicotine during adolescence alters intake of the drug later in adulthood. *National Hispanic Science Network on Drug Abuse, 2009*.
11. **Tejeda, H.A.**, Chefer V.I., Shippenberg T.S. Modulation of dopamine, GABA, and glutamate overflow in the prefrontal cortex by local kappa-opioid receptors. *Society for Neuroscience, 2009*.
12. **Tejeda, H.A.**, Chefer V.I., Shippenberg T.S. Regulation of dopamine, GABA, and glutamate overflow in the prefrontal cortex by local kappa-opioid receptors. *Gordon Research Conference on Catecholamines, 2009*.

13. Torres, O. V., Van Weelden S.A., Natividad, L. A., Beas, B.S., **Tejeda, H. A.**, & O'Dell, L. E. The rewarding effects of nicotine are enhanced in female adolescent rats relative to adults that display rewarding or aversive effects in a hormone-dependent manner. *Society for Neuroscience*, 2008.
14. Natividad, L. N., **Tejeda, H. A.**, Torres, O. V., and O'Dell, L. E. Robust developmental differences to the neurochemical effects of nicotine withdrawal are not observed following nicotine administration in adolescent versus adult rats. *Society for Neuroscience*, 2008.
15. **Tejeda, H.A.**, Natividad, L.N., Torres, O.V., Castaneda E., and O'Dell, L.E. The behavioral and neurochemical effects of kappa-opioid receptor stimulation are age-dependent. *Society for Neuroscience*, 2008.
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19. Byers, D.M. Natividad, L.A., **Tejeda, H.A.**, Torres, O.V., and O'Dell L.E. Developmental and sex differences in the expression of key molecular targets during nicotine withdrawal. *Society for Research on Nicotine and Tobacco*, 2008.
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21. Natividad, L. N., Torres, O. V., **Tejeda, H. A.** and O'Dell, L. E. Pre-exposure to nicotine during adolescence facilitates nicotine self-administration in adult rats given intermittent access to escalating nicotine doses. *Society for Neuroscience*, 2007.
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23. Torres, O. V., **Tejeda, H. A.**, Natividad, L. N., and O'Dell, L. E. The rewarding effects of nicotine are enhanced in female adolescent rats and in adult females in an estrous-dependent manner. *Society for Neuroscience*, 2007.
24. Natividad, L.A., Torres, O.V., **Tejeda, H.A.**, & O'Dell, L.E. Nicotine withdrawal produces a decrease in dopamine release in the nucleus accumbens of adult, but not adolescent rats. *Society for Neuroscience*, 2006.
25. Torres, O. V., **Tejeda, H. A.**, Natividad, L. A., & O'Dell, L. E. Enhanced nicotine reward and diminished nicotine withdrawal in adolescent versus adult rats. *Society for Neuroscience*, 2006.
26. Torres, O. V., **Tejeda, H. A.**, Natividad, L. A., & O'Dell, L. E. Reduced sensitivity to the aversive effects of nicotine withdrawal may contribute to tobacco use during adolescence. *National Hispanic Science Network on Drug Abuse*, 2006.

27. **Tejeda H.A.** & Chester J.A. Effects of adolescent alcohol exposure on alcohol drinking behavior in adulthood in a genetic animal model of alcoholism. *Committee on Institutional Cooperation Conference, 2006.*
28. Torres, O. V., Natividad, L. A., **Tejeda, H. A.**, & O'Dell, L. E. Diminished nicotine withdrawal in adolescent rats: implications of vulnerability to addiction. *Society for Neuroscience, 2005.*
29. O'Dell, L.E., Torres, O.V., Natividad, L.A. and **Tejeda, H.A.** The affective properties of nicotine withdrawal are diminished in adolescent versus adult rats. *College on Problems of Drug Dependence, 2005.*

Abstract

Title: Prefrontal kappa-opioid receptor regulation of local neurotransmission

Hugo Tejada, Doctor of Philosophy, 2013

Dissertation directed by: Dr. Patricio O'Donnell, Professor, Program in Neuroscience

Kappa-opioid receptors (KORs) are mediators of motivational processes, mood/emotion, and stress reactivity. KORs are enriched in brain regions that mediate such behaviors, including the medial prefrontal cortex (mPFC). The mPFC guides behavior through its connections with limbic brain regions such as the amygdala, hippocampus, and ventral tegmental area. A critical knowledge gap exists in our understanding of the role of KOR systems in modulating mPFC afferents. Thus, we designed a series of studies to understand the role of KORs in regulating dopamine (DA) and limbic glutamatergic afferents to the mPFC. First, we determined the role of KOR systems in regulating neurotransmission in the mesocortical DA pathway by utilizing a combination of neurochemical techniques in rats and in transgenic mice. We found that mPFC KOR activation and antagonism decreased and increased DA output, respectively. Genetic ablation of KOR in DA neurons abolished the inhibitory effect of mPFC KOR signaling on local DA output. These findings suggest that mPFC KORs negatively modulate the mesocortical DA pathway by directly acting on DA varicosities in the mPFC. Second, we examined the role of mPFC KOR systems in regulating mPFC extracellular glutamate and glutamatergic limbic inputs utilizing neurochemical and electrophysiological techniques in rats. We found that extracellular glutamate and glutamate-driven GABA levels were inhibited by mPFC KORs. mPFC KOR activation inhibited synaptic

transmission in the BLA to mPFC pathway in a manner not overcome by BLA burst stimulation. KORs do not inhibit the hippocampus to mPFC pathway. These findings show that mPFC KORs inhibit glutamatergic afferents in a pathway-specific manner. Lastly, we characterized heterosynaptic interactions between the BLA and hippocampus in the mPFC, and determined the role of KORs in these interactions. BLA train stimulation inhibits hippocampal inputs to the mPFC in an activity-dependent manner, while hippocampus stimulation is without effect on BLA-evoked responses. KOR antagonism does not modify BLA-evoked heterosynaptic suppression, suggesting that BLA-evoked heterosynaptic suppression is KOR-independent. This series of studies demonstrate that mPFC KORs regulate mesocortical DA and limbic pathways, but may not be involved in limbic interactions. These findings provide a biological framework whereby mPFC KOR signaling alters mPFC-dependent behaviors.

Prefrontal kappa-opioid receptor regulation of local neurotransmission

by
Hugo Alejandro Tejada

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

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Acknowledgements

First and foremost I would like thank God for the many blessings I have received throughout my life. May our scientific observations always give glory to the beauty that is existence, which is a manifestation of our almighty God.

I would not be where I am now were it not for my beautiful wife and all the love and support she provided throughout my career and the support she will provide as I pursue a career as a neuroscientist. I would also like to thank my son Caleb for all his love, smiles, and hugs!

I would like to thank my parents for instilling in me a strong work ethic and encouragement to seek a higher education. Thank you for all the support you both provided over the years. *Mama tu eres la persona mas querida del mundo. ¡Gracias a Dios por haberme bendecido con una madre con tanto amor por sus hijos!* I would like to extend my love to all my friends and extended family for all their support, old and new alike. I love you all!

I would like to thank my graduate co-mentors, Drs. Patricio O'Donnell (UMB now at Pfizer) and Toni S. Shippenberg (NIDA) for all the support through graduate school. Thank you for allowing me to develop into an independent scientist, by letting me learn from both success and mistakes. Special thanks to Dr. Toni Shippenberg for bringing me to UMB and NIDA. May her soul rest in peace. Much appreciation goes to my dissertation committee members Drs. Brad Alger, Antonello Bonci, Joe Cheer, and Greg Elmer for their guidance; especially Drs. Cheer, Alger, and Elmer for providing critical feedback after reading this document. I would also like to thank Dr. Laura E. O'Dell (UTEP) for getting me started in my research career and the support she still provides.

This work would not have been possible without the help of my lab colleagues, past and present. Good people that make work feel like play and make science fun and exciting. Special thanks to Drs. Vladmir Chefer, Danielle Counotte, Agustin Zapata, Carlos Mejias-Aponte, Adam Puche, Sammanda Ramamoorthy, Richard Henriksson, and Eastman Lewis for their much needed advice/help on my thesis studies. Thanks to all who provided technical assistance with collecting dialysis samples while I ran to class, histology and drug pipettes.

I would like to thank the patients at Spring Grove and Springfield Psychiatric Hospitals for keeping my feet grounded by reminding me that beyond the excitement of basic science lies the truth that mental illness can be treated through the compassionate efforts of family, friends, physicians, spiritual leaders, and scientists. They certainly are the major motivator to gain a further understanding of the neural pathology associated with psychiatric disorders. *Gracias a mi gente que sufre en gran parte por la injusticia social por darme el animo todos los días para ayudar al mundo como se pueda. ¡No nos moverán!*

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Chapter I: General Introduction

The Prefrontal Cortex: An Overview

Behavioral Function of the Prefrontal Cortex

The prefrontal cortex (PFC) consists of various neocortical structures that subserve a plethora of behavioral functions that include cognitive behavior, goal-directed behavior, motivation, stress/anxiety, and learning and memory (Goldman-Rakic, 1996; Clark et al., 2004; Schoenbaum et al., 2009; Euston et al., 2012). In primates, PFC consists of, but is not limited to, orbitofrontal (OFC), dorsolateral, and ventralmedial cortices; while in rodents PFC is largely composed of OFC and medial prefrontal cortex (mPFC). Although, the PFC, as a collective unit, plays a role in top-down control of behavior, different PFC sub-regions play different roles in the execution of behavior. For example, both OFC and mPFC play a role in behavioral flexibility, which is the ability to adapt behavior in the face of changing contingencies to enhance survival or beneficial outcomes. However, OFC function is critical for good performance on reversal learning tasks where reward- or aversive-predictive cues lose their salience and a previously non-predictive cue acquires this predictive property. On the other hand, mPFC function is critical for set-shifting behavior where an organism must learn to switch between rules of different dimensions (i.e. visual vs spatial) to guide behavior when rules or dimensional contingencies change. Conversely, the OFC does not play a dominant role in set-shifting and the mPFC is not critical for reversal learning. Thus, the PFC consists of various,

highly developed structures that each control slightly different facets to cognition and motivation, but as a whole have executive control over behavior.

The PFC is not an island in the sea of neuronal structures. The PFC mediates its behavioral function through interconnections with other neocortical and subcortical structures, each of which is involved in different aspects of sensation and perception (i.e. vision or olfaction), memory (i.e. short-term memory), salience attribution, and emotion/mood. Through these interconnections with various neural structures the PFC is capable of formulating a representation of an organism's internal states, its surrounding environment, and experiences, to direct attention or motivation to appropriate stimuli or thoughts. Moreover, the PFC through its direct connections with pre-motor and motor cortices, as well as parallel basal ganglia loops, is able to guide the behavioral output of the organism. PFC function is ultimately governed by its ability to synthesize diverse information arriving from most of the central nervous system and act on that information through effector motor systems, such as the basal ganglia.

Prefrontal Cortical Microcircuits

Like neurons in other neocortical regions, PFC neurons are organized into layers. These layers are numbered I-VI, with layer I being closest to the pial surface (or the midline for mPFC) and layer VI to the external capsule (white matter). The PFC primarily consists of output pyramidal cells, which are located throughout all layers except layer I, but are primarily localized in to two bands in layers II/III and IV/V. Pyramidal cells are morphologically identified by their pyramid-shaped somata, short

basal dendrites, and long apical dendrites that extend to superficial layers, although differences in morphology and size exist between pyramidal cells in different layers. Moreover, dendrites of pyramidal neurons are covered with spines. The functional role of individual spines is not fully understood, but it is now appreciated that individual spines contribute to input-specific encoding of information transfer and synaptic (Yuste, 2011; Chen and Sabatini, 2012). Pyramidal cells are excitatory neurons that synthesize and release glutamate to influence the activity of their downstream targets. Differences exist in the projection of PFC pyramidal neurons of different layers. Pyramidal neurons of layers II/III preferentially project to other neocortical regions, while layer V and layer VI pyramidal neurons form corticostriatal and corticthalamic projections, respectively.

The PFC also consists of a plethora of local circuit interneuron subtypes, which collectively make up about 10-30% of the total neuronal population. The majority of interneurons utilize GABA as their primary neurotransmitter. Different GABA interneurons have been described, based on their morphology, electrophysiological properties, neurochemical contents, regulation by neuromodulators, and the way they innervate pyramidal cells (Markram et al., 2004; Armstrong and Soltesz, 2012; Bartos and Elgueta, 2012; Defelipe et al., 2013). Morphologies of GABA interneurons include basket, chandelier, double bouquet, neurogliaform, and heterogeneous multipolar cells found in layer I. The most predominant morphology of GABA interneurons is the basket cell, which innervates pyramidal neuron somata. Calcium-binding proteins found in GABA interneurons include parvalbumin (PV), calbindin (CB), and calretinin, while interneurons can also be distinguished based on neuropeptide expression including vasoactive intestinal peptide, somatostatin, neuropeptide Y, and cholecystokinin.

Although the majority of GABA interneurons display “fast-spiking” characteristics in response to depolarizing current steps, further breakdown of electrophysiological responses to steady state depolarization include non-accommodating, accommodating, stuttering, irregular spiking, regular spiking, and bursting. It should be noted that there is not a mutually exclusive combination of morphology, neuropeptide/calcium-binding protein, or electrophysiological properties, although some patterns have been observed. For instance, basket cells and chandelier cells tend to express PV or CB. Also PV cells express mu-opioid receptors (MORs), while CCK cells express cannabinoid 1 receptors (CB1). Taken together, GABA interneurons are heterogenous and contribute to the diverse role of inhibition within cortical networks.

Interactions between pyramidal neurons and GABAergic interneurons play an important role in cortical networks. Cortical oscillations play a role in integrating inputs, helping select neuronal ensembles, and synchronizing the output of pyramidal cells. For instance, the hippocampus has a strong oscillation in the theta range (5-10 Hz) and entrains the mPFC local field potentials (LFPs) and single unit activity to hippocampal theta oscillations (Sirota et al., 2008; Young and McNaughton, 2009; Adhikari et al., 2010, 2011), but hippocampal theta also engages gamma oscillations in the mPFC (Sirota et al., 2008). PV interneurons are necessary for pyramidal neuron activation to drive fast gamma oscillations in the LFP (Sohal et al., 2009), and their activation is sufficient to drive these oscillations (Cardin et al., 2009). Thus, interactions between GABA interneurons and pyramidal neurons play an important role in generating local oscillations in the LFP that are crucial for synchronizing activity across structures and

contribute to formation of synchronized pyramidal neuronal ensembles that drive behavior and cognition.

Ventral Tegmental Area Afferents

Ascending neuromodulator systems, such as monoamine transmitters, also innervate the PFC where they play a major role in modulating neuronal excitability and shape excitatory and inhibitory synaptic transmission. One such ascending system is the mesocortical dopamine (DA) system, which consists of DA-containing neurons originating in the ventral tegmental area (VTA) of the midbrain (Van Edén et al., 1987; Sesack and Carr, 2002). Midbrain DA neurons display two modes of neuronal discharge, tonic pacemaker firing and phasic firing (Grace and Bunney, 1984a, b). Although it is not understood what tonic DA neuron activity confers behaviorally, it is now appreciated that phasic midbrain DA neuron discharge encodes various behaviors and processes including sensorimotor function, effort/cost-based behavior, reward-seeking, stress, cognition, reward-prediction error, behavioral flexibility, and risk-based behavior (Schultz et al., 1997; Jentsch et al., 2000; Carelli, 2004; Wise, 2004; Floresco and Magyar, 2006; Grace et al., 2007; Ungless et al., 2010; Salamone and Correa, 2012). Research on the role of DA in the PFC has been heavily focused on working memory. It is now appreciated that DA levels must be optimal for normal working memory performance. Indeed, the effects of PFC DA on working memory follow an inverted-U dose response curve, with both low and high levels of DA receptor stimulation resulting in working memory deficits in animal models (Sawaguchi and Goldman-Rakic, 1994; Murphy et al., 1996b; Murphy et

al., 1996a; Zahrt et al., 1997; Arnsten and Goldman-Rakic, 1998; Seamans et al., 1998; Romanides et al., 1999; Arnsten et al., 2000; Mizoguchi et al., 2000). Thus, DA signaling plays an important role in PFC-dependent processes by modulating the activity of cortical networks.

DA released in the PFC is anatomically poised to modulate network activity by acting on cortical neuronal population via distinct DA receptors. DA released by VTA varicosities in the PFC is thought to act via volume transmission as little DA transporter (DAT) levels, the substrate responsible for reuptake of DA from the extracellular space, are present in the PFC (Sesack et al., 1998b), unlike other DA rich brain regions like the striatum. Unlike DAT-enriched regions, termination of extracellular DA signaling in the PFC is primarily mediated by the enzyme catechol-*O*-methyl-transferase or the nor-epinephrine transporter (Carboni and Silvagni, 2004; Tunbridge et al., 2006). The PFC is enriched in DA D1-like, and to a lesser extent DA D2-like, receptors in rodents and primates (Goldman-Rakic et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991; Smiley et al., 1994). Expression of D1- and D2-like receptors has been localized to PFC pyramidal neurons (Smiley et al., 1994; Bergson et al., 1995). Both D1 and D2 receptors have also been localized to GABA interneurons (Bergson et al., 1995; Le Moine and Gaspar, 1998), including PV interneurons (Le Moine and Gaspar, 1998; Muly et al., 1998), which have been confirmed to receive DA synapses (Sesack et al., 1998a). Additionally, D1-like (both D1 and D5) DA receptors are also localized to presynaptic excitatory terminals (Bergson et al., 1995; Paspalas and Goldman-Rakic, 2005). Thus, DA receptors are strategically situated to modulate PFC outputs by directly acting on pyramidal neurons and GABA interneurons via volume transmission.

The effects of PFC DA signaling on neuronal activity and synaptic function in PFC are complex. The predominant response produced by VTA stimulation or intra-mPFC DA agonists is to decrease spontaneous neuronal activity in the majority of pyramidal neurons *in vivo* (Sesack and Bunney, 1989; Yang and Mogenson, 1990; Godbout et al., 1991; Pirot et al., 1992). This effect may be due in part by recruitment of PFC GABAergic interneurons by the mesocortical DA system. VTA chemical stimulation has been shown to increase the excitability of PFC interneurons *in vivo* (Tseng et al., 2006) and the inhibitory effects of VTA stimulation and intra-mPFC DA on pyramidal neuron activity is mediated by GABA-A and D2-like receptors (Sesack and Bunney, 1989; Godbout et al., 1991; Pirot et al., 1992). This is in agreement with the finding that quinpirole, a D2 agonist, increases excitability of FSI interneurons and recruits a GABA-A mediated inhibition of cortically-evoked EPSPs and decreases pyramidal neuron excitability (Tseng and O'Donnell, 2004, 2007). *In vivo* intracellular recordings have demonstrated that electrical or chemical VTA stimulation elicits silent UP states with decreased action potential firing in PFC pyramidal neurons (Lewis and O'Donnell, 2000), and the elicited UP states are attenuated by a D1-like DA receptor antagonist (Lewis and O'Donnell, 2000; Onn and Wang, 2005). Moreover, co-applied D1-like agonist and NMDA elicit UP states *in vitro* (Tseng and O'Donnell, 2005). Moreover, D1 agonists enhanced NMDA receptor function (Tseng and O'Donnell, 2004). The end result of DA transmission on the PFC microcircuit would be an enhancement in signal to noise ratio, via recruitment of interneurons and D1-like receptor sharpening the excitatory inputs to pyramidal neurons that are able to overcome the inhibition.

The mesocortical system also consists of VTA GABA neurons that innervate both PFC pyramidal neurons and interneurons (Carr and Sesack, 2000). In addition to the prototypical VTA DA and GABA neurons, it is now appreciated that subsets of mesocortical neurons utilize glutamate as a neurotransmitter, based on their expression of the vesicular glutamate transporter 2 (VGLut2), which is a marker of glutamate-releasing neurons (Hur and Zaborszky, 2005; Kawano et al., 2006; Nair-Roberts et al., 2008; Yamaguchi et al., 2011; Li et al., 2012). These neurons can be further subdivided into neurons that express VGLut2 alone or VGLut2 and tyrosine hydroxylase (TH), the rate limiting enzyme necessary for the synthesis of DA. Electrophysiological evidence for glutamatergic signaling by VTA neurons was previously obtained as VTA electrical stimulation or intra-VTA glutamate puff evoked intracellular and LFP EPSPs in the mPFC, even in the presence of DA blockade (Lavin et al., 2005). Thus, the mesocortical system consists of heterogeneous neurons which likely encode different aspects of behavior and perception. The release of DA, GABA, and glutamate from the VTA likely shapes pyramidal neuron output in a complex manner to guide behavior through basal ganglia networks and connections with pre-motor and motor cortices.

Basolateral Amygdala Afferents

A major focus of the work in this thesis involves the pathway originating in the basolateral amygdala (BLA) which sends projections to PFC areas, including the mPFC. The BLA plays a role in processing emotional information, attention, decision-making, motivation, tagging valence or salience to rewarding and aversive events/stimuli, as well

as acquisition, consolidation, extinction, and reinstatement of learned associations (Pelletier and Pare, 2004; Balleine and Killcross, 2006; Barad et al., 2006; Floresco et al., 2008; Roesch et al., 2012). BLA projection neurons synthesize glutamate as their neurotransmitter. BLA axons predominantly form asymmetric synapses (i.e. excitatory synapses) onto mPFC dendritic spines of pyramidal neurons (Bacon et al., 1996; Gabbott et al., 2006; Cunningham et al., 2008), and to a lesser extent onto GABAergic inhibitory interneurons (Gabbott et al., 2006; Cunningham et al., 2008). While this type of anatomical arrangement suggests that activity in the BLA to mPFC pathway should result in excitation, *in vivo* electrophysiological studies have demonstrated that BLA stimulation results in brief inhibition of mPFC in the majority of pyramidal cells (approx. 80%), while only a small subset of neurons (approx. 10%) are excited (Perez-Jaranay and Vives, 1991; Ishikawa and Nakamura, 2003a; Floresco and Tse, 2007). The discrepancy between anatomical and electrophysiological studies may be explained by feedforward inhibition in the mPFC by the BLA via interneuron recruitment. In a study, which I first co-authored, we demonstrated that FSIs in the mPFC were activated by BLA stimulation and synaptic responses pyramidal neurons consisted components mediated by GABA-A receptors (Dilgen et al., 2013). This suggests the BLA can have an inhibitory influence on mPFC function via disynaptic feedforward inhibition.

Endogenous and exogenous DA modulates the BLA to mPFC pathway in a complex manner. VTA stimulation and iontophoretically-applied DA decreased BLA-evoked inhibition in putative PFC pyramidal neurons (Floresco and Tse, 2007). D2/D4 DA receptor agonists generally decreased BLA-evoked inhibition in most neurons, but in some only unmasked a short-latency monosynaptic excitatory response (< 50 ms) without

altering the longer-lasting inhibition (approx. 200 ms). The D1 receptor agonist SKF-81297 was without effect on BLA-evoked inhibition. Moreover, VTA stimulation and iontophoretically-applied DA decreased single pulse BLA-evoked monosynaptic, orthodromic spikes, and this effect was overcome by the 3rd, 4th, and 5th pulses in a 5 pulse, 20 Hz BLA train. D1, but not D2, DA receptor agonists inhibited BLA-evoked excitation of PFC neurons. Thus, DA has complex effects on the BLA to PFC pathway, with D2/D4 and D1 receptor activation decreases BLA-evoked inhibition and excitation, respectively.

Hippocampal Afferents

The hippocampal formation sends glutamatergic afferents to PFC areas. Hippocampal afferents to PFC arise from glutamatergic neurons in the ventral hippocampal formation, including the CA1 and ventral subiculum (Jay and Witter, 1991; Carmichael and Price, 1995; Hoover and Vertes, 2007; Roberts et al., 2007; Burman et al., 2011). These inputs form asymmetrical synapses that produce excitatory responses onto pyramidal neurons via AMPA and NMDA receptors (Carmichael and Price, 1995; Carr and Sesack, 1996). *In vivo* intracellular recordings in pyramidal neurons have demonstrated that electrical stimulation of hippocampal inputs to the mPFC elicits complex synaptic responses consisting of multiple depolarizing and hyperpolarizing components (Degenetais et al., 2003). Moreover, hippocampal electrical stimulation activates mPFC interneurons (Tierney et al., 2008). Hippocampal theta rhythms drive theta oscillations in the mPFC and mPFC neurons phase lock their activity to these

oscillations (Sirota et al., 2008; Young and McNaughton, 2009; Adhikari et al., 2010, 2011), suggesting that synchronized theta activity can help transfer of information between these two structures. Importantly, synchronized theta between hippocampus and PFC is dynamic during cognitive tasks and stress in rodents. This is of importance as dysfunction in the hippocampal-PFC pathway has been implicated in psychiatric disorders such as schizophrenia (Colgin, 2011; Lisman, 2012; O'Donnell, 2012).

The ability of hippocampal inputs to modulate PFC activity is regulated by DA afferents in a behaviorally relevant manner. Concomitant DA transmission and hippocampal-mPFC communication is necessary for optimal performance during a working memory task (Seamans et al., 1998). Although, the majority of hippocampal terminals do not form synapses in close apposition to DA terminals, some instances do occur (Carr and Sesack, 1996), providing a framework whereby volume DA transmission can modulate hippocampal afferents. DAergic signaling inhibits hippocampal-evoked spiking of PFC neurons (Jay et al., 1995; Floresco and Grace, 2003; Tierney et al., 2008). Plasticity in the hippocampus-PFC pathway requires intact DAergic signaling in the PFC (Gurden et al., 1999; Gurden et al., 2000; Jay et al., 2004). Intra-PFC DA microinjections increase coherence in theta oscillations between hippocampus and PFC and shift phase locking of PFC neuronal spiking to theta (Benchenane et al., 2010). This provides a mechanism by which DA's effects on excitation/inhibition and plasticity can contribute to synchronized PFC ensemble activity and hippocampal theta activity. Thus, behavioral events engaging both hippocampal PFC afferents and VTA DA neurons will help encode information processing between neural ensembles that beat to the same theta rhythm.

The Dynorphin/Kappa-Opioid Receptor System: An Overview

The dynorphin (DYN)/kappa-opioid receptor (KOR) system consists of the opioid peptides, the DYNs, and their cognate receptor, the KOR (Chavkin et al., 1982). DYNs were discovered by Avram Goldstein (1919-2012). The name DYN comes from the Greek word dynamis (meaning powerful) referring to its potent effects on the ileum longitudinal muscle that was routinely used to study neuromodulators and orphin relating it to its opioid structure (Goldstein et al., 1979). The KOR was named after the first letter (in Greek) for the first ligand that was identified as selective this receptor, ketocyclazocine. However, at the time it was not clear what the endogenous ligand/s for KOR at the time. It was not until 1982, when two seminal papers provided evidence that DYNs were endogenous ligands of the KOR (Chavkin et al., 1982; Yoshimura et al., 1982).

Dynorphins

DYN peptides consist of the basic the basic enkephalin peptide (Tyr-Gly-Gly-Phe) with various additional amino acid residues depending on the kind of DYN peptide. DYN peptides are derived from the polypeptide precursor prodynorphin (also known as proenkephalin B), which can be processed to yield various opioid peptides including the dynorphins, leu-enkephalin, and α - and β -neoendorphin (Kakidani et al., 1982). The processing of prodynorphin to DYN peptides can occur in axon terminals and dendrites, as well as somatic golgi apparatus (Yakovleva et al., 2006), suggesting that DYN

peptides can be synthesized locally and on-demand. Although dynorphin A (1-17) is considered the endogenous ligand for the KOR (Chavkin et al., 1982), various biologically active DYNs have also been identified including DYN A (1-8), DYN A (1-13), DYN B (1-13; rimorphin), DYN B (1-29; leumorphin), and DYN A/B (1-32; big DYN) (Goldstein et al., 1979; Minamino et al., 1980; Fischli et al., 1982; Kilpatrick et al., 1982). The number consists of the number of amino acid residues in the peptide, including the basic enkephalin building block. The affinity and potency of these peptides at the KOR differ with DYN A being the most potent and β -neoendorphin the least (Naqvi et al., 1998; Chen et al., 2007). Although DYNs are capable of binding other opioid receptors (μ - and δ -opioid receptors), it is much more selective for KOR than for the other opioid receptors (Garzon et al., 1983; Mansour et al., 1995). Conversely, the affinity of non-DYN opioid peptides for the KOR is orders of magnitude lower than for μ - and δ -opioid receptors (Li et al., 1993; Meng et al., 1993). Although DYN peptides do not solely have an affinity for KOR, their specificity can also be dictated by their proximal localization to KOR relative to μ - and δ -opioid receptors (MORs and DORs, respectively). It should be noted that DYN peptides can also exert non-opioid actions at the NMDA receptor (Shukla and Lemaire, 1994). DYN peptides decrease NMDA receptor function independent of the KOR by decreasing channel open time (Chen et al., 1995b, a; Brauneis et al., 1996). However, potentiation of NMDA receptor function by DYNs has also been reported (Lai et al., 1998). It is not clear whether endogenous DYNs act on NMDA receptors *in vivo*. Thus, DYNs consists of various opioid peptides that predominantly target the KOR.

Kappa-Opioid Receptors

The KOR is a seven trans-membrane domain G-protein coupled receptor (Tejeda et al., 2012a), which shares a high homology to the MOR and DOR. KOR primarily couples to inhibitory Gi/o G-proteins, resulting in activation of G-protein-coupled inwardly-rectifying potassium (GIRK) channels or decreasing presynaptic voltage-gated Ca²⁺ channel function (Bruchas and Chavkin, 2010; Tejeda et al., 2012a). The former and the latter result in decreased membrane excitability and decreased probability of neurotransmitter release, respectively. In addition to coupling to canonical G-protein signaling mechanisms, KOR activation also initiates intracellular signaling cascades including ERK/MAP kinases (Bruchas and Chavkin, 2010). Thus, the primary role of KOR is to decrease neuronal output of the cell in which it is expressed by decreasing excitability and/or decreasing neurotransmitter release.

Functional Dynorphin/Kappa-Opioid Receptor Neuroanatomy

DYN and KOR expression is widespread throughout the central nervous system. DYN and KOR expression is observed in the cerebral cortex, basal ganglia, hippocampus, amygdala, thalamus, as well as monoaminergic centers in the midbrain and brainstem. The following sections will provide an overview of the anatomical studies of the DYN/KOR system in brain regions that are essential for proper PFC function and downstream targets of the PFC.

Dynorphin/Kappa-Opioids in the Neocortex

Relative to the DYN/KOR system in other structures, such as the striatum (see below), little is known about the DYN/KOR system in the cerebral cortex. *In situ* hybridization and immunocytochemical studies have demonstrated that sparse layer II/III and layer V cortical pyramidal and nonpyramidal cells (based on cell morphology) express PDYN and DYN peptides in rodents and non-human primates, while DYN fibers have been consistently reported throughout all layers, except layer I (Khachaturian et al., 1982; Vincent et al., 1982; Weber et al., 1982; Weber and Barchas, 1983; Khachaturian et al., 1985; Fallon and Leslie, 1986). Studies examining tissue content of DYN-immunoreactivity in frontal cortex or neocortex have also found significant levels, albeit lower than areas such as the NAcc and hypothalamus in rodents and man (Gramsch et al., 1982; Cone et al., 1983; Zamir et al., 1983, 1984a; Zamir et al., 1984b; Ji et al., 2010; Goldstein and Volkow, 2011). KOR binding is present in the mPFC and to a larger extent the OFC in rodents, while surrounding frontal cortical areas display less KOR binding (Morris and Herz, 1986; Van't Veer et al., 2013). KOR binding in frontal cortical regions mirrors that of DYN-fiber localization, with laminar distributions in layers II/III and deeper layer (V/VI). Binding of tritiated KOR ligands has demonstrated that in man, KOR binding in the cortex is among the highest in the brain with similar laminar distributions as those observed in animal models but with more localized to deep layers (Pilapil et al., 1987; Quirion et al., 1987). PDYN mRNA expression and DYN-immunoreactivity has also been reported in tissue from the frontal cortex of man (Bazov et al., 2013).

Very little is known about the functional role the DYN/KOR system plays in the mPFC. One electron microscopy study demonstrated that the majority of KOR-immunoreactivity is restricted to presynaptic elements (Svingos and Colago, 2002). These include axonal varicosities, which may contain neuromodulators such as DA, and terminals of symmetric and asymmetric synapses, which are indicative of inhibitory and excitatory synapses, respectively. Consistent with these anatomical findings that KORs play a role in regulating presynaptic neurotransmitter release, *in vitro* studies have demonstrated that KOR agonists decrease [3H] DA, [3H] 5-HT, GABA, and glutamate release from frontal cortical mouse and rat slices or synaptosomal preparations (Heijna et al., 1990; Sbrenna et al., 1999; Grilli et al., 2009).

Since little is known about the role of DYN/KOR systems in the frontal cortex, one can begin to gain insight about what role these systems play in information processing by examining what is known about DYN/KORs in other neural structures, especially those that send afferents to the PFC, and which receive projections from the PFC. The following sections briefly summarize functional neuroanatomical findings in such structures.

Dorsal and Ventral Striatal Kappa-Opioid Receptors

KOR function and localization has been well studied in the dorsal and ventral striatum, which consists of the NAcc and olfactory tubercles. This may be because the dorsal and ventral striatum are some of the brain regions with the highest levels of

expression of DYN and KOR in the brain (Fallon and Leslie, 1986; Meng et al., 1993) and the role of the ventral and dorsal striatum in motivation/affect and action selection/habit formation, respectively. In both the ventral and dorsal striatum DYN is expressed in GABAergic medium-sized spiny neurons (MSNs) that preferentially express the DA D1 receptor (Steiner and Gerfen, 1998), which suggests that the primary source in this structure may arise from local D1-expressing MSNs. Ultrastructural studies have shown that, DYN-immunoreactive axon terminals appose KOR immunoreactive terminals, DYN-containing KOR-immunoreactive terminals, and to a lesser extent soma/dendrites (Svingos et al., 1999). Previous work has shown that processing of PDYN to DYN can occur locally at axons and dendrites in the striatum and this processing and release occurs on-demand in response to depolarization (Yakovleva et al., 2006). Thus, DYN/KOR systems are found in the striatum and anatomically poised to regulate neurotransmission therein.

KORs negatively regulate DA transmission in the striatum. Electron microscopy studies have shown that striatal KORs are localized on DA varicosities in close apposition to the dopamine transporter (DAT), suggesting that KORs may strategically positioned to inhibit DA transmission by decreasing release (Svingos et al., 2001). In agreement with an inhibitory role of presynaptic KORs on DA terminals in the striatum are findings demonstrating that systemic or local administration of KOR agonists into either striatal sub-region decreases basal and stimulated DA efflux *in vivo* (Spanagel et al., 1992; Devine et al., 1993; Maisonneuve et al., 1994; Chefer et al., 2005; Zhang et al., 2005; Ebner et al., 2010). Similarly, KOR agonists decrease K⁺- or electrically-evoked

DA release in striatal and NAcc slices, suggesting that effects observed *in vivo* are not due to a circuit effect of KOR activation, but rather, via direct action of KOR in the NAcc (Mulder et al., 1984, 1989; Heijna et al., 1992b; Heijna et al., 1992a; Schoffelmeer et al., 1992). Furthermore, striatal and NAcc KORs tonically inhibit basal dopamine overflow *in vivo* such that intra-striatal or intra-accumbal KOR blockade elevates DA overflow (Spanagel et al., 1992; You et al., 1999). Thus, KORs negatively regulate striatal DA transmission, and this system is tonically active.

KOR immunoreactivity is also present on terminals of asymmetric synapses, which are presumably excitatory synapses (Svingos et al., 1999; Meshul and McGinty, 2000). Consistent with the localization of NAcc KORs on asymmetric synapses, *in vitro* electrophysiological studies have shown that exogenous and endogenous KOR agonists inhibit presynaptic glutamate release onto MSNs (Hjelmstad and Fields, 2001, 2003; Mu et al., 2011). Moreover, *in vitro* and *in vivo* neurochemical studies have demonstrated that KOR activation decreases evoked, but not basal, glutamate overflow (Rawls and McGinty, 1998; Gray et al., 1999; Hill and Brotchie, 1999; Rawls et al., 1999). It should be noted that it is currently not clear which glutamatergic afferents into the dorsal and ventral striatum express KOR.

Although the majority of KOR immunoreactivity in the NAcc is limited to presynaptic sites, a small proportion of KOR immunoreactivity is also observed in somata and proximal dendrites of MSNs (Svingos et al., 1999). Immunoreactivity in somata is typically associated with cytosolic organelle membranes such as the endoplasmic reticulum and Golgi apparatus, while dendritic KORs are associated with the plasma membrane. It should be noted that DYN-containing terminals do not tend to

appose KOR-positive somata and dendrites, making it unclear whether these receptors receive DYN tone via more extended volume transmission or are being trafficked to DYN-targeted sites.

It is hypothesized that the DYN/KOR receptor system in the dorsal and ventral striatum acts as a negative feedback system in response to environmental or pharmacological stimuli that strongly activate ascending DA pathways and/or strong glutamatergic drive of the system (Nestler and Carlezon, 2006; Shippenberg et al., 2007). As mentioned above, both the dorsal and ventral striatum contain DYN-expressing MSNs, and this expression is limited to D1-expressing MSNs. DA D1 and NMDA receptors have been shown to positively regulate prodynorphin synthesis, DYN content, and DYN release in both striatal compartments, and to mediate psychostimulant-induced increases in PDYN expression (Smiley et al., 1990; Johnson et al., 1991a, b; Singh et al., 1991; You et al., 1994; Hanson et al., 1995; Drago et al., 1996; Wang and McGinty, 1999; Zhang et al., 2004; Isola et al., 2009). For instance, intra-striatal perfusion of the D1 receptor agonist SKF38393 produces a concentration-dependent increase in DYN B dialysate levels, which correlated with a decrease in DA levels (You et al., 1994), presumably due to DYN's action on KORs regulating DA overflow. An ultra-structural study found that prodynorphin and DYN are in close proximity to D1 receptors in dendrites and axons (Hara et al., 2006). Moreover, D1 DA receptors, which have extrasynaptic localizations, have a high degree of co-localization with NMDA receptors, suggesting that extrasynaptic glutamate overflow may be critical for D1/NMDA receptor interactions (Hara and Pickel, 2005). This provides an anatomical framework whereby DYNs are released from terminals and possibly from dendritic sites in response to strong

D1 and/or NMDA receptor activation. Collectively, these studies suggests that in response to behavioral stimuli that concomitantly activate ascending DA systems and strong glutamatergic inputs to D1-expressing MSNs, DYNs will be released, act on presynaptic KORs on DA and glutamate terminals, and depress signaling from these inputs.

Ventral Tegmental Area/Substantia Nigra Kappa-Opioid Receptors

KOR expression is also observed in both the VTA and SN (Meng et al., 1993). KOR activation decreases the firing rate of VTA DA neurons, presumably by hyperpolarizing neurons via activation of GIRK channels (Margolis et al., 2003; Ford et al., 2007). These results suggest that in the VTA KORs may have direct post-synaptic effects on DA neurons. Mesocortical and mesoamygdalar, but not mesolimbic, DA neurons are directly hyperpolarized by U69,593 (Margolis et al., 2006; Margolis et al., 2008). In agreement with these findings, *in vivo* microdialysis studies demonstrated that intra-VTA administration of KOR agonists does not decrease basal NAcc DA levels (Spanagel et al., 1992; Devine et al., 1993; Margolis et al., 2006), but decreases mPFC DA efflux (Margolis et al., 2006). Collectively, these studies provide evidence that whereas functional KOR expression is not present in mesoaccumbal DA soma, it is present in mesocortical DA cell bodies. At odds with these observations, U69,593 directly hyperpolarized mesolimbic and mesoamygdalar DA neurons in mice (Ford et al., 2006). Factors that may account for the discrepancies include, but are not limited to, differences in species used (rat vs. mouse). Unlike the mesoaccumbens DA pathway, the

nigrostriatal DA pathway appears to be regulated by KORs at the levels of the cell bodies, as administration of nor-BNI into the SNc via reverse dialysis increases local and dorsal striatal DA overflow. This suggests that KORs in the SNc tonically inhibit DA neuron activity *in vivo*. Moreover, KORs in the midbrain are capable of inhibiting somatodendritic DA release (Ford et al., 2007). DYN and U69,593 inhibit electrically-evoked, D2-mediated IPSCs in DA neurons in both the VTA and SNc, but are without effect on D2-mediated IPSCs evoked by iontophoretically applied DA. In addition to direct action of KORs on DA neurons, KOR activation decreases VTA glutamate release (Margolis et al., 2005). Inhibition of glutamate release by presynaptic KORs is more robust onto cells directly hyperpolarized by the mu-opioid receptor (MOR) agonist, DAMGO, (putative GABAergic neuron), suggesting that endogenous DYN differentially regulates presynaptic glutamate release onto midbrain DA and GABAergic neurons. Thus, KORs play a critical role in VTA DA neuron activity by directly inhibiting their excitability, DA autocrine function, and glutamatergic drive.

Dynorphin/Kappa-Opioid Receptor Systems in Amygdaloid Structures

DYN is expressed in GABAergic MSNs localized to lateral portions of the central nucleus of the amygdala (CeA). DYN-positive neurons are segregated from enkephalin-positive GABAergic neurons, which are localized to medial portions of the CeA (Fallon and Leslie, 1986; Ma et al., 2003). This arrangement is consistent with the notion that the CeA is a striatal-like structure (i.e. GABAergic medium-sized spiny projection neurons that express either dynorphins or enkephalins). Moreover, subsets of CeA neurons that

are hyperpolarized by met-enkephalin are not by U69,593, and vice versa, suggesting that these two opioid systems may also segregate in the CeA terms of their postsynaptic action (Zhu and Pan, 2004). DYN fibers from the CeA innervate the VTA (Fallon et al., 1985; Code and Fallon, 1986). There is also evidence that CeA DYN neurons innervate the locus coeruleus (LC). Electrolytic CeA lesions decrease DYN immunoreactivity in the LC suggesting that DYN-positive CeA neurons innervate the LC (Reyes et al., 2008). A moderate proportion of CeA DYN-containing neurons (approx. 30–40%) co-express corticotropin-releasing factor (CRF) but do not co-express enkephalin (Marchant et al., 2007; Reyes et al., 2008). Findings from ultrastructural experiments mirror the latter observations demonstrating that DYN immunoreactivity is co-localized in LC CRF-positive axon terminals (Reyes et al., 2008). This raises the possibility that some CeA neurons co-release CRF and DYN in monoaminergic nuclei such as the LC and VTA.

KOR expression is present in the BLA of animal models and man (Meng et al., 1993; DePaoli et al., 1994; Paxinos and Franklin, 2001; Van't Veer et al., 2013). The functional role of KOR in the BLA is not understood. However, U50,488, a synthetic KOR agonist, decreased evoked-LFP amplitude and blocked LTP induction in a nor-BNI sensitive manner in slices (Huge et al., 2009b), suggesting that KORs in the BLA play an inhibitory role in synaptic transmission within the BLA.

Dynorphin/Kappa-Opioid Receptor Systems in the Hippocampus

DYN immunoreactivity in the hippocampus is densest in dentate gyrus neurons and the mossy fiber pathway from the dentate gyrus to CA3 with little or no expression in other

hippocampal regions (McGinty et al., 1983; Chavkin et al., 1985). KOR activation by endogenous or exogenous ligands decreases mossy fiber pathway synaptic transmission in guinea pigs, Long–Evans rats, DBA/2 and C57BL/6 mice, and hamster (Wagner et al., 1992; Wagner et al., 1993; Weisskopf et al., 1993; Salin et al., 1995; Castillo et al., 1996), an effect that is absent in Sprague–Dawley rats. This suggests the existence of strain differences in hippocampal DYN/KOR systems in rats. Nor-BNI bath application promotes induction of LTP of the mossy fiber pathway when sub-threshold tetanic stimulation protocols are utilized (Weisskopf et al., 1993). DYN inhibition of mossy fiber transmission is enhanced after tetanic stimulation of mossy fibers and this enhancement of inhibition is specific to tetanized, but not non-tetanized, mossy fiber pathway synapses in the same preparation (Weisskopf et al., 1993). Moreover, DYN peptides are recruited to produce heterosynaptic inhibition in the mossy fiber pathway. Thus, DYNs released in response to strong stimuli may decrease function of mossy fiber pathway synapses that have low levels of activity, suggesting hippocampal DYN/KOR modulation of synaptic transmission appears to be synapse-selective and highly dependent on activity. DYN released from dendrites and/or local collaterals of dentate gyrus granule cells may act in a retrograde fashion and activate KORs on perforant path terminals arising from the entorhinal cortex into the dentate gyrus (Wagner et al., 1992; Yizhar et al., 2011). Retrograde signaling by DYN has been reported in the hypothalamus (Iremonger and Bains, 2009; Iremonger et al., 2011), suggesting that retrograde signaling may extend to other DYN-rich regions. Collectively, these studies provide evidence that DYNs are recruited by sustained mossy fiber or perforant path activity under normal physiological conditions to control information relayed to the hippocampus from the entorhinal cortex.

Behavioral Control by the Dynorphin/Kappa-Opioid Receptor System

Psychoactive Effects of Kappa-Opioid Receptor Ligands in Humans

In healthy humans, KOR agonists produce behavioral effects akin to those associated with schizophrenia, drug addiction, and bipolar disorder. For instance, intravenous administration of the preferential KOR agonist, (-) MR2034, produces psychotomimetic, anxiogenic, and sedative effects (Pfeiffer et al., 1986). Psychotomimetic effects include perceptual distortion of sensory stimuli, depersonalization, speech and language impairments, and thought disorganization. Collectively, these behavioral effects may be perceived as dysphoric behavioral states. Importantly, these effects are stereo-selective and naloxone reversible, suggesting opioid receptor-mediation. Intramuscular administration of enadoline, a synthetic KOR agonist, produces effects perceived as “bad” in humans and psychotomimetic effects at higher doses (Walsh et al., 2001). Spiradoline, a synthetic KOR agonist, produces dysphoria and sedation (Chappell et al., 1993; Rimoy et al., 1994; Ur et al., 1997), and psychotomimetic effects in some patients (Chappell et al., 1993). *Salvia divinorum* is a potent hallucinogenic herb increasingly used recreationally. The psychoactive compound in *S. divinorum* is salvinorin A, a selective, highly potent KOR agonist (Roth et al., 2002). *S. divinorum* use in humans produces psychotomimesis, sedation, speech and language impairments, and uncontrollable laughter, effects similar that of synthetic KOR ligands (Lange et al., 2010; Johnson et al., 2011; Maclean et al., 2012; Ranganathan et al., 2012). However, the effects are more rapid and transient as *S. divinorum* is usually smoked. Interestingly, a substantial proportion of *S. divinorum* users report that “entering another reality” (e.g., psychotomimesis) is the “best thing” about *S. divinorum*

intoxication, while only a small proportion report unpleasant physical after-effects and “bad things” about intoxication (Gonzalez et al., 2006). This is in stark contrast to observations in laboratory settings where most subjects report dysphoric or unpleasant effects, suggesting that expectancy of a “trip” may determine whether the experience is deemed a positive or negative outcome. However, it should also be noted that most *S. divinorum* users have a high degree of cannabis and nicotine consumption (Gonzalez et al., 2006). Therefore, it is possible that self-reported effects of *S. divinorum* are influenced by interactions of several psychoactive compounds. This is of particular importance since cannabinoids can have pro-psychotic or anti-psychotic effects, depending on the cannabis composition and developmental period (e.g., adolescence) of exposure (Fernandez-Espejo et al., 2009; Malone et al., 2010). Recently, case reports have described recurrent psychosis-like symptoms days after *S. divinorum* toxicity in adolescents (Singh, 2007; Paulzen and Grunder, 2008; Przekop and Lee, 2009), which could precipitate symptoms resembling psychiatric disorders not under the direct pharmacological influence of *S. divinorum*. Recurrent psychosis is likely not frequent in *S. divinorum* users, but may be exhibited in subset of users with genetic predisposition or heightened risk to develop schizophrenia or other affective disorders.

Motivational Effects of Kappa-Opioid Receptor Ligands in Animal Models

In rodents KOR activation produces negative affective states. Pavlovian procedures, such as the conditioned place aversion (CPA) paradigm, have been utilized to determine the motivational effects of KOR ligands in rodents. In these procedures, drug

is repeatedly paired with a conditioned stimulus (i.e., a discrete compartment) and approach (i.e., preference) or avoidance (i.e., aversion) behavior to the conditioned stimulus in the absence of drug is assessed during testing. Mucha and Herz first demonstrated that systemic KOR agonist administration produces conditioned aversive effects in rodents using CPA and conditioned taste aversion paradigms (Mucha and Herz, 1985). Conditioned aversive effects produced by KOR ligands is a phenomenon that has been widely replicated in both mice and rats (Bechara and van der Kooy, 1987; Bals-Kubik et al., 1993; Zhang et al., 2005; Braida et al., 2008; Land et al., 2008; Michaels and Holtzman, 2008; Land et al., 2009; Wiley et al., 2009; Tejada et al., 2012b; Tejada et al., 2013). Microinjection of the synthetic KOR agonist U50,488 into medial PFC, midbrain, NAcc, or lateral hypothalamus, but not to the SN or dorsal striatum, is sufficient to produce CPA (Bals-Kubik et al., 1993). This suggests that KORs in the mesolimbic DA pathway (VTA and NAcc), hypothalamic, and prefrontal cortical sites may be responsible for conditioned aversive effects produced by systemically administered KOR agonists. Intracranial self-stimulation (ICSS) procedures are sensitive to manipulations that decrease motivation or decrease brain reward function (Carlezon and Chartoff, 2007). Rate-frequency ICSS procedures have been widely utilized to assess the motivational and anhedonic effects of drugs. In this paradigm, stable ICSS thresholds are established and persist for days to weeks. Anhedonic behavioral states (i.e., drug withdrawal) or drugs that produce conditioned aversive effects reliably increase ICSS thresholds, suggesting that more ICSS is needed to reach “normal” brain reward levels. Systemic administration of KOR agonists increases thresholds in rats (Todtenkopf et al., 2004; Carlezon et al., 2006; Ebner et al., 2010; Negus et al., 2010), whereas KOR

antagonists are without effect, suggesting that endogenous KOR signaling does not tonically inhibit brain reward function (Mague et al., 2003; Todtenkopf et al., 2004). Collectively, Pavlovian and ICSS procedures demonstrate that activation of DYN/KOR systems produce negative motivational effects.

The ability of systemic KOR activation to produce CPA is dependent on intact NAcc DA signaling. NAcc DA D1 receptor antagonism and mesolimbic, but not nigrostriatal or mesocortical, DA pathway denervation blocks KOR agonist-induced CPA (Shippenberg and Herz, 1987; Shippenberg et al., 1993). As KORs decrease mesolimbic DA output, these effects are consistent with findings that decreases in DA signaling encode negative affective states (Acquas et al., 1989; Liu et al., 2008). Thus, modulation of NAcc DA is one mechanism by which DYN/KORs exert negative motivational effects.

The Dynorphin/Kappa-Opioid Receptor System and Behavioral Stress

The DYN/KOR system has also been implicated in mediating many facets of behavioral stress in animal models (Bruchas et al., 2010). Learned helplessness and repeated forced swim stress robustly increases DYN immunoreactivity in hippocampus (CA3, dentate gyrus) and NAcc (Shirayama et al., 2004). Increased hypothalamic and decreased striatal DYN A immunoreactivity after context-induced immobility (paired with electric shock) and a single forced swim exposure have also been reported (Nabeshima et al., 1992). Thus, KOR systems may initially be recruited by stress systems. CRF is a powerful mediator of behavioral stress responses. CRF promotes DA-

dependent release of DYN peptides in striatal regions (Sirinathsinghji et al., 1989; Sirinathsinghji et al., 1990). Moreover, enhanced phospho-KOR immunoreactivity, an index of putative KOR activation, is increased in the NAcc, hippocampus, BLA, bed nucleus of the stria terminalis, DRN, VTA, and ventral pallidum following central CRF administration (Land et al., 2008). Together, these data indicate that repeated stress and the ensuing increase in CRF enhance DYN release and KOR activation, which then modulates stress reactivity. Consistent with this view, systemically administered KOR agonists increase immobility in repeated forced swim procedures whereas rodents treated with KOR antagonists and pdyn KO mice exhibit decreased immobility (Mague et al., 2003; McLaughlin et al., 2003; Shirayama et al., 2004; Carr et al., 2010). Nor-BNI-treated wild-type mice exhibit decreased social defeat stress-induced behaviors (i.e., defeat postures)(McLaughlin et al., 2006; Bruchas et al., 2011). Pre-clinical studies demonstrating that dynorphin/KOR systems are mediators of anxiety-like behavior are consistent with clinical work demonstrating that KOR agonists increase anxiety (Pfeiffer et al., 1986). In tests of anxiety in animal models, endogenous and exogenous KOR agonists and KOR antagonism produces anxiogenic and anxiolytic effects, respectively (Narita et al., 2006; Knoll et al., 2007; Bruchas et al., 2009; Wittmann et al., 2009; Carr and Lucki, 2010; Tejada et al., 2012b). The BLA appears to be a critical site where DYN/KORs regulate anxiety-like behavior as intra-BLA nor-BNI blocks CRF-induced anxiogenesis (Bruchas et al., 2009), and intra-BLA DYN A microinjections are sufficient to produce anxiogenic effects (Narita et al., 2006). Collectively, these results suggest that the DYN/KOR system is engaged by stress and anxiety, and this may possibly occur via CRF systems.

Dynorphin/Kappa-Opioid Dysfunction in Disease

In drug addiction, DYN/KOR systems have been hypothesized to counteract neuroplastic adaptations produced by drugs of abuse in reward-related brain regions, such as the NAcc (Nestler and Carlezon, 2006; Shippenberg et al., 2007; Wee and Koob, 2010; Tejada et al., 2012a). Drug addiction has been hypothesized to be primarily driven by positive reinforcement early on in the disease. The hypothesis that DYN/KOR systems counteract neuroplastic adaptations produced by drugs of abuse is consistent with observations that KOR agonists prevent behavioral effects produced by acute and sensitizing regimens of psychostimulants. With the development of drug addiction, drug use is shifted from being primarily driven positive reinforcement to being driven by compulsive behaviors, negative reinforcement (i.e. increased likelihood of drug use to remove a negative stimuli such as drug withdrawal or alleviate stress), and stress-driven drug use. With repeated engagement of DYN/KOR systems by drugs of abuse, dysregulation of DYN/KOR system dynamics have been hypothesized to contribute to allostatic changes in synaptic activity and DA regulation in mesocorticolimbic circuits resulting in negative affective states, heightened stress and anxiety reactivity, and decreased social volition (Nestler and Carlezon, 2006; Bruchas et al., 2010; Wee and Koob, 2010). Consistent with this notion, KOR antagonists have been effective in blocking drug use driven by stress and negative affect in animal models, presumably by targeting activated KOR systems in affect-related brain regions. Thus, DYN/KOR systems appear to play multiple roles in drug addiction, with an anti-addiction role early on that switches to pro-addictive as drug use shifts from positive reinforcement drive to negative reinforcement drive.

Hypotheses and Specific Aims

It is known that the KORs are present in mPFC circuits, with the vast majority of KOR-immunoreactivity localized to presynaptic localizations. KOR mRNA-expressing and KOR-immunoreactive neurons in the mPFC are sparse, suggesting that the majority of presynaptic KOR may be expressed on terminals of mPFC afferents. Indeed, KOR expression is found in the cell bodies of different brain regions that innervate the mPFC, such as the VTA and BLA. It is conceivable that VTA and BLA neurons may express KOR on their mPFC terminals. Thus, mPFC KORs may be playing an important role in presynaptic control of the mesocortical and amygdalo-cortical pathways, both of which are critical for mPFC-dependent behavior. As KORs inhibit both DA and glutamate release elsewhere in the brain it is also plausible that KORs may have an inhibitory function in the mPFC on DA release from the VTA inputs and glutamate release from BLA inputs. However, the role of KORs regulating neurotransmission into the mPFC in these pathways is not known. Here we designed a series of studies to test the hypothesis that *KORs negatively regulate synaptic inputs from afferents structures into the mPFC*. We sought to test this hypothesis with three specific aims. In Specific Aim 1 we determined whether KORs on DA varicosities in the mPFC inhibited local DA output. In Specific Aim 2 we determined whether KORs were inhibiting glutamate transmission in the mPFC and if this disruption was present in the BLA-mPFC pathway. DYN signaling via KOR is believed to be recruited in response to strong stimuli. DYN/KOR systems within the hippocampus are recruited during strong stimulation to mediate heterosynaptic suppression. It is currently unclear whether KOR signaling in the mPFC can mediate inhibitory heterosynaptic interactions in the mPFC. In Specific Aim 3 we determined

whether we could recruit the DYN/KOR system to mediate inhibitory heterosynaptic interactions between the BLA and hippocampus in the mPFC. Below are listed the specific aims and associated predictions.

Specific Aim 1: mPFC KOR systems negatively modulate mesocortical dopamine signaling by directly inhibiting DA terminals.

Prediction 1a: If KOR systems negatively regulate mesocortical DA systems, then systemic U69,593 administration should inhibit extracellular mPFC DA levels.

Prediction 1b: If KORs in mesocortical DA terminal regions inhibit extracellular DA levels, then intra-mPFC KOR agonists will decrease local extracellular DA levels.

Prediction 1c: If PFC KORs inhibit DA overflow and this system is tonically active, then reverse dialysis of nor-BNI should elevate local extracellular DA levels.

Prediction 1d: If KORs tonically inhibit DA release via a direct action on DA terminals, then basal DA levels should be enhanced in mice lacking KOR in DA neurons.

Prediction 1e: If the effects of U69,593 on DA are mediated by KORs on mPFC DA terminals, then intra-mPFC administration of U69,593 should be without effect in mice lacking KOR in DA neurons.

Specific Aim 2: mPFC KOR systems negatively modulate glutamatergic BLA inputs to the mPFC.

Prediction 2a: If KORs are found on terminals of excitatory synapses, then intra-mPFC U69,593 should decrease basal extracellular glutamate levels.

Prediction 2b: No change in basal glutamate can be indicative of a lack of KOR regulation of glutamate transmission or because extracellular glutamate does not come from synaptic sources. If KORs do not affect basal glutamate levels and basal glutamate levels do not reflect synaptic sources of extracellular glutamate, then unmasking synaptic sources of glutamate using the glutamate reuptake blocker tPDC should reveal an inhibitory role of U69,593 on glutamate transmission.

Prediction 2c: If tPDC-evoked elevations in glutamate increase extracellular GABA, and U69,593 inhibits tPDC-evoked glutamate elevations, then U69,593 pretreatment will block tPDC-evoked elevations in extracellular GABA by inhibiting rises in glutamate.

Prediction 2d: If KORs are expressed by BLA, but not mPFC-projecting hippocampal neurons, then systemic U69,593 treatment should inhibit BLA-evoked, but not fornix-evoked fEPSP responses.

Prediction 2e: If KORs inhibit BLA inputs to the mPFC by acting on KORs on BLA terminals, then intra-mPFC U69,593 pressure ejection should decrease the fEPSP slope, in a nor-BNI-dependent manner.

Prediction 2f: If mPFC KOR inhibition of BLA-evoked fEPSP can be overcome in an activity-dependent manner, then U69,593 inhibition of BLA-evoked responses should be decreased with increasing pulses associated with a BLA pulse train.

Prediction 2g: If mPFC KORs inhibit synaptic efficacy of the BLA to mPFC pathway, then short-term synaptic plasticity of BLA-evoked responses within a train should be decreased by intra-mPFC U69,593 pressure ejection.

Prediction 2h: If mPFC KORs inhibit glutamatergic neurotransmission in the BLA to mPFC pathway, the intra-mPFC U69,593 will decrease optically-evoked BLA synaptic responses in the mPFC

Specific Aim 3: mPFC KOR systems mediate heterosynaptic interactions between the BLA and hippocampus

Prediction 3a: If the BLA provides heterosynaptic feedforward inhibition, then BLA stimulation should suppress hippocampus-evoked synaptic responses in an activity- and time-dependent manner.

Prediction 3b: If BLA-evoked heterosynaptic suppression is mediated by changes in membrane potential induced by the BLA train, then baseline and test pulses to the fornix should be similar at similar membrane potentials.

Prediction 3c: If BLA-evoked suppression of fornix-evoked responses is not due to inhibition of hippocampal afferents in the mPFC, then BLA-evoked heterosynaptic suppression of optogenetically activated hippocampal afferents in the fornix should not be present.

Prediction 3d: If the hippocampus also provides heterosynaptic feedforward inhibition, then fornix stimulation should suppress BLA-evoked synaptic responses in an activity- and time-dependent manner as well.

Prediction 3e: If heterosynaptic suppression is mediated by KOR systems then local or systemic nor-BNI administration should decrease heterosynaptic suppression.

Chapter II: Prefrontal Cortical Kappa-Opioid Receptors Tonicly Inhibit Mesocortical Dopamine Neurotransmission by Directly Acting on Dopamine Varicosities¹

Introduction

Although there is strong evidence for an inhibitory role of KORs in regulating mesolimbic DA neurotransmission, little is known about their role in regulating mPFC DA neurotransmission. KORs directly inhibit mesocortical DA neurons thereby reducing mPFC DA overflow (Margolis et al., 2006). KOR activation decreases DA efflux in frontal cortex synaptosomes and slices (Heijna et al., 1990; Grilli et al., 2009). In the mPFC, KOR immunoreactivity is present in axonal varicosities (Svingos and Colago, 2002), providing an anatomical framework for mPFC KOR systems to locally regulate DA neurotransmission. However, whether mPFC KORs modulate extracellular DA dynamics *in vivo* has not been studied. Furthermore, it is currently unclear if the inhibitory effect of KOR activation on DA was due to direct action of KOR on DA terminals. Here we carried out studies in Specific Aim 1: *mPFC kappa-opioid receptors*

¹ This chapter was formatted for this thesis from the previously published article:

“Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion.”

Tejeda H.A., Counotte D.S., Oh E., Ramamoorthy S., Schultz-Kuszak K.N., Bäckman C.M., Chefer V., O'Donnell P., Shippenberg T.S. (2013). *Neuropsychopharmacology*. doi: 10.1038/npp.2013.76. ¹

tonically inhibit mesocortical dopamine neurotransmission by directly acting on dopamine varicosities using a combination of *in vivo* microdialysis and conditional knock-out of KOR in DA neurons.

Methods

Subjects: Adult male Sprague Dawley rats (Charles River) weighing 300-400 g (> PND 60) were utilized. For conditional knock-out of KOR in DA neurons, we utilized a DAT promoter-driven Cre transgenic mouse line [DAT^{Cre}; Slc6a3Cre; (Backman et al., 2006)] and KOR^{loxP} mice (Dr. Jennifer Whistler; University of California, San Francisco). DAT^{Cre} and KOR^{loxP} mice maintained on a pure C57BL/6J background were cross bred to obtain control mice (DAT^{Cre/wt}; KOR^{wt/wt}) and knockout mice lacking KOR in DA neurons: DAT-KOR KO mice (DAT^{Cre/wt}; KOR^{loxP/loxP}). KOR^{loxP} mice were generated by flanking Exon 2, containing the start codon, of the Oprk1 gene with loxp sequences. DAT^{Cre} mice were generated by inserting an internal ribosome entry-linked Cre recombinase gene downstream from the Slc6a3 gene stop codon. Both mouse strains were in congenic C57BL/6 background at the time of crossbreeding. KOR knock-out in DAT-expressing neurons was confirmed by PCR analysis of DNA from olfactory bulb and VTA/substantia nigra tissue, and was absent in cortex, striatum, or cerebellum. Additionally, double fluorescent in situ hybridization (FISH) was performed to determine the specificity of the mutation in the SN / VTA region. Adult male mice (20-30 g; >PND 60) were housed in temperature- and humidity-controlled facilities accredited by the

American Association for the Accreditation of Laboratory Animal Care under a 12 h light/dark cycle with *ad-libitum* chow and water. Experiments were conducted in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse (NIDA).

Surgery: Rats were anesthetized with Equithesin (1% pentobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol, 3 ml/kg, i.p.) and implanted with a unilateral microdialysis guide cannula (CMA11; CMA Microdialysis) aimed at the mPFC (3.2 mm rostral to bregma (AP); ± 0.6 mm from midline (ML); 2.3 mm from brain surface (DV)). Stereotaxic coordinates are based on the atlas of Paxinos and Watson (1988). A separate group of rats was implanted with a silastic tubing catheter (inner diameter, 0.508 mm; outer diameter, 0.940 mm; Dow Corning) into the right jugular vein, as previously described (Zapata et al., 2010), concomitantly with a unilateral microdialysis guide cannula in the mPFC for systemic drug administration without disturbing the animal during *in vivo* microdialysis sampling. Mice were anaesthetized with a mixture of ketamine (80 mg/kg; i.p.) and xylazine (8 mg/kg; i.p.) and implanted with a unilateral microdialysis guide cannula aimed at the mPFC (CMA7; CMA Microdialysis; AP +1.8 -1.9 mm; ML: -0.3 mm; DV: -1.75 mm) according to the stereotaxic coordinates of Paxinos and Franklin (2001).

In vivo microdialysis: Rats or mice were allowed to recover from the surgery for 5-7 days before dialysis. The evening before dialysis testing (approximately 14 hr), animals were

lightly restrained and a microdialysis probe (CMA/11; membrane dimension 0.24 x 3 mm for rat; CMA/7; membrane dimension 0.24 x 2 mm for mouse; CMA Microdialysis) was inserted through the guide cannula manually. The inlet tubing of the probe was connected to a microinfusion pump (CMA 102; CMA Microdialysis) via a dual quartz-lined swivel (Instech Laboratories) and the animal was placed into a Plexiglas test chamber (40 x 40 x 35 cm). Microdialysis probes were perfused with aCSF (145 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 5.4 mM glucose, adjusted to pH 7.2-7.4 using NaOH) overnight at a flow rate of 0.3 µl/min using a CMA102 syringe pump (CMA Microdialysis). The following day, syringes were replaced with fresh aCSF and the flow rate was changed to 0.6 µl/min. Following a 2 hr equilibration period, three to four 15 min baseline samples were collected prior to experimental manipulation. For experiments examining the effects of acute systemic U69,593 administration on mesocortical DA transmission, rats were challenged with repeated intravenous vehicle injections (heparinized saline, pH 7.2-7.4) or a vehicle injection followed by escalating doses of U69,593 (0.02 and 0.04 mg/kg). For experiments examining the role of mPFC KOR in regulating local neurotransmission, perfusate containing U69,593 (0.5 and 1.0 µM), nor-BNI (0.5 and 1.0 µM), DAMGO (100 µM), and/or 1-trans-pyrrolidine-2,4-dicarboxylate (tPDC; 1 mM) were administered locally via reverse dialysis. Drug effects were subsequently washed out by replacing the perfusate containing drug/s with normal aCSF. No-net-flux *in vivo* microdialysis experiments were conducted in a separate group of mice to quantify basal mPFC DA dynamics. Five different concentrations of DA were included in the perfusate (DA_{in} : 0, 1.25, 2.5, 5, and 10 nM) in a pseudo-random order to determine extracellular DA (DA_{ext} ; x-intercept) and extraction fraction (E_d ; slope), which

is an indicator of DA uptake. After a 20 min equilibration period, three 15 min dialysis samples were collected at each DA_{in} concentration to determine DA in the perfusate (DA_{out}).

High Performance Liquid Chromatography: Dialysate DA concentrations were analyzed using high performance liquid chromatography (HPLC) with electrochemical detection. Aliquots from dialysate samples (8 μ l) were injected into either a BAS HPLC system (Bioanalytical Systems, West Lafayette, IN) consisting of a BAS-Phase II HPLC column (100 x 3.2 mm, inner diameter, C-18, 3 μ m particulate silica gel), a BAS PM-92e HPLC pump, and a BAS LC-4C amperometric detector or an Eicom HTEC-500 HPLC system with an integrated amperometric detector (San Diego, CA), consisting a PP-ODS HPLC column (30 x 4.6 mm, inner diameter, C-18, 2 μ m particulate silica gel) and a HTEC-500 pump. BAS HPLC system mobile phase consisted of 150 mM NaH_2PO_4 , 1.0 mM EDTA, 0.03 mM sodium dodecyl sulfate, 20% MeOH, pH 5.0, resulting in retention time of 3-4 min at a pump rate of 0.5 ml/min. The Eicom HPLC system mobile phase consisted of 100 mM NaH_2PO_4 , 1.3 mM EDTA, 2.0 mM decane-1-sulfonate, 1% MeOH, pH 6.0, resulting in retention time of 2.5-3.0 min at a pump rate of 0.5 ml/min. Applied potentials were set at +700 and +400 mV versus a Ag/AgCl reference electrode in BAS and Eicom systems, respectively. Concentrations of dialysate DA levels were estimated using calibration curves obtained from external standards. Detection limit of DA using these conditions was approximately 50 pM. Dialysate samples were analyzed within 24 h of collection to prevent degradation.

Drugs: U69,593, nor-BNI, and DAMGO were supplied by the Research Technology Branch of the National Institute of Drug Abuse (Rockville, MD). Stock solutions of U69,593 (10 mM) were dissolved in 0.1 N HCl and diluted in either sterile saline or aCSF. Nor-BNI and L-trans-Pyrrolidine-2,4-dicarboxylic acid (tPDC) were obtained from Tocris (Ellisville, MO) and dissolved in aCSF. The pH of saline or aCSF containing drugs was adjusted to 7.2-7.4 using NaOH.

Histology: Upon termination of *in vivo* microdialysis testing, animals were anesthetized with Equithesin and a microdialysis probe with dye on the active membrane was manually inserted into the microdialysis cannula to determine membrane location. Brains were removed, frozen, and sectioned (40 μ m). Histological verification of the location of the active membrane of the microdialysis probe in rats and mice was obtained from coronal sections (Fig. 2.1).

Statistical Analysis: Data were analyzed using repeated measures ANOVA with sample-type (i.e. baseline, drug infusion, washout samples) and time (i.e. consecutive samples within a sample-type; e.g. baseline 1, baseline 2, baseline 3, etc) as within-subjects factors and drug dose, treatment, or genotype as a between subjects factor. Since we were interested in changes in response to pharmacological manipulations only sample-type (i.e. baseline, drug infusion, washout samples) and treatment, dose, or genotype effects were further explored. Area under the curve (AUC) values was obtained using a standard

trapezoidal method from an equal number of basal and drug samples represented (Rawls and McGinty, 1998; Chefer et al., 2005). For experiments examining the effects of systemic U69,593, AUC were calculated for each three sample period following a challenge (i.e. vehicle or U69,593). For experiments examining the effects of intra-mPFC U69,593 or nor-BNI, the first three samples of the drug challenge were utilized to calculate the AUC. For DAMGO/nor-BNI experiments, AUC was calculated for the 3-sample period corresponding to DAMGO infusion. AUC values were analyzed using one-way or repeated measures two-way ANOVA, or Student's t-test where appropriate. Post-hoc analyses were carried out using Fisher's LSD test or a paired t-test, where appropriate. DA_{ext} and E_d from no-net-flux experiments were analyzed using a Student's t-test.

Results

For in vivo microdialysis experiments, microdialysis probe membranes spanned anterior cingulate, prelimbic, and infralimbic cortexes in rats and mice (Fig 2.1).

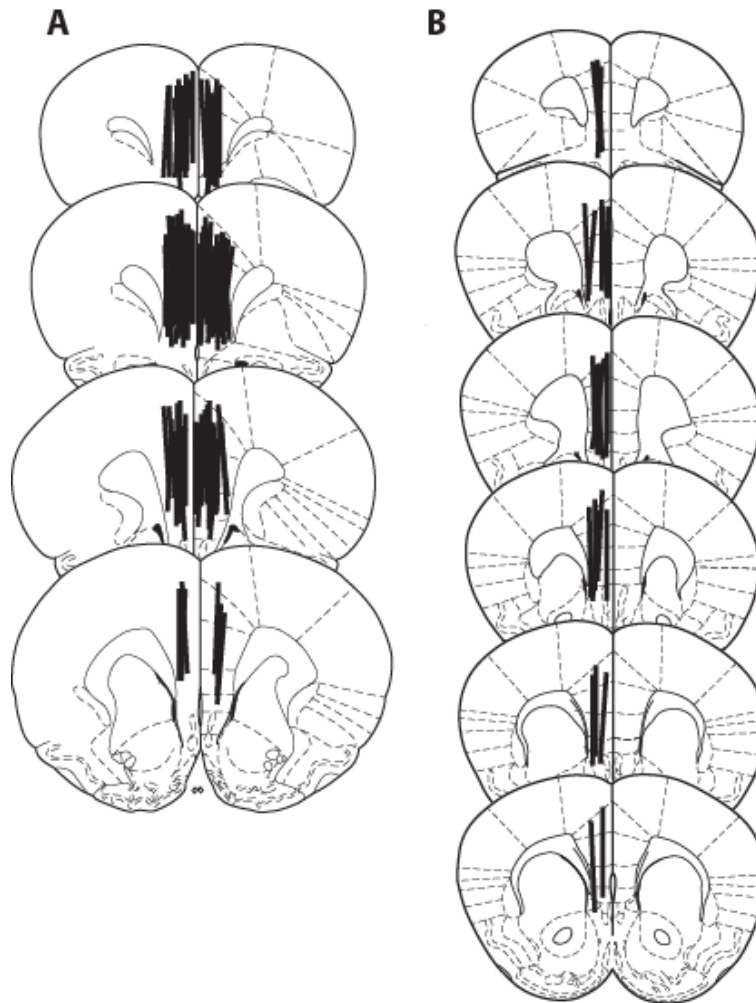


Figure 2.1: *A*, Placements of microdialysis probes (|) in the mPFC for all in vivo microdialysis studies conducted in rats and mice. The active membrane of the microdialysis probe primarily spanned the prelimbic and infralimbic cortex with a small proportion in the anterior cingulate. Diagrams were adapted from Paxinos and Watson (1987). *B*, Placements of microdialysis probes (|) in the mPFC of mice. Diagrams were adapted from Paxinos and Franklin (2001).

Systemic administration of the selective KOR agonist U69,593 decreases extracellular DA levels

To determine whether global activation of KOR systems modulate mesocortical DA output, we challenged rats with an intravenous challenge of ascending doses of U69,593 or repeatedly with vehicle. Acute intravenous administration of U69,593 decreased mPFC DA overflow in rats (Fig 2.2). Analysis of DA levels revealed a main effect of sample-type (baseline, vehicle infusion, U69,593 infusion; $F_{(3,66)} = 10.09$; $p < 0.001$), no main effect of treatment (controls treated with repeated vehicle vs U69,593-treated; $F_{(1,11)} = 4.02$; $p = 0.07$) and a significant sample-type x treatment interaction ($F_{(3,66)} = 4.38$; $p = 0.011$; Fig 2.2A). AUC analysis (of the three samples corresponding to the each challenge) also revealed a main effect of sample-type ($F_{(2,22)} = 12.21$; $p < 0.001$), no main effect of treatment ($F_{(1,11)} = 1.32$; $p = .27$), and a significant sample-type x treatment interaction ($F_{(2,22)} = 5.47$; $p = 0.03$; Fig 2.2B). Post-hoc analysis revealed a significant decrease in DA after administration of 0.04 mg/kg U69,593 relative to vehicle administration ($p < 0.05$) and vehicle-treated control rats ($p < 0.05$). These findings confirm that KOR systems negatively control extracellular DA levels in the mPFC.

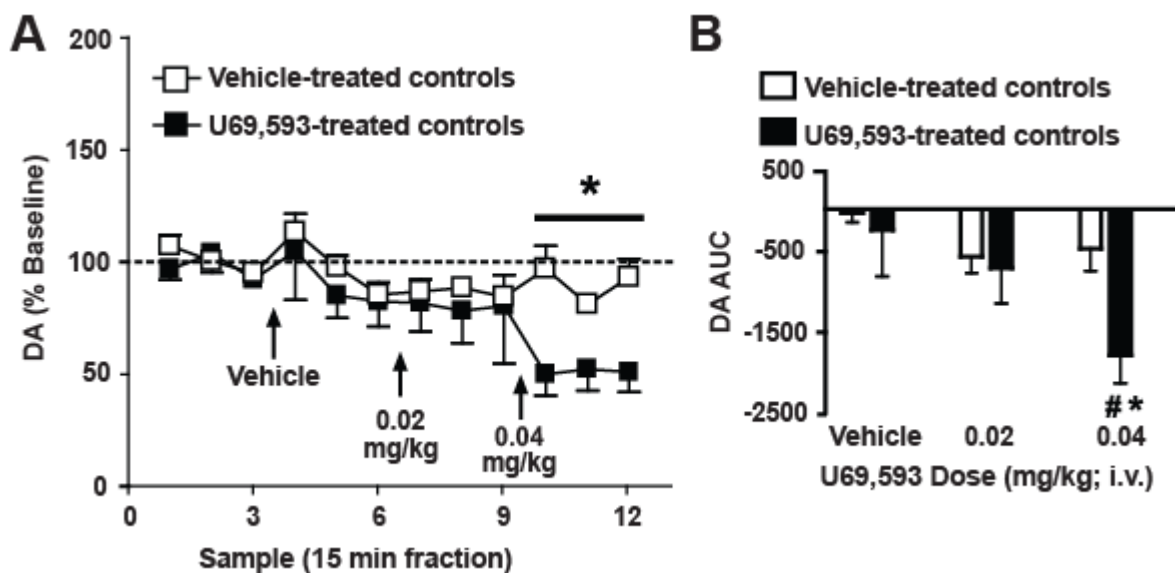


Figure 2.2: Systemic administration of a KOR agonist decreases mesocortical DA neurotransmission in rats. **A**, Time course of mPFC dialysate DA levels after repeated challenge with systemic administration of vehicle (open squares; n=7) or escalating doses of U69,593 (filled squares; n=7). Vertical arrows depict the time of vehicle or U69,593 (0.02 mg/kg then subsequently 0.04 mg/kg). (*) reflects a significant sample-type x treatment interaction. **B**, AUC values of DA levels after vehicle or U69,593 challenge in control (open bars) and U69,593-treated (filled bars) groups. (*) and (#) reflect a significant difference from baseline and from vehicle controls after intravenous administration of 0.04 mg/kg at that corresponding time point, respectively. **A**, Abscissa, microdialysis fractions (15 min). Data points reflect the mean \pm SEM.

mPFC KOR activation inhibits local DA overflow

It is unclear whether activation of mPFC KOR is sufficient to inhibit local extracellular DA levels. Reverse dialysis of U69,593 decreased mPFC DA levels in rats (Fig 2.3). ANOVA revealed a main effect of sample-type (baseline, U69,593 infusion, washout; $F_{(2,78)}=14.88$; $p<0.001$), no main effect of dose (0, 0.5, 1.0 μM ; $F_{(1,13)}=0.17$; $p=0.84$), and a sample-type x dose interaction ($F_{(4,78)}=6.15$; $p<0.01$; Fig 2.3A). AUC (of the first three U69,593 samples) comparisons indicated an effect of dose ($F_{(2,22)}=8.021$; $p=0.01$; Fig 2.3B). Post-hoc analysis demonstrated that intra-mPFC U69,593 (0.5 and 1.0 μM) administration decreased DA levels relative to aCSF controls ($p<0.01$). These results demonstrate that activation of mPFC decreases DA output in the mPFC.

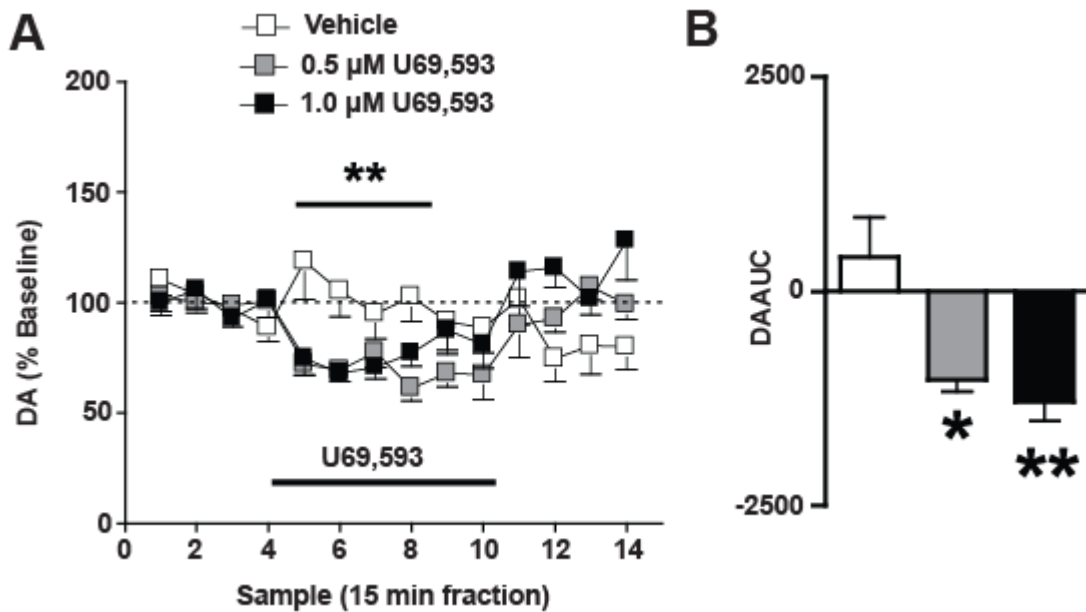


Figure 2.3: mPFC KORs inhibit local DA levels. *A*, Time course of mPFC dialysate DA levels after reverse dialysis administration of vehicle (white squares; $n=8$), 0.5 μM U69,593 (grey squares; $n=8$), or 1.0 μM U69,593 (black squares; $n=8$). (**) reflects a significant sample-type x treatment interaction. *B*, AUC values of DA levels after vehicle or U69,593 challenge. (*) and (**) reflect a significant difference from vehicle controls. *A*, Abscissa, microdialysis fractions (15 min). Data points reflect the mean \pm SEM. Black bar depicts period of infusion of U69,593.

mPFC KORs tonically inhibit local DA overflow

To determine whether KORs in the mPFC were constitutively inhibiting local DA, we examined the effects of intra-mPFC KOR antagonism on DA. Reverse dialysis of the KOR antagonist nor-BNI significantly enhanced basal dialysate DA levels in rats (Fig 2.4). Repeated measures ANOVA revealed an effect of sample-type (baseline, nor-BNI, washout; $F_{(2,108)}=6.80$; $p=0.013$; Fig 2.4A), but no main effect of dose ($F_{(2,108)}=1.73$; $p=0.21$) or sample-type x dose interaction ($F_{(4,108)}=1.53$; $p=0.22$). ANOVA comparing AUC values (of the first three nor-BNI samples) across nor-BNI doses revealed an effect of dose ($F_{(2,20)}=3.709$; $p=0.045$; Fig 2.4B). Post-hoc analysis showed that reverse dialysis of 1.0 μM nor-BNI enhanced extracellular DA levels relative to aCSF controls ($p<0.05$). These results indicate that mPFC KORs negatively modulates mPFC extracellular DA levels and this system is tonically active.

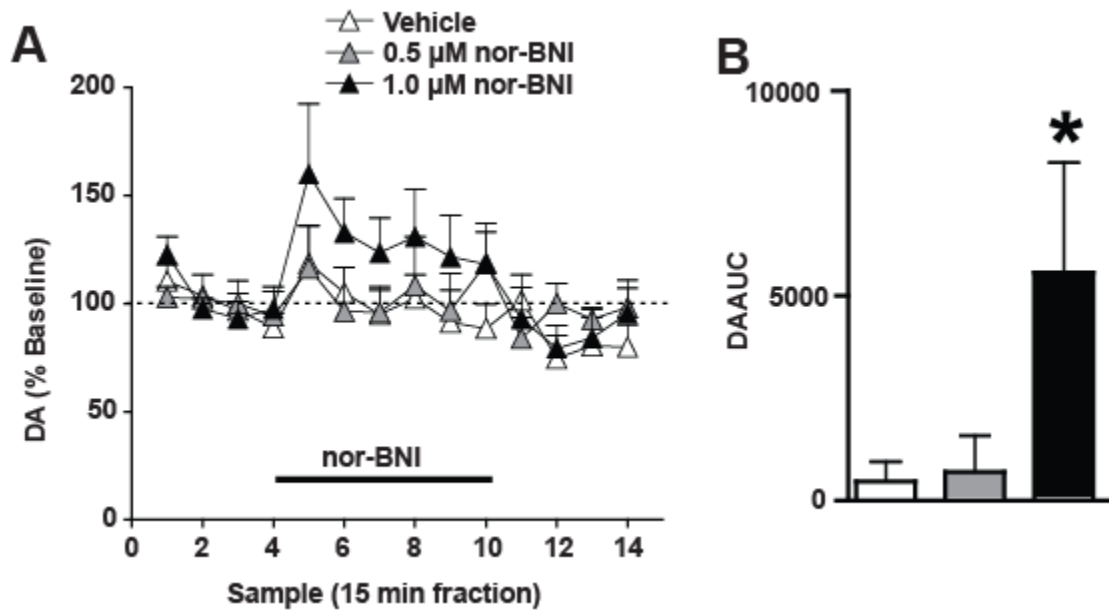


Figure 2.4: KORs in the mPFC tonically inhibit mPFC DA levels. *A*, Time course of mPFC dialysate DA levels after reverse dialysis administration of vehicle (white triangles; n=8), 0.5 μM nor-BNI (grey triangles; n=7), or 1.0 μM nor-BNI (black triangles; n=6). *B*, AUC values of DA levels after vehicle or nor-BNI challenge. (*) reflects a significant difference from vehicle controls. *A*, Abscissa, microdialysis fractions (15 min). Data points reflect the mean ± SEM. Black bar depicts period of infusion of nor-BNI.

Nor-BNI does not enhance extracellular DA in the mPFC by acting as a functional KOR antagonist.

In addition to antagonizing KOR, nor-BNI may initially act as a functional MOR antagonist as it is able to block mu-opioid receptor (MOR)-induced analgesia within two hours after systemic nor-BNI administration (Endoh et al., 1992). Therefore, we determined whether nor-BNI would antagonize the effects of intra-mPFC administration of a MOR agonist, DAMGO, on local DA levels. Nor-BNI failed to antagonize the effects of intra-mPFC perfusion of the selective MOR agonist, DAMGO (100 μ M), on DA overflow in rats (Fig 2.5). Analysis revealed a main effect of sample-type (baseline, nor-BNI and/or DAMGO infusion; $F_{(1,34)}=30.504$; $p<0.001$; Fig 2.5A), no main effect of treatment (nor-BNI alone, DAMGO alone, nor-BNI/DAMGO; $F_{(2,17)}=0.87$; $p=0.44$) or sample-type x treatment interaction ($F_{(2,34)}=0.66$; $p=0.53$). ANOVA comparing AUC values across different treatments during the 3 sample period corresponding to DAMGO infusion, revealed an effect of treatment ($F_{(2,20)}=3.709$; $p=0.045$; Fig 2.5B), with significantly enhanced DA in DAMGO and nor-BNI/DAMGO groups relative to nor-BNI alone ($p<0.05$). Thus, nor-BNI does not enhance DA via MOR antagonism.

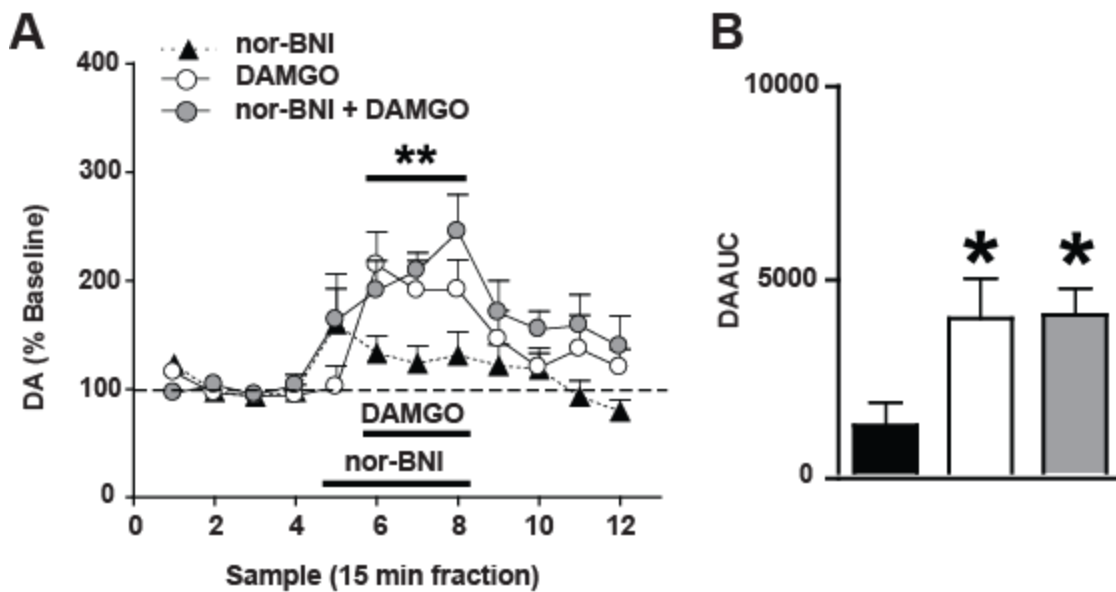


Figure 2.5: Nor-BNI does not enhance extracellular DA in the PFC by acting as a functional MOR antagonist. **A**, Time course of mPFC DA overflow after reverse dialysis administration of 1.0 μ M nor-BNI (black triangles; n=6), 100 μ M DAMGO (white squares; n=6), or 100 μ M DAMGO/1.0 μ M nor-BNI (black squares; n=8). (***) reflects a main effect of sample-type. **B**, AUC values of DA overflow after nor-BNI and/or DAMGO challenge. (*) reflects a significant difference from rats challenged with nor-BNI alone. **A**, Abscissa, microdialysis fractions (15 min). Data points reflect the mean \pm SEM. Black bar depicts period of infusion of the indicated drug.

Conditional knockout of KOR in DA neurons of the VTA

Mating of DAT^{Cre/wt} mice with KOR^{loxP/loxP} resulted in Cre-mediated recombination of the loxP sites of the KOR gene in neurons that express DAT of offspring. Thus, KOR expression was knocked down in DA neurons expressing DAT. To verify that KOR mRNA expression was knocked down in DAT-expressing neurons, our collaborator Dr. Cristina Bäckman at NIDA conducted dual label FISH in midbrain sections from DAT-KOR control and DAT-KOR KO mice. KOR mRNA was present in DAT mRNA-positive and negative neurons in the VTA of DAT-KOR control mice (Fig. 2.6). DAT-KOR KO mice did not express KOR mRNA in DAT mRNA-positive cells, whereas KOR mRNA expression in DAT mRNA-negative neurons was unaffected. This provides anatomical evidence that KOR expression is lacking in DA neurons that express DAT.

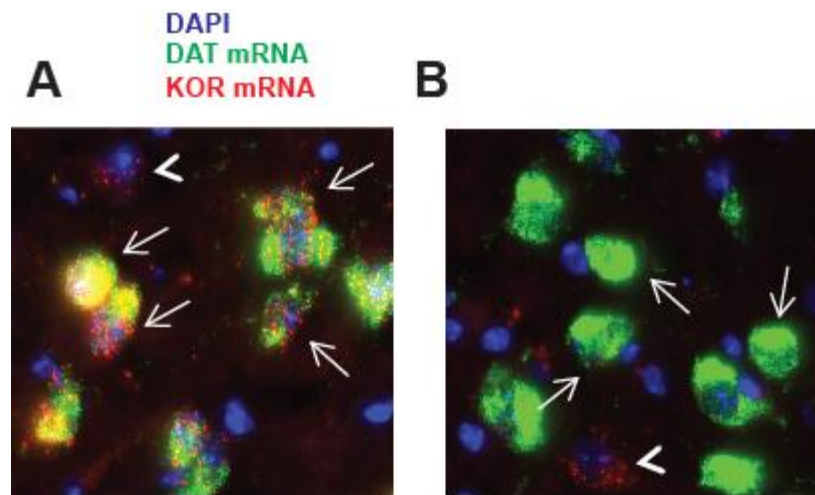


Figure 2.6: KOR mRNA expression is absent in VTA DA neurons in DAT-KOR KO mice. A, B, Representative images of dual fluorescence in situ hybridization of DAT mRNA (green) and KOR mRNA (red) in the VTA of a control (**A**) and DAT KOR KO (**B**) mouse. Blue, green, and red signals represent DAPI nucleic acid stain, DAT mRNA, and KOR mRNA, respectively. Arrows indicate cells expressing DAT mRNA. DAT positive neurons co-express KOR mRNA in control animals (**A**). As predicted DAT positive neurons do not express KOR mRNA in DAT-KOR KO mice (**B**). Additionally, KOR mRNA was present in DAT negative neurons in both control and KO animals (arrowheads in **A** and **B**).

Selective deletion of KOR in DAT-expressing neurons does not modify basal DA dynamics in the PFC

If mPFC KOR signaling negatively regulates basal DA levels, then conditional knockout of KOR in DA neurons should result in enhanced DA levels in the mPFC. We determined whether basal DA dynamics in the mPFC were altered in DAT-KOR KO mice utilizing no-net-flux in vivo microdialysis. No-net-flux microdialysis allows determination of extracellular DA levels, DA uptake, and allows release to be inferred from these parameters (Chefer et al., 2006). No-net-flux microdialysis experiments revealed no difference in basal DA dynamics between control and DAT-KOR KO mice (Fig 2.7). There was not a significant difference between control and DAT-KOR KO mice in E_d (Fig 2.7B; $t_{16}=-0.533$, ns) or mPFC DA_{ext} (Fig 2.7C; $t_{24}=0.011$, ns). These results suggest that genetic deletion of KOR in DAT-expressing neurons does not modify basal mPFC DA levels and clearance, suggesting that developmental compensation to mPFC DA systems may occur in DAT-KOR KO mice.

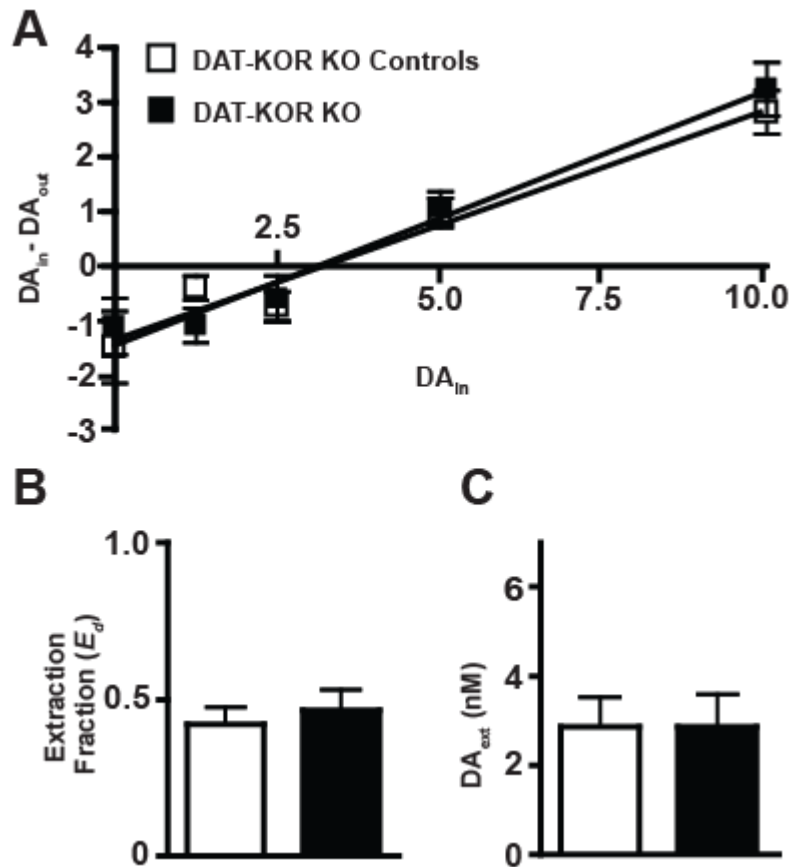


Figure 2.7: Basal DA dynamics are not altered in DAT-KOR KO mice. *A*, $DA_{in} - DA_{out}$ plotted against DA_{in} with fitted regression lines for control (open squares; $n=9$) and DAT-KOR KO (filled squares; $n=9$) mice depicting similar x-intercepts (DA_{ext}) and slopes (E_d). *B*, E_d (an indirect measure of DA uptake) in control (open bar) and DAT-KOR KO (filled bar) mice. *C*, mPFC DA_{ext} in control (open bar) and DAT-KOR KO (filled bar) mice.

KORs on mPFC DA terminals directly inhibit mPFC DA overflow

It is possible that U69,593 may inhibit DA levels indirectly by modulating systems regulating DA terminals and/or mesocortical DA neuron activity. To elucidate if KORs on DA terminals mediate inhibition of DA by intra-mPFC U69,593, we determined whether intra-mPFC U69,593-induced decreases in local DA levels were absent in DAT-KOR KO mice. Reverse dialysis of U69,593 inhibited mPFC DA overflow in control mice, an effect that was absent in DAT-KOR KO mice (Fig 2.8). Analysis revealed a sample-type (baseline, U69,593 infusion, washout) x genotype (control and DAT-KOR KO) interaction (Fig 2.8A; $F_{(2,36)}=3.825$; $p=0.041$). Comparison of AUC values (of the first three U69,593 samples) revealed a significant difference between the control and DAT-KOR KO mice (Fig. 2.8B; $t_9=2.867$, $p=0.019$). Thus, U69,593 inhibits mPFC DA levels by acting on mPFC DA varicosity KORs. This also provides functional evidence that DAT-KOR KO mice lack functional KORs in mesocortical DA terminals.

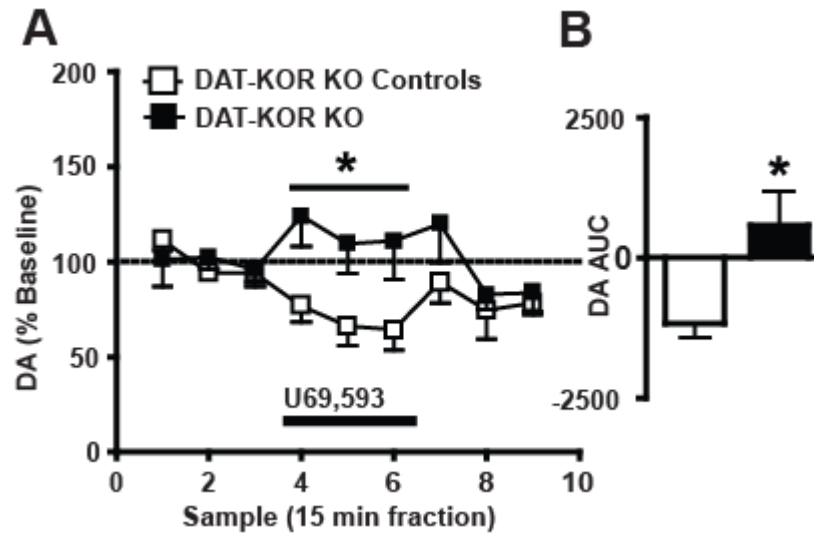


Figure 2.8: KORs inhibit extracellular DA levels by acting directly on DA varicosities in the mPFC. *A*, Time course of mPFC dialysate DA levels after reverse dialysis administration of 1.0 μ M U69,593 in control (open squares; n=6) and DAT-KOR KO (filled squares; n=5) mice. (*) reflects a significant sample-type x treatment interaction. *B*, AUC values of DA levels after 1.0 μ M U69,593 challenge in control (open bar) and DAT-KOR KO (filled bar) mice. (*) reflects a significant difference from control mice. Data points reflect the mean \pm SEM. Black bar depicts period of infusion of U69,593.

Chapter II Summary

Our findings are consistent with the notion that PFC KOR systems negatively modulate mesocortical dopamine signaling by directly inhibiting local DA terminals and this system is tonically active. The finding that systemic U69,593 inhibited extracellular PFC DA was consistent with the hypothesis that mPFC KORs inhibit VTA DA inputs to the mPFC, but did not rule out the possibility U69,593 was acting in the VTA to inhibit mesocortical DA neurons. Reverse dialysis of U69,593 while monitoring extracellular PFC DA levels decreased DA tone. Moreover, intra-PFC nor-BNI administration elevated local DA overflow. These results suggest that mPFC KORs tonically decrease DA levels. Our laboratory generated conditional knock-out mice lacking KOR in DAT-expressing neurons by crossing $\text{DAT}^{\text{Cre/wt}}$ and $\text{KOR}^{\text{loxP/loxP}}$ mice. Using these mice we probed the role of KOR in mesocortical DA neurons on mesocortical DA transmission. Basal DA dynamics were not altered in DAT-KOR KO mice, which is not consistent with our prediction that basal DA levels would be enhanced in these mice. However, it is also possible that there may be developmental compensation/s in mPFC DA systems in response to loss of KOR in DA neurons. Intra-PFC U69,593 inhibition of DA levels was present in control mice, but was lacking in DAT-KOR KO mice, consistent with the notion that U69,593 inhibits mesocortical DA output by activating KORs on DA varicosities. Collectively, these studies demonstrate that mPFC KOR systems negatively modulate mesocortical dopamine signaling by directly inhibiting local DA terminals and this system is tonically active.

Chapter III: Prefrontal Cortical Kappa-Opioid Receptors Inhibit Glutamatergic Transmission in the Amygdala to Prefrontal Cortex Pathway²

Introduction

KORs negatively regulate glutamatergic activity in a wide variety of brain regions (Tejeda et al., 2012a). KOR-immunoreactivity is present in presynaptic entities of excitatory synapses in the rat mPFC (Svingos and Colago, 2002), suggesting they may regulate glutamatergic activity in this brain region as well. To date, the role of KOR in regulating glutamatergic neurotransmission in the mPFC has been limited to an *in vitro* study where KOR activation inhibited K⁺-evoked glutamate release from synaptosomes (Sbrenna et al., 1999). However, evidence of an inhibitory role of KORs in glutamate

² A portion of this chapter was formatted for this thesis from the previously published article:

“Prefrontal cortical kappa-opioid receptor modulation of local neurotransmission and conditioned place aversion.”

Tejeda H.A., Counotte D.S., Oh E., Ramamoorthy S., Schultz-Kuszkak K.N., Bäckman C.M., Chefer V., O'Donnell P., Shippenberg T.S. (2013). *Neuropsychopharmacology*. doi: 10.1038/npp.2013.76.

A portion of this chapter was formatted for this thesis from a manuscript prepared for *Neuropsychopharmacology*:

“Regulation of limbic inputs to the mPFC by kappa-opioid receptors”.

Tejeda H.A., Mejias-Apontes C., O'Donnell P.

neurotransmission in intact mPFC cortical circuits containing all local and long-distance connections is lacking.

The mPFC receives glutamatergic inputs from distal limbic regions, including the BLA (McDonald, 1996). The BLA is a brain region that has a rich density of KOR receptor mRNA and immunoreactivity (Meng et al., 1993; DePaoli et al., 1994; Paxinos and Franklin, 2001; Van't Veer et al., 2013). Moreover, KOR inhibits evoked synaptic potentials and LTP induction in slices containing the BLA (Huge et al., 2009a). These results suggest that the BLA and its efferents to downstream targets, such as the mPFC, may be modulated by KORs. Here we utilized *in vivo* microdialysis and *in vivo* electrophysiological techniques combined with optogenetics to explore *Specific Aim 2: mPFC KORs presynaptically inhibit glutamatergic limbic inputs, including the BLA, into the mPFC in a pathway-specific manner.*

Methods

Subjects: For neurochemical and electrophysiological studies, adult male Sprague Dawley and Long-Evans rats (Charles River), respectively, weighing 300-400 g (> PND 60) were utilized. These animals were housed in temperature- and humidity-controlled facilities accredited by the *American Association for the Accreditation of Laboratory Animal Care* under a 12 h light/dark cycle with *ad-libitum* chow and water. Experiments were conducted in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse (NIDA) and the University of Maryland, Baltimore.

In vivo microdialysis: Sprague-Dawley rats were anesthetized with Equithesin (1% pentobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol, 3 ml/kg, i.p.) and implanted with a unilateral microdialysis guide cannula (CMA11; CMA Microdialysis) aimed at the mPFC (3.2 mm rostral to bregma (AP); ± 0.6 mm from midline (ML); 2.3 mm from brain surface (DV)). Stereotaxic coordinates are based on the atlas of Paxinos and Watson (1988). Rats were allowed to recover from the surgery for 5-7 days before dialysis. The evening before dialysis testing (approximately 14 hr), animals were lightly restrained and a microdialysis probe (CMA/11; membrane dimension 0.24 x 3 mm for rat; CMA/7; membrane dimension 0.24 x 2 mm for mouse; CMA Microdialysis) was inserted through the guide cannula manually. The inlet tubing of the probe was connected to a microinfusion pump (CMA 102; CMA Microdialysis) via a dual quartz-lined swivel (Instech Laboratories) and the animal was placed into a Plexiglas test chamber (40 x 40 x 35 cm). Microdialysis probes were perfused with aCSF (145 mM NaCl, 2.8 mM KCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 5.4 mM glucose, adjusted to pH 7.2-7.4 using NaOH) overnight at a flow rate of 0.3 μ l/min using a CMA102 syringe pump (CMA Microdialysis). The following day, syringes were replaced with fresh aCSF and the flow rate was changed to 0.6 μ l/min. Following a 2 hr equilibration period, four to six 10 min baseline samples were collected prior to experimental manipulation. For experiments examining the role of mPFC KOR in regulating local glutamate and GABA levels, perfusate containing U69,593 (0.5 and 1.0 μ M), nor-BNI (0.5 and 1.0 μ M), and/or 1-trans-pyrrolidine-2,4-dicarboxylate (tPDC; 1 mM) were administered locally via reverse dialysis. Drug effects were subsequently washed out by replacing the perfusate containing drug/s with normal aCSF.

Capillary Electrophoresis with Laser-Induced Fluorescence: GABA and glutamate dialysate concentrations were estimated using a capillary electrophoresis P/ACE™ MDQ system (Beckman, USA) coupled to an external ZETALIF laser-induced fluorescence detector (Picometrics, France), as previously described (Chefer et al., 2009). Separations were carried out in a fused-silica capillary (50 µm ID, 350 µm OD, Polymicro Technologies, Phoenix, AZ) that was 62 cm long (46 cm from injection to detection window). Sampling and derivatization procedures were automated and carried out by the P/ACE MDQ system. Hydrodynamic injection running buffer and reagents was performed via application of positive or negative pressure at the capillary inlet. At the onset of analysis, sample tubes contained 2 µl of dialysate. The capillary was flushed with 0.9 µl of H₂O applying pressure in the H₂O vial. Next, the capillary was loaded with 0.020 µl of sodium cyanide (300 mM in 0.5 M Borate buffer, pH 10.5) and 0.040 µl of naphthalene-2,3-dicarboxaldehyde (15 mM in 75% DMSO). Then, 0.33 µl of the contents of the capillary (including sodium cyanide, naphthalene-2,3-dicarboxaldehyde, and 0.27 µl of water) were delivered into the dialysate by applying negative pressure in the sample vial. A brief pressure pulse was delivered into the sample vial in order to push all the solutions to the bottom of the vial and ensure proper mixing. The capillary was then conditioned by flushing with 0.1 M NaOH (4 µl) followed by H₂O (3 µl) and then filled with running buffer (3 µl). After approximately 5 minutes of derivatization, 0.015 µl of the mixture were injected into the capillary. Separation was achieved by applying a 24 kV potential at 33°C. The running buffer consisted of sodium borate buffer (75 mM, pH 9.2) including 10 mM hydroxypropyl-β-cyclodextrine and 70 mM sodium dodecyl sulfate; to which 5% methanol was added daily. Under these conditions, GABA and

glutamate were resolved within 11 minutes and the limit of detection was below 1 nM for both analytes. Fluorophore excitation at the detection window was achieved via a laser diode (Picometrics, France) with a wavelength at 410 nm, while the emission wavelength was 490 nm.

***In vivo* extracellular electrophysiology**

A subset of rats were treated with saline or nor-BNI (10 mg/kg; s.c.) 24 hr prior to recording procedures. Long-Evans rats were anesthetized with chloral hydrate (400 mg/kg, i.p) and fixed on a stereotaxic apparatus (Kopf Instruments). Anesthesia was maintained throughout the remainder of the experiment using continuous chloral hydrate (24-30 mg/kg/h) via an intraperitoneal catheter. Body temperature was maintained at approximately 37°C using a thermal probe-controlled heat pad (Fine Science Tools). Concentric bipolar stimulating electrodes (0.5 mm diameter, 0.5 mm pole separation; Rhodes Medical Instruments Inc.) were lowered into the left BLA (AP: -3.0 mm; ML: 4.9-5.0 mm; DV: -7.4 mm from dura) and in some cases left fornix (AP: -3.0 mm; ML: 3.9-4.0 mm; DV: -3.1-3.2 mm from dura) as well. Coordinates were based on the rat brain atlas of Paxinos and Watson (1998).

Extracellular recording microelectrodes were pulled on a vertical Stoelting puller from 1.5 mm diameter borosilicate glass (World Precision Instruments), and tips were broken against a glass slide to 2-3 μm . This resulted in a tip resistance of 5-15 M Ω *in situ*. We utilized custom-built double barrel glass pipettes for local drug pressure ejection as previously described on a published paper on which I was co-first author (Dilgen et al., 2013). Pressure ejection pipettes were pulled from 1.0 mm diameter borosilicate glass

(0.25 mm I.D.; A-M Systems). The drug pipette was filled with aCSF containing the AMPA and NMDA receptor antagonists CNQX (50 μ M) and AP-5 (100 μ M), respectively, or the KOR agonist U69,593 (1000 μ M), and <0.01% Chicago Sky Blue to help visualize drug delivery. Extracellular recording microelectrodes were filled with 0.5 M sodium chloride. Microelectrodes were lowered into the superficial cortex using a hydraulic manipulator (Trent Wells, Coulterville, CA) and electrode resistance was determined. Signals were passed through a headstage to a Neurodata Intracellular Amplifier (Cygnus), subsequently amplified, and continuously monitored on a digital oscilloscope (Fluke), a multimeter (Tektronix), and an audio monitor (Grass). Signals were digitized at 20 kHz using a Digidata (Molecular Devices), and acquired with Axoscope 9 software (Molecular Devices) for offline analysis. Local field potential (LFP) signal was filtered between 1 Hz and 100 Hz.

Microelectrodes were lowered through the mPFC while the fornix or BLA was stimulated (0.1-0.5 ms duration; 0.4-1.0 mA; 0.2 Hz) until a consistent monosynaptic evoked field excitatory postsynaptic potential (fEPSP) with > 0.1 mV in amplitude was encountered in response to single pulse fornix or BLA electrical stimulation. A response was monosynaptic if the onset latency was < 10 ms and had a negative-going peak \leq 25 ms from stimulation. To measure glutamatergic transmission in the BLA to mPFC pathway, we measured the slope of the fEPSP as we have previously demonstrated that early components of evoked synaptic potentials are composed of excitatory responses using *in vivo* intracellular recordings (Dilgen et al., 2013). In experiments assessing the effects of systemic U69,593 on fornix- and BLA-evoked responses, the fornix or BLA were stimulated every 30 sec with a 15 sec delay between BLA and fornix. A 30 min

baseline was assessed followed by systemic injection of the KOR agonist U69,593. For pressure ejection experiments, baseline was assessed for 5 min while the BLA was electrically stimulated at 0.2 Hz (single pulse time course experiments) or 0.05 Hz (BLA train experiments). aCSF, CNQX/AP-5, or U69,593 was pressure ejected using a Toohey Pressure System IIe (Toohey Systems; 40 psi; 5-10 ms duration for single pulse experiments; 5-20 ms for BLA train experiments) 500 ms before BLA stimulation over a 5 min period. This resulted in delivery of approximately 45 nl of aCSF or drug. A maximum of two sites were sampled from each rat in local drug experiments. At least 45 min were allowed after drug delivery before advancing the micropipette >750 μ m ventrally to search for another recording site.

Optogenetics

Rats were anesthetized with 1-2% isoflurane. An adeno-associated virus expressing channelrhodopsin-2-YFP under the control of the CaMKII promoter was bilaterally microinjected into the BLA (AP: -3.0 mm; ML: \pm 5.0 mm; DV: -7.4 mm from dura; 0.5 μ l). This viral construct has been previously shown to infect excitatory projection neurons in the amygdala (Yizhar et al., 2011). Electrophysiological experiments were conducted 7-9 weeks after viral infection. These experiments were conducted as described above with the exception that an optical fiber (100 μ m) coupled to a laser diode (472 nm emission; Thor Labs) was fixed to the drug pipette (350-400 μ m above the recording electrode tip) to optically activate ChR2-expressing BLA terminals in the mPFC and concomitantly eject drug near the recording site as described above. Drug was ejected similar to electrical stimulation experiments with the exception that Chicago Sky Blue was not included to circumvent any effects of the dye on light spread. Optical

stimulation consisted of a square pulse of blue light (8-15 mW) with a duration of 1 ms delivered at 0.2 Hz.

Drugs

U69,593, nor-BNI were supplied by the Research Technology Branch of NIDA (Rockville, MD). CNQX and AP-5 were purchased from Sigma-Aldrich. Stock solutions of U69,593 were dissolved in 0.1 N HCl, then subsequently brought to a pH of 7.4 with NaOH. CNQX, AP-5, and U69,593 were diluted from stock solutions into aCSF. Nor-BNI was dissolved in saline.

Histology

Upon termination of *in vivo* microdialysis testing, animals were anesthetized with Equithesin and a microdialysis probe with dye on the active membrane was manually inserted into the microdialysis cannula to determine membrane location. Brains were removed, frozen, and sectioned (40 μ m). Histological verification of the location of the active membrane of the microdialysis probe in rats and mice was obtained from coronal sections (Fig. 2.1). For *in vivo* electrophysiology experiments, brains were postfixed overnight in 4% paraformaldehyde, rinsed in PBS, and switched to 30% sucrose with 1% azide. Sections (40-50 μ m) were obtained using a freezing microtome. During sectioning, the location of Chicago Sky Blue as an index of the recording (not drug diffusion) site was verified. Sections were Nissl-stained, cover-slipped, and examined under a microscope to verify stimulating electrode placements and recording microelectrode track placement.

Data Analyses

In vivo microdialysis data were analyzed using repeated measures ANOVA with sample-type (i.e. baseline, drug infusion, washout samples) and time (i.e. consecutive samples within a sample-type; e.g. baseline 1, baseline 2, baseline 3, etc) as within-subjects factors and drug/treatment as a between subjects factor. Since we were interested in changes in response to pharmacological manipulations only sample-type (i.e. baseline, drug infusion, washout samples) and drug effects were further explored. Area under the curve (AUC) values was obtained using a standard trapezoidal method from an equal number of basal and drug samples represented (Rawls and McGinty, 1998; Chefer et al., 2005). AUC values were analyzed using one-way ANOVA. Post-hoc analyses were carried out using Fisher's LSD.

In-vivo electrophysiology time-course experiments for systemic administration of U69,593 was analyzed using a two-way ANOVA with time as a within subjects factor and stimulation site as a between subjects factor. The average percent change from baseline for the 15 min to 45 min period after systemic U69,593 treatment was analyzed using Student's *t*-test. Time-course experiments for pressure ejection experiments were analyzed using a two-way ANOVA with time as a within subjects and drug as a between subjects factor. A one-way ANOVA or Student's *t*-test was conducted on the average percent of baseline in the five minute period after the last pressure ejection. Train stimulation inhibition data were analyzed using repeated measures ANOVA with pulse number as a within subjects factor and frequency as a between subjects factor. BLA train plasticity results were analyzed using repeated measures ANOVA with drug and pulse number as within subjects factors. Post-hoc analyses were carried out utilizing a Bonferroni correction.

Results

mPFC KORs do not modulate basal, extracellular glutamate and GABA levels

We first determined whether KOR activation or antagonism would modify basal mPFC glutamate and GABA levels. Intra-mPFC perfusion of U69,593 (1 μ M) and nor-BNI (1 μ M) were without effect on local glutamate and GABA overflow in rats (Fig 3.1). Analysis of extracellular GABA (Fig 3.1A) did not reveal a main effect of sample-type (i.e. baseline, drug infusion, washout samples; $F_{(2,140)}=0.947$; $p=0.401$), treatment ($F_{(1,14)}=0.108$; $p=0.748$), or a sample-type x treatment interaction ($F_{(2,140)}=2.456$; $p=0.105$). Analysis of extracellular glutamate (Fig 3.1B) did not reveal a main effect of sample-type (i.e. baseline, drug infusion, washout samples; $F_{(2,140)}=2.672$; $p=0.087$), treatment (U69,593 or nor-BNI; $F_{(1,14)}=0.27$; $p=0.87$), or a sample-type x treatment interaction ($F_{(2,140)}=1.093$; $p=0.349$).

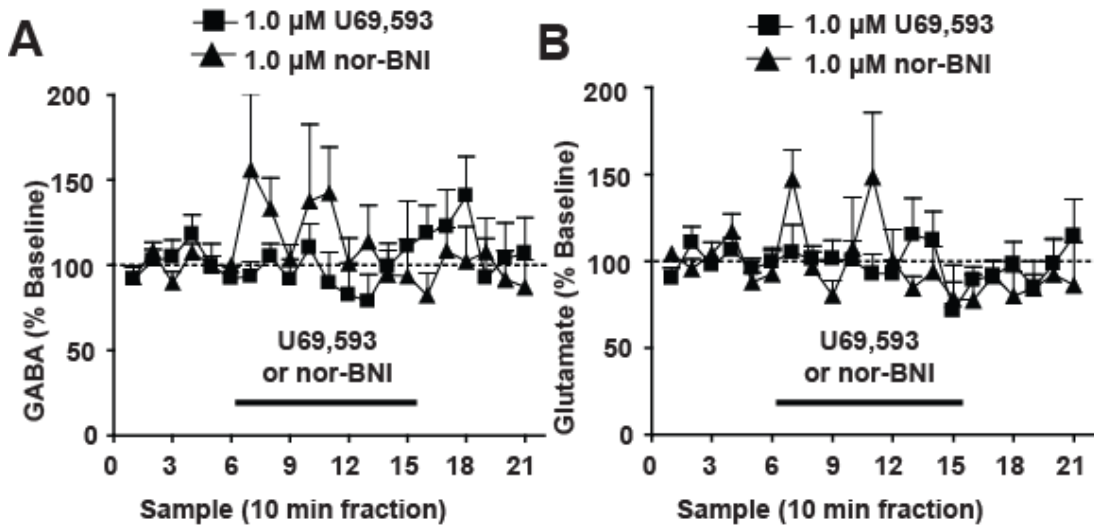


Figure 3.1: *mPFC KORs do not modulate basal dialysate GABA and glutamate levels in the mPFC.* **A**, Time course of mPFC extracellular GABA levels after reverse dialysis administration of 1.0 μ M U69,593 (filled squares; $n=10$) or 1.0 μ M nor-BNI (filled triangles; $n=6$). **B**, Time course of mPFC basal dialysate glutamate levels after reverse dialysis administration of 1.0 μ M U69,593 (filled squares; $n=10$) or 1.0 μ M nor-BNI (filled triangles; $n=6$).

mPFC KORs inhibit tPDC-evoked glutamate overflow

Extracellular glutamate levels are buffered by rapid reuptake potentially masking inhibitory effects of KORs (Herrera-Marschitz et al., 1996; Rawls and McGinty, 1997). The glutamate reuptake blocker tPDC produces TTX- and Ca^{2+} -sensitive elevations in dialysate glutamate (Herrera-Marschitz et al., 1996; Rawls and McGinty, 1997; Kreuter et al., 2004). Therefore, we assessed the effects of mPFC U69,593 on tPDC-evoked elevations in extracellular glutamate. Intra-mPFC perfusion of U69,593 (0.5 and 1.0 μM) decreased tPDC-induced elevation in dialysate glutamate in rats (Fig. 3.2). Repeated measures ANOVA revealed a main effect of sample-type (baseline, tPDC perfusion period; $F_{(1,51)}=50.368$; $p<0.001$), no main effect of treatment (tPDC alone, 0.5 or 1.0 μM U69,593/tPDC; $F_{(1,51)}=2.371$; $p=0.124$), and a significant sample-type x treatment interaction ($F_{(2,51)}=7.066$; $p=0.006$; Fig 3.2A). ANOVA of AUC values (of the tPDC sample period) revealed an effect of treatment ($F_{(2,19)}=6.984$; $p=0.006$; Fig 3.2B). Post-hoc analysis revealed that both reverse dialysis of 0.5 and 1.0 μM U69,593 attenuated tPDC-evoked glutamate levels ($p<0.05$). Thus, glutamate reuptake blockade unmasks inhibitory effects of mPFC KORs on local extracellular glutamate.

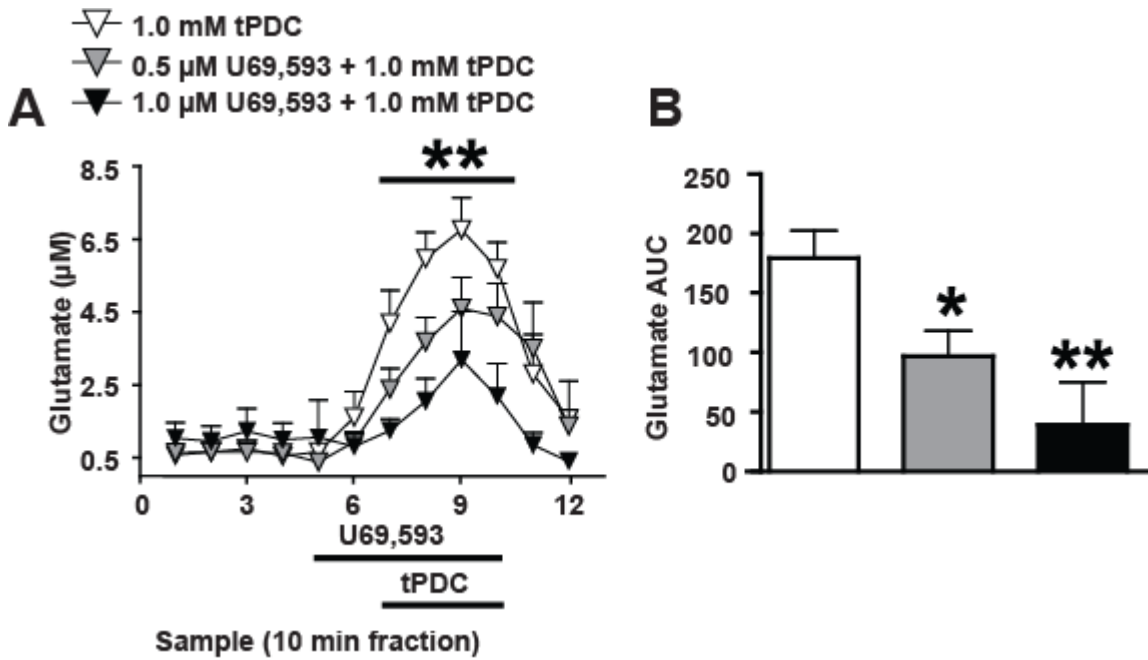


Figure 3.2: mPFC glutamate reuptake blockade unmasks the inhibitory role of KOR on extracellular glutamate levels. *A*, Time course of mPFC extracellular glutamate levels in response to local administration of tPDC in rats pretreated with vehicle (white triangles; n=8), 0.5 μM U69,593 (grey triangles; n=8), or 1.0 μM U69,593 (black triangles; n=4). (**) reflects a significant sample-type x treatment interaction. *B*, AUC values of tPDC-evoked elevations in dialysate glutamate levels after vehicle, 0.5 μM U69,593, or 1.0 μM U69,593 pretreatment. (*) and (**) reflect a significant difference from vehicle controls.

KOR activation attenuates tPDC-evoked elevations in extracellular GABA

If KOR is inhibiting tPDC-induced elevations in extracellular glutamate then subsequent changes in mPFC GABA will be prevented by KOR activation. Interestingly, tPDC did elevate extracellular GABA levels, an effect blocked by 0.5 and 1.0 μM U69,593 pretreatment (Fig. 3.3). Repeated measures ANOVA showed no main effect of sample-type (baseline, tPDC perfusion period; $F_{(1,51)}=1.265$; $p=0.276$), a main effect of treatment (tPDC alone, 0.5 or 1.0 μM U69,593/tPDC; $F_{(2,17)}=4.343$; $p=0.03$), and a sample-type x treatment interaction ($F_{(2,51)}=4.492$; $p=0.027$). Thus, mPFC KORs inhibit local glutamate overflow, and prevent glutamate-induced elevations in extracellular GABA levels.

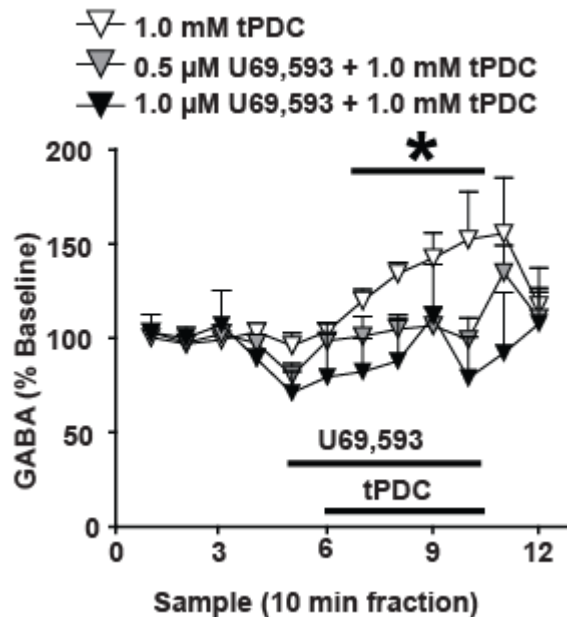


Figure 3.3: KOR activation blocks tPDC-evoked elevations in mPFC GABA levels. Time course of mPFC GABA dialysate levels in response reverse dialysis of tPDC in animals pretreated with vehicle (white triangles; $n=8$), 0.5 μM U69,593 (grey triangles; $n=8$), or 1.0 μM U69,593 (black triangles; $n=4$). (**) reflects a significant sample-type x treatment interaction. Data points reflect the mean \pm SEM. Black bar depicts period of infusion of the indicated drug.

KOR antagonism fails to modify tPDC-induced elevation in extracellular glutamate

To determine whether KOR tonically inhibited mPFC glutamate levels, we administered nor-BNI via reverse dialysis. Nor-BNI infusion (1.0 μ M) in the presence of the glutamate reuptake blocker tPDC, failed to modify the tPDC-induced extracellular glutamate response (Fig. 3.4). This would suggest that a dose of nor-BNI effective in revealing tonic KOR regulation of DA did not modify tPDC-evoked elevations in extracellular glutamate. It is also possible that there is a ceiling effect and nor-BNI is not able to further elevate glutamate levels beyond that of tPDC alone.

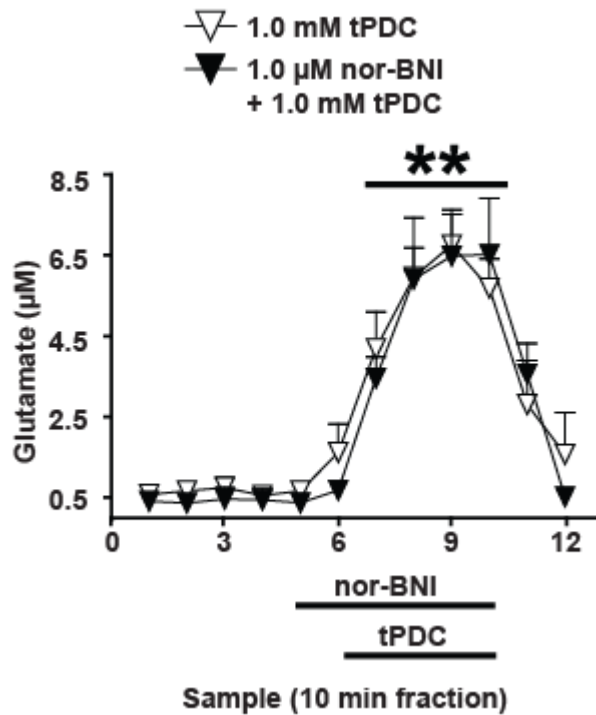


Figure 3.4: KOR antagonism fails to modify tPDC-evoked elevations in dialysate glutamate. Time course of mPFC glutamate dialysate levels in response reverse dialysis of tPDC in animals pretreated with vehicle (white triangles; n=8) or 1.0 μ M U69,593 (black triangles; n=6). Data points reflect the mean \pm SEM. Black bar depicts period of infusion of the indicated drug.

The glutamatergic BLA to mPFC pathway

Our results that U69,593 decreased tPDC-evoked elevations in dialysate glutamate suggest that KORs inhibit synaptic sources of glutamate. Dr. Danielle Counotte, a post-doctoral fellow in the O'Donnell laboratory, utilizing whole-cell recordings from layer V pyramidal neurons in mPFC slices demonstrated that U69,593 produced a decrease in mEPSP frequency, but not amplitude, in a nor-BNI-sensitive manner (Tejeda et al., 2013). This suggests KORs do indeed inhibit glutamate synaptic transmission via a presynaptic site of action. However, from these results it is not clear which glutamate terminals were being inhibited by mPFC KORs. We sought to focus on the BLA-mPFC pathway as the BLA is rich in KOR mRNA, and thus could be a pathway that is modulated by this system.

Anatomical evidence suggests that BLA afferents in the mPFC form asymmetric synapses onto post-synaptic targets, suggesting that this pathway utilizes glutamate to influence mPFC activity (McDonald, 1996). We have also shown that BLA electrical stimulation evokes EPSPs in pyramidal neurons with early components that do not have a clear reversal potential even at depolarized states (Dilgen et al., 2013), which is consistent with the notion that the BLA to mPFC pathway releases glutamate onto its targets in the mPFC. However, the glutamatergic nature of the BLA-mPFC pathway had not been previously tested *in vivo*. We first determined the effect of CNQX/AP-5 pressure ejection on BLA-evoked responses to confirm that BLA-evoked fEPSP responses in mPFC were mediated by glutamatergic transmission. The onset latency of BLA-evoked responses was less than 10 ms, consistent with a monosynaptic response. Pressure ejection of approximately 45 nl of CNQX/AP-5 significantly decreased the fEPSP slope, while aCSF

pressure ejection was without effect. Repeated measures ANOVA revealed a main effect of time ($F_{(49,686)}=8.08$; $p<0.0001$), a main effect of drug ($F_{(1,686)}=24.95$; $p=0.0002$), and a time x drug interaction ($F_{(49,686)}=4.54$; $p<0.0001$). A comparison of the fEPSP slope (expressed as a percentage of baseline) in the 5 min bin after pressure ejection between aCSF and CNQX/AP-5 groups revealed a significant difference ($t_{(16)}=6.117$; $p<0.0001$). Thus, pressure ejection confirmed that the slope of the BLA-evoked response is mediated by glutamatergic transmission, consistent with *in vivo* intracellular recordings (Dilgen et al., 2013) and anatomical evidence (McDonald, 1996). These results also suggest that this approach for local drug delivery reliably affects the area where the fEPSP is originated, suggesting that CNQX/AP-5 was able to reach the majority of synapses that were mediating the glutamatergic synaptic response.

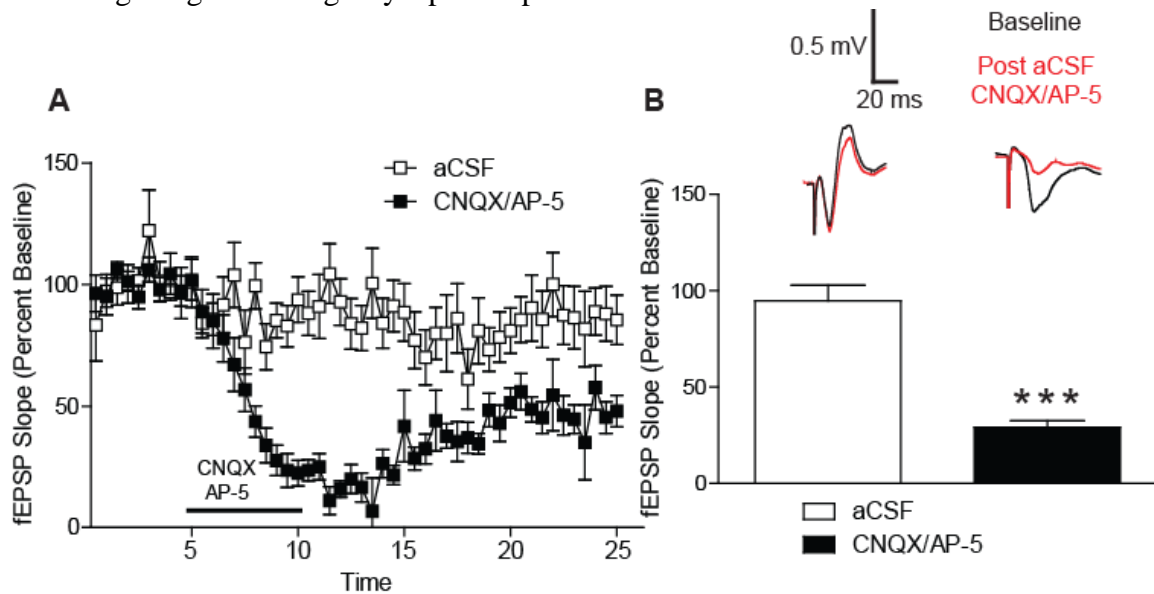


Figure 3.5: BLA-evoked synaptic responses are blocked by pressure ejected glutamate receptor antagonists. **A**, Time course of the effects of pressure ejected aCSF (white squares; n=11 sites in 7 rats) or CNQX/AP-5 (black squares; n=7 sites in 5 rats) on BLA-evoked fEPSP slope. **B**, *Top*, Representative traces of BLA-evoked fEPSPs at baseline (black trace) and after aCSF or CNQX/AP-5 (red trace) above their corresponding group. Scale bars: 0.5 mV and 20 ms. *Bottom*, Mean fEPSP slope expressed as a percentage of baseline in the 5 min window after the last puff of aCSF or CNQX/AP-5. (***) reflects a significant difference from aCSF controls. Data points reflect the mean \pm SEM. Black bar depicts period of CNQX/AP-5 pressure ejection.

Systemic KOR activation inhibits the BLA to PFC, but not the hippocampus to PFC, pathway.

We next determined the effects of systemic U69,593 on glutamatergic synaptic transmission the BLA-mPFC. We rationalized that since KOR mRNA expression is largely absent in the output regions of the hippocampal formation (Meng et al., 1993; DePaoli et al., 1994; Paxinos and Franklin, 2001; Van't Veer et al., 2013) that this pathway would not be modulated by KORs. Thus, the hippocampus to mPFC pathway was utilized as a within subjects control. In these experiments, the fornix, the fiber bundle containing ventral hippocampal axons that innervate the mPFC, were stimulated. Systemic U69,593 administration inhibited glutamatergic synaptic transmission in the BLA to mPFC pathway, without modifying synaptic transmission in the hippocampus to mPFC pathway (Fig. 2). Repeated measures ANOVA revealed no effect of time ($F_{(5,95)}=1.76$; $p=0.1286$), a main effect of stimulation site ($F_{(1,95)}=9.58$; $p=0.006$), and a time x stimulation site interaction ($F_{(5,95)}=4.74$; $p=0.0007$). Post-hoc analysis of the mean percent of baseline fEPSP slope of BLA and fornix responses 15-45 min after systemic U69,593 administration revealed that BLA synaptic responses were inhibited relative to fornix responses ($t_{(19)}=3.06$; $p<0.0064$). These results suggest that KORs in the PFC inhibit glutamatergic synaptic transmission from limbic afferents in a pathway-specific manner.

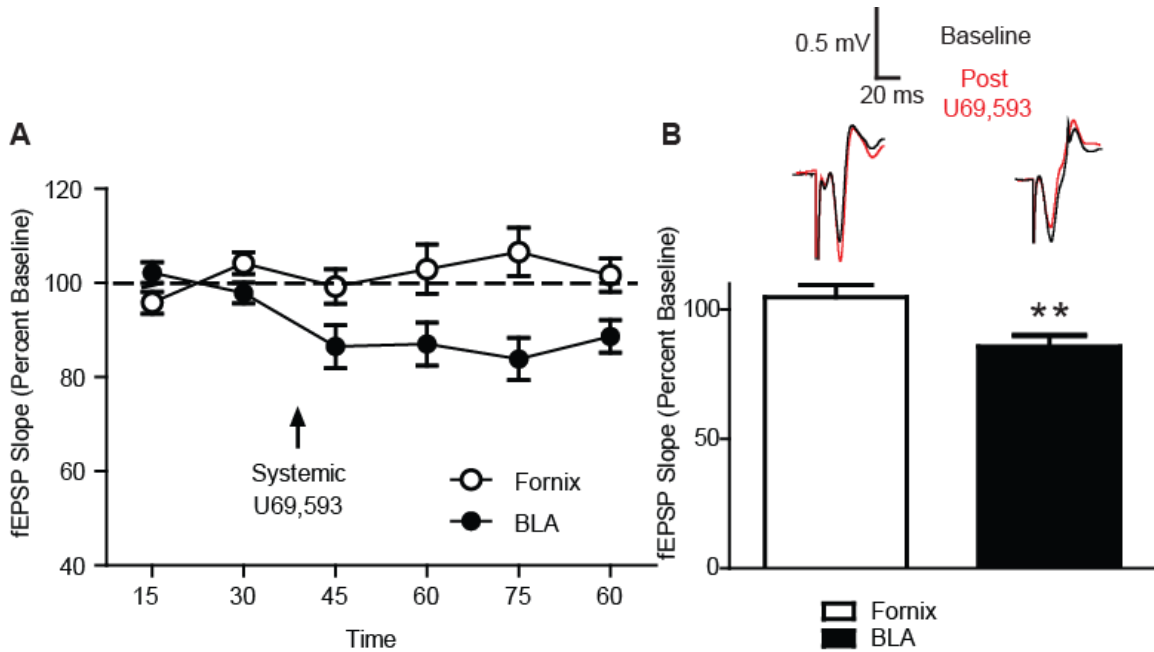


Figure 3.6: Systemic administration of a KOR agonist inhibits glutamatergic fEPSPs in a pathway-specific manner. *A*, Time course of the effects of systemic U69,593 on fornix-evoked (white circles; n=11 rats) and BLA-evoked (black circles; n=10 rats) fEPSP slope. *B*, *Top*, Representative traces of fornix- and BLA-evoked fEPSPs at baseline (black trace) and after systemic U69,593 administration (red trace) above their corresponding group. Scale bars: 0.5 mV and 20 ms. *Bottom*, Mean fEPSP slope expressed as a percentage of baseline in the 30 min window after systemic U69,593 injection. (**) reflects a significant difference from fornix-evoked synaptic responses. Data points reflect the mean \pm SEM.

mPFC KORs inhibit the BLA-PFC pathway

KOR activation in has been shown to inhibit synaptic transmission and plasticity *ex vivo* in slices containing the BLA (Huge et al., 2009a). U69,593 may be inhibiting BLA-mPFC pathway synaptic transmission by decreasing the excitability of BLA neurons. To determine whether mPFC KORs negatively modulated glutamatergic transmission in the BLA-mPFC pathway we determined the effects of U69,593 pressure ejection in the mPFC on BLA-evoked fEPSPs. We also determined whether the effect of pressure ejected U69,593 was due to selective action at the KOR in rats that had been pretreated with systemically-administered nor-BNI approximately 24 hrs prior to electrophysiological recordings. Intra-mPFC U69,593 pressure ejection inhibited BLA-evoked synaptic responses in a nor-BNI-sensitive manner (Fig. 3.7). Repeated measures ANOVA revealed a main effect of time ($F_{(49,1421)}=3.31$; $p<0.0001$), no main effect of drug ($F_{(2,1421)}=1.13$; $p=0.34$), and a significant time x drug interaction ($F_{(98,1421)}=1.33$; $p=0.02$). One-way ANOVA of the mean percent of baseline for the 5 min following U69,593 pressure ejection showed a significant effect ($F_{(2,35)}=5.191$; $p=0.011$). Post-hoc analysis showed that U69,593 inhibited BLA-evoked fEPSP slope relative to aCSF ($p<0.05$). Pretreatment with the KOR antagonist, nor-BNI, blocked the ability of pressure ejected U69,593 to inhibit BLA-evoked synaptic responses in the mPFC ($p<0.05$), suggesting that U69,593-mediated inhibition of BLA-evoked fEPSPs is due to action at the KOR. These results suggest that KORs in the mPFC negatively modulate BLA glutamatergic synaptic transmission in the BLA to mPFC pathway.

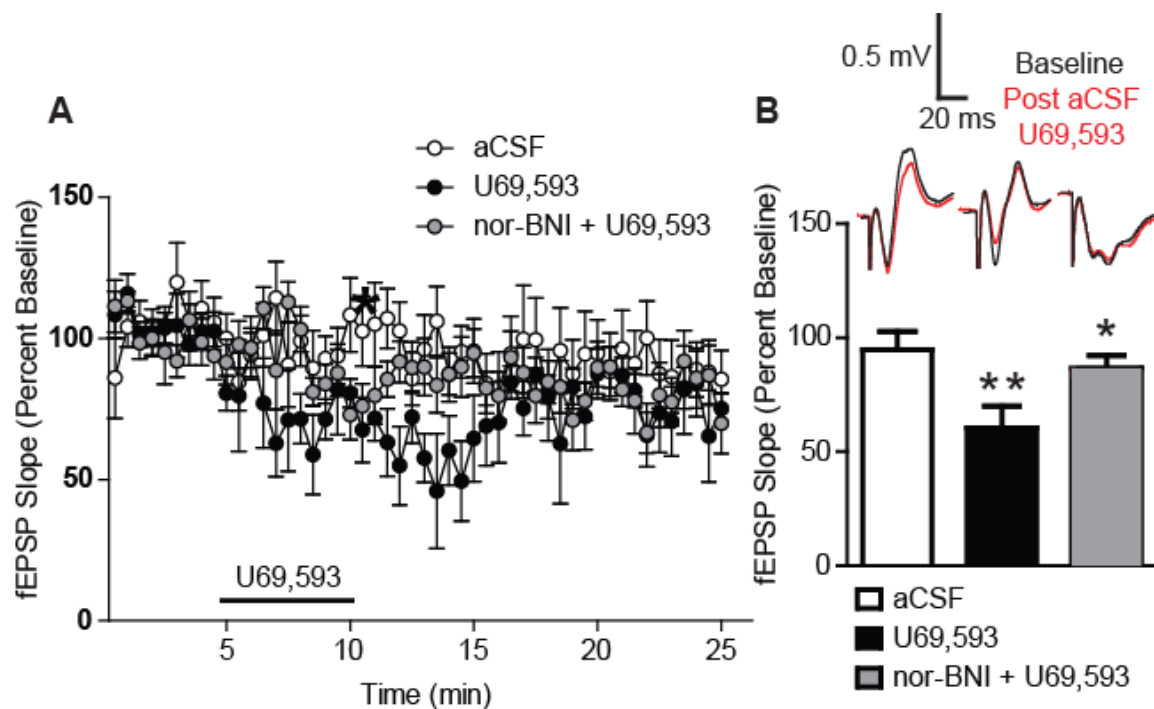


Figure 3.7: mPFC KORs negatively modulate glutamatergic synaptic transmission in the BLA to PFC pathway. *A*, Time course of the effects of pressure ejected aCSF (white circles; n=11 sites in 7 rats), U69,593 (black circles; n=13 sites in 7 rats), or U69,593 in nor-BNI pre-treated rats (grey circles; n=12 sites in 7 rats) on BLA-evoked fEPSP slope. *B*, *Top*, Representative traces of BLA-evoked fEPSPs at baseline (black trace) and after aCSF or U69,593 (red trace) above their corresponding group. Scale bars: 0.5 mV and 20 ms. *Bottom*, Mean fEPSP slope expressed as a percentage of baseline in the 5 min window after the last puff of aCSF or U69,593 in rats pretreated with saline or nor-BNI approximately 24 hrs prior to electrophysiological recordings. (**) reflects a significant difference from aCSF controls. (*) reflects a significant difference between saline- and nor-BNI-pretreated rats that were challenged with U69,593 pressure ejection. Data points reflect the mean \pm SEM. Black bar depicts period of U69,593 pressure ejection.

mPFC KOR activation produces a downward shift in the mPFC response curve to BLA stimulation.

The inhibitory effects of mPFC KOR activation may potentially be different depending on stimulation intensity. We therefore determined the effects of mPFC KOR activation on increasing stimulation intensities. Increasing current intensities increased the slope of BLA-evoked fEPSPs (Fig. 3.8), with maximal responses at 1.0 mA. U69,593 produced a downward shift in the mPFC response curve to increasing intensities of BLA stimulation. Repeated measures ANOVA revealed a main effect of stimulation intensity ($F_{(4,36)}=102.54$; $p<0.0001$), a main effect of drug ($F_{(1,36)}=13.33$; $p=0.005$), and a stimulation intensity x drug interaction ($F_{(4,36)}=6.98$; $p<0.0001$). Post hoc test revealed a significant difference at 0.6-1.0 mA intensities ($p<0.05$), but not at lower current intensities. This suggests that increasing current intensity does not overcome intra-mPFC U69,593-mediated inhibition of the BLA to mPFC pathway.

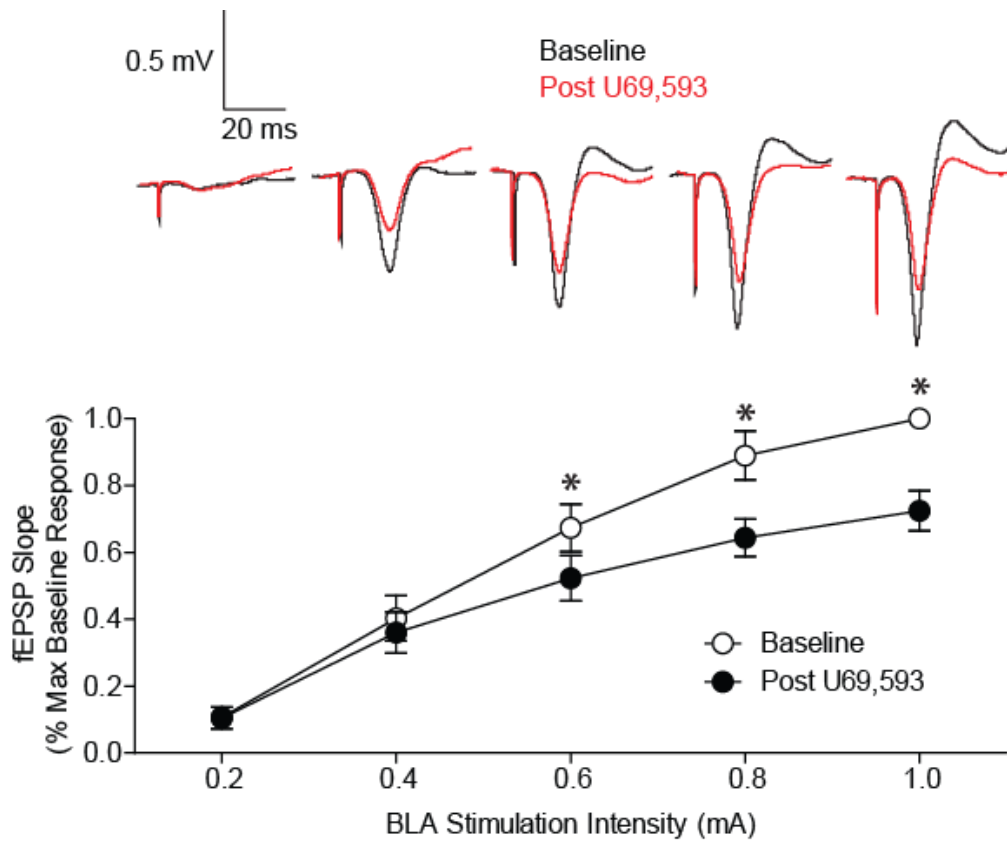


Figure 3.8: mPFC KOR activation produces a downward shift in the mPFC response curve to increasing BLA stimulation intensities. *Top*, Representative traces of BLA-evoked fEPSPs in the mPFC with increasing BLA stimulation intensity (0.2-1.0 mA) at baseline (black trace) or after U69,593 administration (red trace). Scale bars: 0.5 mV and 20 ms. *Bottom*, Mean BLA-evoked fEPSP normalized to maximal response during baseline at different stimulation intensities (n=10 sites in 10 rats). Asterisks (*) reflect a significant difference from baseline post U69,593 pressure ejection. Data points reflect the mean \pm SEM.

BLA pulse train stimulation does not overcome the inhibitory effects of mPFC KOR activation.

It is possible that mPFC KORs may negatively modulate the BLA-mPFC in an activity-dependent manner. We determined the effects of U69,593 pressure ejection on BLA train-evoked fEPSPs. Intra-mPFC U69,593 pressure ejection similarly inhibited each pulse in a 20 Hz and 40 Hz, BLA train (10 pulses; Figure. 3.9). Repeated measures ANOVA of normalized responses did not reveal an effect of frequency ($F_{(1,171)}=1.28$; $p=0.27$), pulse number ($F_{(9,171)}=0.97$; $p=0.47$), or frequency x pulse number interaction ($F_{(9,171)}=1.2$; $p=0.3$). These results suggest that the inhibitory effects of mPFC activation cannot be overcome by BLA pulse train stimulation. Moreover, the inhibitory effects of U69,593 on BLA-evoked fEPSPs were similar for both the first pulse of the 20 and 40 Hz BLA train and that of single pulse stimulation ($F_{(2,34)}=0.16$; $p=0.85$), suggesting that the stimulation protocol itself does not modify KOR modulation of the BLA to mPFC pathway.

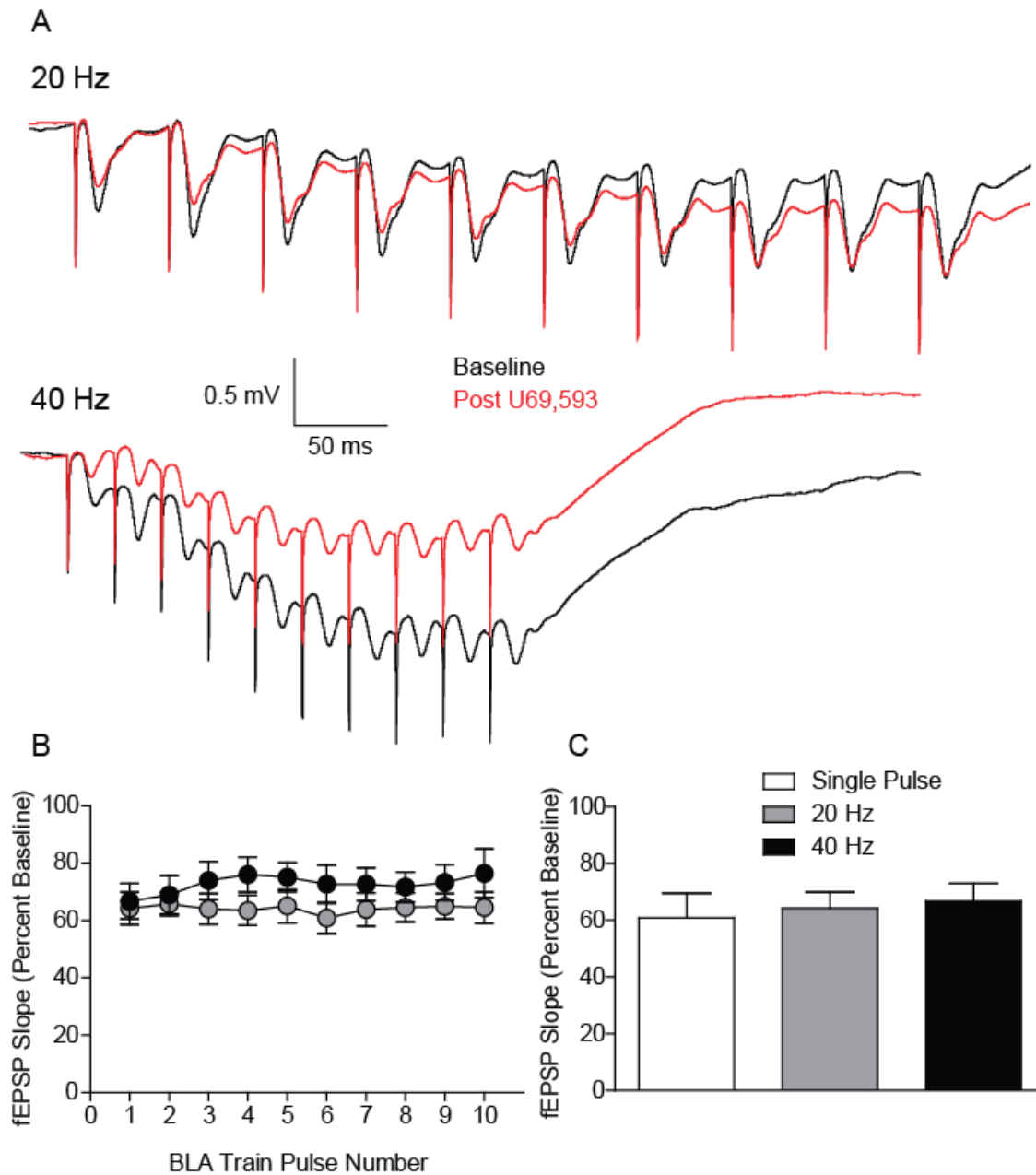


Figure 3.9: KOR-mediated inhibition is not overcome by BLA pulse train stimulation. **A**, Top, Representative traces of 20 and 40 Hz BLA train-evoked fEPSPs in the mPFC during baseline (black traces) or after U69,593 pressure ejection (red traces). Scale bars: 0.5 mV and 20 ms. **B**, The fEPSP slope of each pulse in the BLA train after drug was normalized to the corresponding baseline pulse (i.e. $P1_{\text{postU69,593}}/P1_{\text{baseline}}$, $P2_{\text{postU69,593}}/P2_{\text{baseline}}$, etc.). Mean normalized BLA-evoked fEPSP slope for each pulse in the 20 Hz (n=11 sites in 8 rats) or 40 Hz (n= 10 sites in 10 rats) BLA train. **C**, Mean normalized BLA-evoked fEPSP slope for single pulse BLA stimulation and the first pulse in the 20 or 40 Hz BLA train. Data points reflect the mean \pm SEM.

Intra-mPFC U69,593 administration does not modify short-term plasticity in BLA-evoked synaptic responses.

We subsequently determined whether U69,593 modified short-term plasticity in the BLA-mPFC pathway elicited by either 20 or 40 Hz BLA train stimulation. Facilitation of the fEPSP slope was observed at early pulses in the 20 Hz train (Fig. 3.10). U69,593 pressure ejection did not modify short-term synaptic plasticity evoked by a 20 Hz BLA train. Repeated measures ANOVA revealed no main effect of drug ($F_{(1,80)}=0.52$; $p=0.49$), an effect of pulse number ($F_{(8,80)}=4.99$; $p<0.0001$), but no drug x pulse number interaction ($F_{(8,80)}=1.03$; $p=0.42$). Longer-lasting facilitation of BLA-evoked synaptic responses was present with 40 Hz train stimulation at baseline compared to facilitation observed with a 20 Hz BLA train. U69,593 enhanced short-term plasticity of fEPSPs elicited by a 40 Hz BLA pulse train. Repeated measures revealed a main effect of drug ($F_{(1,72)}=6.71$; $p=0.03$), a main effect of pulse ($F_{(8,72)}=14.76$; $p<0.0001$), but not a drug x pulse interaction ($F_{(8,72)}=1.26$; $p=0.27$). Therefore, mPFC KOR activation appears to produce a slight, but significant, enhancement in facilitation during a 40 Hz, but not 20 Hz, BLA pulse train. These results suggest that although mPFC KORs uniformly inhibit fEPSPs evoked by a 20 and 40 Hz BLA train, it only modifies plasticity produced by the 40 Hz BLA train.

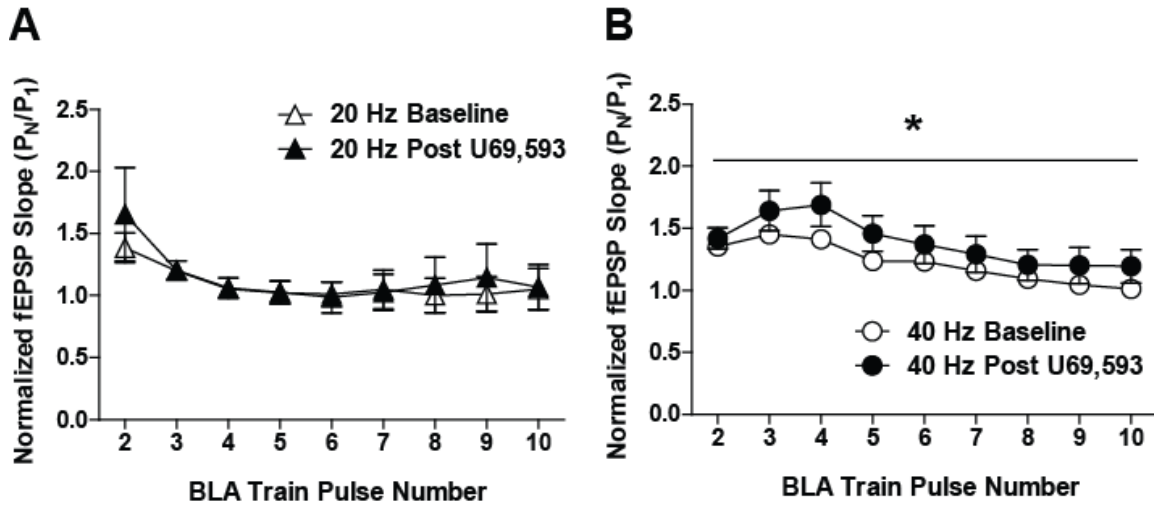


Figure 3.10: BLA-evoked short-term synaptic plasticity is not modulated by mPFC KORs. *A*, The slope of each pulse in the BLA train was normalized to the slope of the first pulse of the train. The normalized slope is plotted against the pulse number of the train (n=11 sites in 8 rats). *B*, The slope of each pulse in the BLA train was normalized to the slope of the first pulse of the train (n= 10 sites in 10 rats). Asterisk (*) reflects a main effect of drug, indicating that U69,593 significantly increased facilitation with the BLA train at 40 Hz independent of pulse number. Scale bars 0.5 mV, 20 ms. Data points reflect the mean \pm SEM.

Optically-evoked BLA to mPFC synaptic responses are glutamatergic

Intra-BLA ChR2-AAV injection resulted in robust expression of YFP in the BLA. Optical stimulation near the recording site evoked fEPSPs with a shorter onset-latency and time-to-peak than fEPSPs evoked by BLA electrical stimulation presumably due to a lack of action potential conduction from the soma to the terminal. We verified that optically-evoked responses were glutamatergic in nature by determining the effects of pressure ejection of CNQX/AP-5. CNQX/AP-5 pressure ejection inhibited optically-evoked fEPSPs (Fig. 3.12). Repeated measures ANOVA revealed a main effect of time ($F_{(49,539)}=12.35$; $p<0.0001$), a main effect of drug ($F_{(1,539)}=22.77$; $p=0.0006$), and a significant time x drug interaction ($F_{(49,539)}=6.28$; $p<0.0001$). A comparison of the fEPSP slope (expressed as a percentage of baseline) in the 5 min bin after pressure ejection between aCSF and CNQX/AP-5 groups revealed a significant difference ($t_{(11)}=6.659$; $p<0.0001$). Thus, optical stimulation of ChR2-expressing BLA terminals evokes monosynaptic fEPSPs dependent on fast glutamatergic signaling.

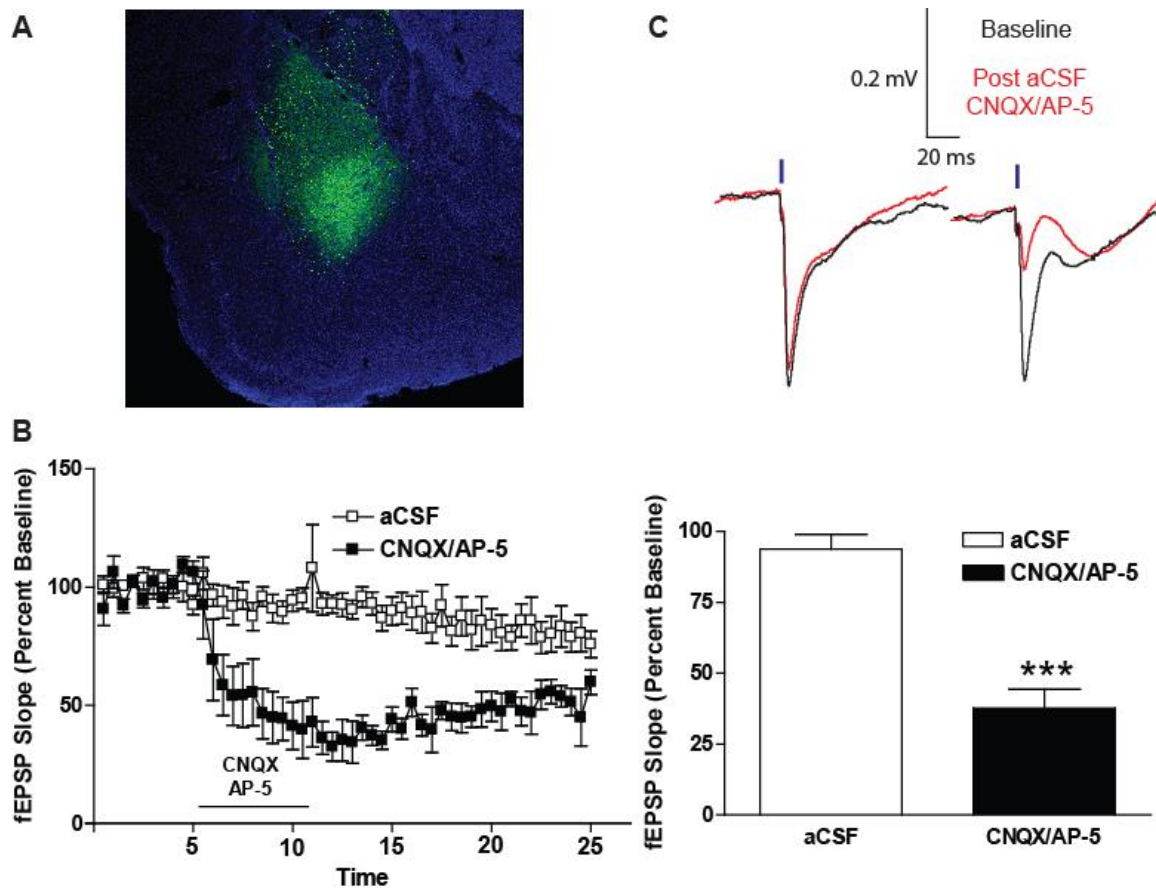


Figure 3.11: Optically-evoked synaptic responses are blocked by CNQX/AP-5 pressure ejection. **A**, Photomicrograph of ChR2-YFP expression in the BLA (Green) and DAPI-stained nuclei (Blue). **B**, Time course of the effects of pressure ejected aCSF (white squares; n=8 sites in 4 rats) or CNQX/AP-5 (black squares; n=5 sites in 3 rats) on BLA optically-evoked fEPSP slope. **B, Top**, Representative traces of BLA-evoked fEPSPs at baseline (black trace) and after aCSF or CNQX/AP-5 (red trace) above their corresponding group. Scale bars: 0.5 mV and 20 ms. **Bottom**, Mean fEPSP slope expressed as a percentage of baseline in the 5 min window after the last puff of aCSF or CNQX/AP-5. (***) reflects a significant difference from aCSF controls. Data points reflect the mean \pm SEM. Black bar depicts period of CNQX/AP-5 pressure ejection. Blue line depicts 1 ms blue light pulse.

mPFC KOR activation inhibits optogenetic activation of the BLA to mPFC pathway

BLA electrical stimulation likely results in activation of fibers of passage and/or antidromic activation of mPFC efferents in addition to activation of BLA projection neurons, which may contribute to the fEPSP slope of the evoked response in the mPFC. It is possible that U69,593 is inhibiting glutamatergic transmission in fibers of passage or antidromically activated mPFC pyramidal neurons. To determine whether mPFC KORs specifically inhibit BLA to mPFC synaptic transmission we determined the effects of U69,593 pressure ejection on synaptic responses evoked by optical activation of BLA terminals in the mPFC. U69,593 pressure ejection inhibited optically-evoked fEPSPs (Fig. 3.13). Repeated measures ANOVA revealed a main effect of time ($F_{(49,735)}=9.83$; $p<0.0001$), a main effect of drug ($F_{(1,735)}=12.76$; $p=0.0028$), and a significant time x drug interaction ($F_{(49,735)}=3.11$; $p<0.0001$). A comparison of the fEPSP slope (expressed as a percentage of baseline) in the 5 min bin after pressure ejection between aCSF and U69,593 groups revealed a significant difference ($t_{(15)}=5.099$; $p<0.0001$). These results suggest that mPFC KORs selectively inhibit BLA afferents to the mPFC.

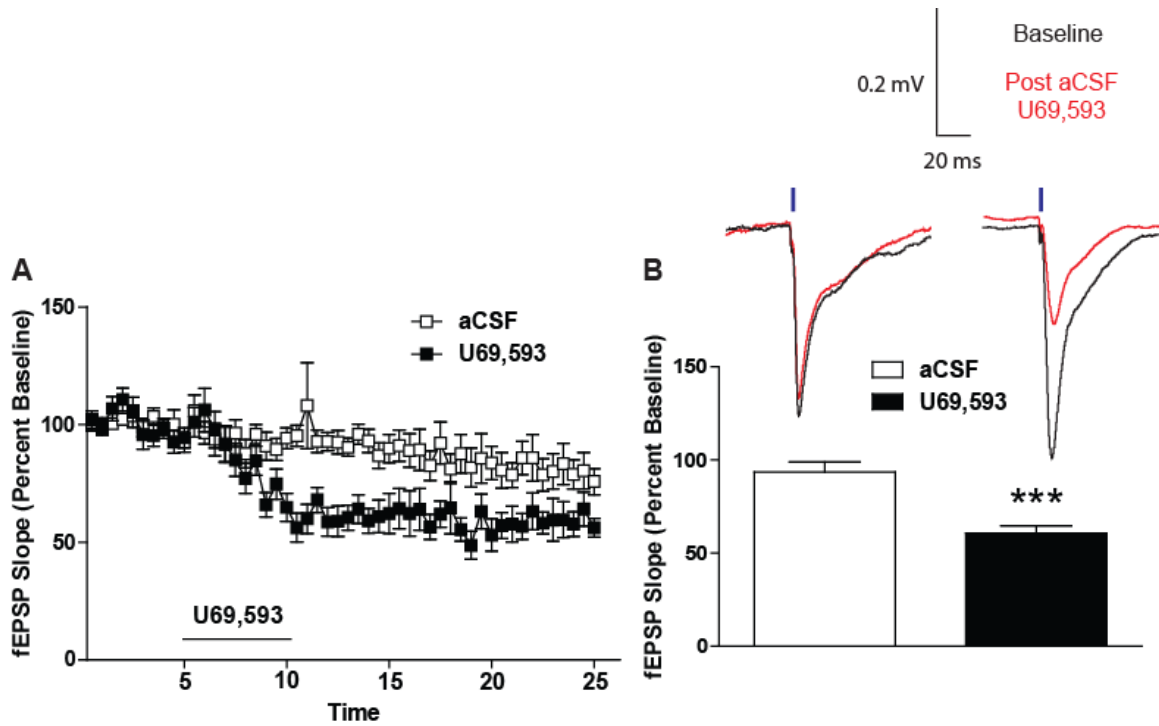


Figure 3.12: 2 A, Time course of the effects of pressure ejected aCSF (white circles; n=8 sites in 4 rats) or U69,593 (black circles; n=9 sites in 6 rats), on BLA optically-evoked fEPSP slope. **B**, *Top*, Representative traces of BLA-evoked fEPSPs at baseline (black trace) and after aCSF or U69,593 (red trace) above their corresponding group. Scale bars: 0.5 mV and 20 ms. *Bottom*, Mean fEPSP slope expressed as a percentage of baseline in the 5 min window after the last puff of aCSF or U69,593. (***) reflects a significant difference from aCSF controls. Data points reflect the mean \pm SEM. Black bar depicts period of U69,593 pressure ejection. Blue line depicts 1 ms blue light pulse.

Chapter III Summary

Here we find that mPFC KOR systems negatively modulate glutamatergic BLA inputs to the mPFC. We rationalized that if KORs were found on glutamatergic terminals from afferents in the mPFC then extracellular glutamate should be negatively regulated by KOR activation. We first found that intra-mPFC U69,593 did not modify basal levels of extracellular glutamate. However, dialysate glutamate levels may not be indicative of synaptic sources of glutamate due to rapid reuptake of glutamate by excitatory amino acid transporters. Using the glutamate transporter blocker, tPDC, we unmasked the inhibitory effects of U69,593 on extracellular glutamate. Moreover, reverse dialysis U69,593 prevented tPDC-induced rises in mPFC GABA levels, suggesting that glutamate-driven increases in GABA could be blocked by local KORs. Dr. Danielle Counotte in our laboratory collaborated with me on my project by conducting whole-cell recordings in mPFC slices and determining the effects of U69,593 on spontaneous mEPSPs. Dr. Counotte demonstrated that KOR activation inhibits glutamatergic synapses via a presynaptic site of action. Collectively, these results suggested that KORs inhibit glutamatergic synaptic transmission in the mPFC. However, from these results it was not clear which glutamatergic afferents are inhibited by KOR.

We predicted that the glutamatergic BLA inputs to the PFC would be inhibited by KOR systems, as high levels of KOR mRNA expression and immunoreactivity are present in the BLA (Meng et al., 1993; DePaoli et al., 1994; Paxinos and Franklin, 2001; Van't Veer et al., 2013). We confirmed the glutamatergic nature of the BLA to mPFC pathway using glutamate receptor antagonists with either electrical stimulation of the

BLA or optical stimulation of Chr2-expressing BLA terminals in the mPFC. Our finding that systemically-administered U69,593 inhibited BLA-evoked synaptic responses was consistent with the hypothesis that KOR signaling would play an inhibitory function in the BLA to PFC pathway. Moreover, this effect was not present in the hippocampus to mPFC pathway, suggesting that KOR regulation of glutamatergic limbic afferents to the mPFC is pathway specific. To determine whether KOR activation in the mPFC would be sufficient to inhibit glutamatergic synaptic transmission in the BLA to mPFC pathway we examined the effects of mPFC KOR activation on BLA to mPFC glutamatergic transmission. Pressure ejection of U69,593 inhibited BLA-evoked glutamatergic fEPSPs in the mPFC in a nor-BNI-sensitive manner and produced a downward shift in the input-output curve of mPFC responses to BLA stimulation. The inhibitory effects of KOR stimulation of BLA to mPFC glutamatergic transmission is not overcome by BLA train stimulation. Intra-mPFC U69,593 uniformly inhibited evoked responses associated with each pulse of the 20 or 40 Hz BLA train. Medial PFC KOR activation did not modify short-term plasticity of synaptic efficacy within a 20 Hz BLA pulse train, while a slight enhancement of facilitation was observed with a 40 Hz BLA train. As BLA electrical stimulation can recruit non-BLA-mPFC excitatory responses in the mPFC we determined whether mPFC KOR activation would inhibit mPFC synaptic responses evoked by optogenetic activation of local BLA terminals. Similar to electrical stimulation, U69,593 pressure ejection inhibited optically-evoked BLA synaptic responses in the mPFC, suggesting that U69,593 action is specific to BLA terminals. Collectively, these results indicate that KORs in the mPFC negatively regulate extracellular glutamate levels and long-range glutamatergic synaptic transmission in a pathway-specific manner.

Chapter IV: Heterosynaptic Interactions Between Temporal Cortical Afferents in the Prefrontal Cortex: the Role of Kappa-Opioid Receptor Systems.³

Introduction

Finely-tuned prefrontal cortical PFC function is critical for cognition, executive function, and regulation of emotional processing (Bechara and Van Der Linden, 2005; Chudasama and Robbins, 2006; Townsend and Altshuler, 2012). Dysfunction of PFC areas has been implicated in cognitive deficits and aberrant emotional processing in psychiatric disorders (O'Donnell, 2011; Townsend and Altshuler, 2012; Chen et al., 2013). The ability of the mPFC to drive cognition and executive function is dependent on interactions with limbic and cortical structures, including the basolateral amygdala (BLA), hippocampus, and associative cortices. The BLA is critical for processing of emotional and motivational information (Bechara et al., 2003; Phelps and LeDoux, 2005). The interplay between the BLA and PFC is critical for decision making, behavioral control, and emotional processing (Floresco and Ghods-Sharifi, 2007; Churchwell et al., 2009; Kim et al., 2011b; Sotres-Bayon et al., 2012). Communication between the hippocampus and PFC is critical for cognition and requires coordinated

³ This chapter was formatted for this thesis from a manuscript prepared for *The Journal of Neuroscience*:

“Amygdala-mediated prefrontal cortex deactivation disrupts information flow in the hippocampus to prefrontal cortex pathway. Manuscript in preparation.”

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hippocampal and PFC activity (Eichenbaum, 2000; Gordon, 2011). Thus, PFC neurons must be able to drive higher-order behaviors by integrating information arriving from limbic and cortical substrates.

Activity in the amygdala and PFC exhibits an inverse relationship. Increases in BLA firing are associated with decreased PFC activity (Kim et al., 2011a; Sripada et al., 2012; Townsend and Altshuler, 2012). Opposing changes in amygdala and PFC activation have been observed during emotional processing, anxiety, and stress (Kim et al., 2011a; Veer et al., 2012). This inverse relationship has been suggested to reflect a “top-down” BLA regulation by the PFC, as cognitive control of emotional processing is associated with increased PFC activation and decreased amygdala activity (Townsend and Altshuler, 2012), although serial dependence is not clear from clinical studies. BLA stimulation inhibits the activity of the majority of PFC neurons (Perez-Jaranay and Vives, 1991; Ishikawa and Nakamura, 2003a; Floresco and Tse, 2007; Dilgen et al., 2013) via a feedforward inhibition mechanism (Dilgen et al., 2013). Indeed, BLA stimulation enhances the activity of PFC GABAergic interneurons and evokes inhibitory synaptic responses in PFC pyramidal neurons. This interaction provides a framework whereby “bottom-up” inhibition of the PFC by the BLA can occur in response to emotionally arousing stimuli via recruitment of feedforward inhibition.

Individual PFC neurons must be able to integrate hippocampus information with synaptic inputs from the BLA, but it is not clear whether strong amygdala activation affects responses to other inputs in the PFC. Both BLA and hippocampal inputs converge onto individual pyramidal neurons (Ishikawa and Nakamura, 2003a; Esmaeili and Grace, 2013), providing a means by which PFC networks can synthesize inputs from afferent

structures. Here, we investigated whether BLA-evoked inhibition in the PFC results in disruption of communication between the hippocampus and PFC and in an associative cortical-PFC pathway. We utilized *in vivo* intracellular and extracellular recordings with optogenetics to determine sub-threshold synaptic interactions in PFC neurons between BLA and hippocampus and to preserve long-range connections between these interacting networks. We tested whether the inhibitory nature of the BLA to PFC pathway results in heterosynaptic suppression of hippocampus-evoked synaptic responses, and vice-versa. As KORs mediate heterosynaptic suppression in the hippocampus we investigated the role of KOR systems in mediating inhibitory heterosynaptic interactions between the BLA and hippocampus in the mPFC.

Methods

Subjects

Male Long Evans rats weighing 300-450 g (Charles River Laboratories) were kept in an AALAC-accredited institution with food and water available *ad-libitum*. Rats were kept in a temperature- and humidity-controlled environment with lights on at 7 am and lights off at 7pm. All experiments were conducted in accordance with guidelines published in the United States Public Health Service *Guide for the Use and Care of Animals*, and all procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee.

In vivo intracellular electrophysiology

Rats were anesthetized with choral hydrate (400 mg/kg, i.p). Anesthesia throughout the experiment was maintained through recording procedures using continuous choral hydrate (24-30 mg/kg/h) via an intraperitoneal catheter. Body temperature was maintained at approximately 37°C using a thermal probe-controlled heat pad (Fine Science Tools). Rats were fixed on a stereotaxic apparatus (Kopf Instruments). Concentric bipolar stimulating electrodes (0.5 mm diameter, 0.5 mm pole separation; Rhodes Medical Instruments Inc.) were lowered into the left BLA (AP: -3.0 mm; ML: 4.9-5.0 mm; DV: -7,4 mm from dura) and left fornix (AP: -3.0 mm; ML: 3.9-4.0 mm; DV: -3.1 mm from dura). Coordinates were based on the rat brain atlas of Paxinos and Watson (1998). Recording sharp microelectrodes were pulled from borosilicate glass (1 mm O.D.; World Precision Instruments) on a horizontal Flaming-Brown puller (Sutter

Instruments). Sharp electrodes were filled with 2% Neurobiotin (Vector Laboratories) in 2 M potassium acetate. Microelectrodes were lowered into the superficial cortex using a hydraulic manipulator (Trent Wells, Coulterville, CA) and electrode resistance was determined (50-110 M Ω). Recordings were made in current clamp. Signals were passed through a headstage to a Neurodata Intracellular Amplifier (Cygnus) and continuously monitored on a digital oscilloscope (Fluke), a multimeter (Tektronix), and an audio monitor (Grass). Signals were digitized at 10 kHz using a Digidata (Molecular Devices), and acquired with Axoscope 9 software (Molecular Devices) for offline analysis.

Microelectrodes were advanced through the medial PFC while passing a square pulse current (0.2 nA; 100 ms) to continuously monitor electrode resistance. After a neuron was impaled, baseline activity was recorded for >3 min before determining the input resistance of the neuron. Neurons included in this study had a resting membrane potential more negative than -60 mV and action potentials with amplitudes \geq 40 mV from threshold. To determine heterosynaptic influences BLA activation may have on hippocampal inputs to the medial PFC, we utilized a stimulation protocol wherein a baseline pulse (F1; 0.5 ms duration; 0.3-1.0 mA) was applied to the fornix, which is composed of hippocampal fibers innervating the forebrain. After a 500 ms delay a conditioning pulse train was applied to the BLA (10 pulses; 10, 20, or 50 Hz). A test pulse (F2; 0.5 ms duration; 0.3-1.0 mA) was subsequently applied to the fornix at varying delays after the last BLA train pulse (BLA_{train}; 50, 100, 150, 300, or 500 ms) in either ascending or descending order. The order in which the F2 test pulse was applied was counterbalanced between cells, with approximately half being presented with F2 pulse in ascending delays and the other half being presented with descending F2 delays.

Additionally, we also determined whether a single, conditioning pulse to the BLA (F1-BLA_{single}-F2) would modify a subsequent F2 test pulse using the same protocol with the exception that the BLA pulse train is replaced by a single pulse to the BLA. To determine whether alterations in F2 responses were not due to non-heterosynaptic short-term facilitation, we conducted the same stimulation protocol as described above (F1-BLA_{train}-F2) with the exception that BLA train stimulation was omitted (F1-NoBLA-F2). In some cases the AP (AP1 and AP2) was stimulated instead of the fornix to evaluate effects of BLA train stimulation on synaptic responses in cortical-PFC pathways. To determine heterosynaptic influences of hippocampus inputs to the PFC on BLA-evoked synaptic responses, we conducted similar stimulating protocols, with the exception that baseline and test pulses (BLA1 and BLA2) were applied to the BLA and conditioning pulse train or single pulse was applied to the fornix (F_{train} or F_{single}).

Optogenetics

Rats were anesthetized with 1-2% isoflurane. An adeno-associated virus expressing channelrhodopsin-2/YFP under the control of the CaMKII promoter was microinjected into the left ventral hippocampus at two sites (AP: -5.6 mm; ML: 5.0 mm; DV: - 6.5 mm and -5.0 mm from dura; 0.5 ul per site). Electrophysiological experiments were conducted 6-8 weeks after viral infection. These experiments were conducted as described above with the exception that an optical fiber (200 μ m) coupled to a laser diode (472 nm emission; Thor Labs) was inserted into the fornix to optically activate transfected ventral HIPPOCAMPUS fibers instead of a stimulating electrode. Optical stimulation consisted of a square pulse of blue light (8-15 mW) with durations of 1-5 ms.

***In vivo* extracellular electrophysiology**

Extracellular recording microelectrodes coupled to drug pressure ejection were constructed in a similar manner to those in Chapter III. Microelectrodes were pulled on a vertical Stoelting puller from 1.5 mm diameter borosilicate glass (World Precision Instruments), and tips were broken against a glass slide to 2-3 μm . This resulted in a tip resistance of 5-15 $\text{M}\Omega$ *in situ*. Pressure ejection pipettes were pulled from 1.0 mm diameter borosilicate glass (0.25 mm I.D.; A-M Systems). The drug pipette was filled with aCSF containing the KOR antagonists nor-BNI (100 μM) and <0.01% Chicago Sky Blue to help visualize drug delivery. Extracellular recording microelectrodes were filled with 0.5 M sodium chloride. Microelectrodes were lowered into the superficial cortex using a hydraulic manipulator and electrode resistance was determined. Signals were passed through a headstage to a Neurodata Intracellular Amplifier (Cygnus), subsequently amplified, and continuously monitored on a digital oscilloscope (Fluke), a multimeter (Tektronix), and an audio monitor (Grass). Signals were digitized at 20 kHz using a Digidata (Molecular Devices), and acquired with Axoscope 9 software (Molecular Devices) for offline analysis. Local field potential (LFP) signal was filtered between 1 Hz and 100 Hz.

Microelectrodes were lowered through the mPFC while the fornix was stimulated (0.1-0.5 ms duration; 0.4-0.7 mA; 0.2 Hz) until a consistent monosynaptic evoked field excitatory postsynaptic potential (fEPSP) with > 0.1 mV in amplitude was encountered in response to single pulse fornix electrical stimulation. A response was monosynaptic if the onset latency was < 10 ms and had a negative-going peak \leq 25 ms from stimulation. Baseline BLA-evoked heterosynaptic suppression was determined in a similar fashion as

in vivo intracellular experiments. Nor-BNI was then pressure ejected using a Toohey Pressure System IIe (Toohey Systems; 40 psi; 5-10 ms duration) over a 5 min period. This resulted in delivery of approximately 45 nl of aCSF or drug. BLA-evoked heterosynaptic suppression was re-assessed 5 min after nor-BNI pressure ejection. A maximum of two sites were sampled from each rat in local drug experiments. At least 45 min were allowed after drug delivery before advancing the micropipette >750 μ m ventrally to search for another recording site.

Histology

Upon completion of stimulation protocols, Neurobiotin was ejected into cells using a 2 Hz current pulse (100 ms; 0.2-1.2 nA) through the recording electrode for 5-20 minutes. In the case where cells were filled with Neurobiotin, rats were given an overdose of chloral hydrate and transcardially perfused with cold saline followed by 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde, rinsed in PBS, and switched to 30% sucrose with azide. Sections (40-50 μ m) were obtained using a freezing microtome. Neurobiotin was visualized using Vectastain Elite ABC reagent (Vector Laboratories), followed by Fast-DAB (Sigma Aldrich). Section were Nissl-stained, cover-slipped, and examined under a microscope to verify stimulating electrode placements, recovered cell or recording microelectrode track placement, and/or LFP recording site as assessed by presence Chicago Sky Blue.

Statistical Analysis.

Heterosynaptic suppression data were analyzed using repeated measures ANOVA with F2 or BLA2 delay as a within subjects factor. Heterosynaptic suppression data where F1

and F2 Vm was matched was analyzed using repeated measures ANOVA with both F2, AP2, or BLA2 delay and pulse type (i.e. F1 vs F2) as within subjects factors. Frequency-dependent effects of BLA pulse train stimulation were analyzed using repeated measures ANOVA with both F2 delay and frequency (10, 20, 50 Hz) as within-subject factors. Post-hoc tests were conducted using paired-t-test or Students' t-test with Bonferroni corrections, where appropriate.

Results

BLA train stimulation evokes-heterosynaptic suppression of hippocampal inputs to the mPFC

We utilized a stimulation protocol wherein a test pulse (F2) to the fornix, the fiber bundle carrying hippocampal fibers innervating the forebrain, was delivered at varying delays after a BLA pulse train (5 pulses at 50 Hz; Fig. 4.1 A). BLA train stimulation decreased F2 excitatory post-synaptic potential amplitude (EPSP) relative to a baseline pulse (F1) in a time-dependent manner in all recorded cells (Fig. 4.1 A,B). Repeated measures ANOVA revealed an effect of pulse delay (time between F2 and the last BLA train pulse; $F_{(5,125)}=39.098$; $p<0.0001$). Post-hoc analysis revealed that BLA train stimulation decreased F2 responses relative to F1 at all intervals ($p < 0.05$), except the 500 ms delay. Shorter intervals displayed more robust heterosynaptic suppression than longer delays. Heterosynaptic suppression persisted for at least 500 ms in a subset of cells (Fig. 4.1A, bottom example). To determine whether BLA-evoked membrane potential changes

contributed to heterosynaptic suppression, we restricted our analysis to sweeps where the membrane potential during F1 was similar to that of F2 by taking advantage of the oscillations of the cells between “down” and “up” states or by depolarizing or hyperpolarizing the membrane via intracellular injection of positive or negative current. BLA-evoked heterosynaptic suppression was present even when F2 and F1 were measured at similar membrane potentials (Fig 4.1C,D). Repeated measures ANOVA revealed a main effect of delay ($F_{(4,60)}=14.526$; $p<0.001$), a main effect of pulse ($F_{(1,60)}=52.213$; $p<0.001$), and a delay by pulse interaction ($F_{(4,60)}=23.44$; $p<0.001$). Post-hoc analysis revealed a significant decrease in F2 EPSP amplitude relative to F1. Moreover, heterosynaptic suppression was still present in neurons in which F2 occurred at more negative membrane potentials than F1, which typically occurred with longer delays. These results indicate that BLA train stimulation elicits heterosynaptic suppression of hippocampal afferents to the mPFC during a window lasting up to several hundred ms.

It is possible that attenuation of F2 EPSP amplitude may be due to short-term depression induced by the relatively short F1-F2 interval. We tested for this option using the same stimulation protocol as described above, but omitting the BLA train. We did not observe a significant difference between F1 and F2 responses at any delay (Fig. 4.1 E,F; $F_{(5,35)}=2.318$; $p=0.064$). This suggests that BLA stimulation is required to elicit heterosynaptic suppression. We have previously demonstrated that single pulse stimulation of the BLA drives mPFC interneurons and produces hyperpolarizing, inhibitory responses associated with loss of the UP-states for 100-600 ms (Dilgen et al., 2013). To determine whether single pulse BLA stimulation was sufficient to evoke

heterosynaptic suppression of hippocampal inputs we repeated experiments using a single BLA pulse. BLA-evoked heterosynaptic suppression of F2 EPSP amplitude was not observed with BLA single pulse stimulation (Fig. 4.1 G,H). ANOVA revealed a main effect of delay ($F_{(4,36)}=9.603$; $p<0.001$), no main effect of pulse ($F_{(4,36)}=0.372$; $p=0.557$), and a delay x pulse interaction ($F_{(4,36)}=2.917$; $p=0.035$). Post-hoc analysis failed to reveal any significant difference between F1 and F2 at any of the time points. These results suggest that train BLA stimulation is required to induce heterosynaptic suppression.

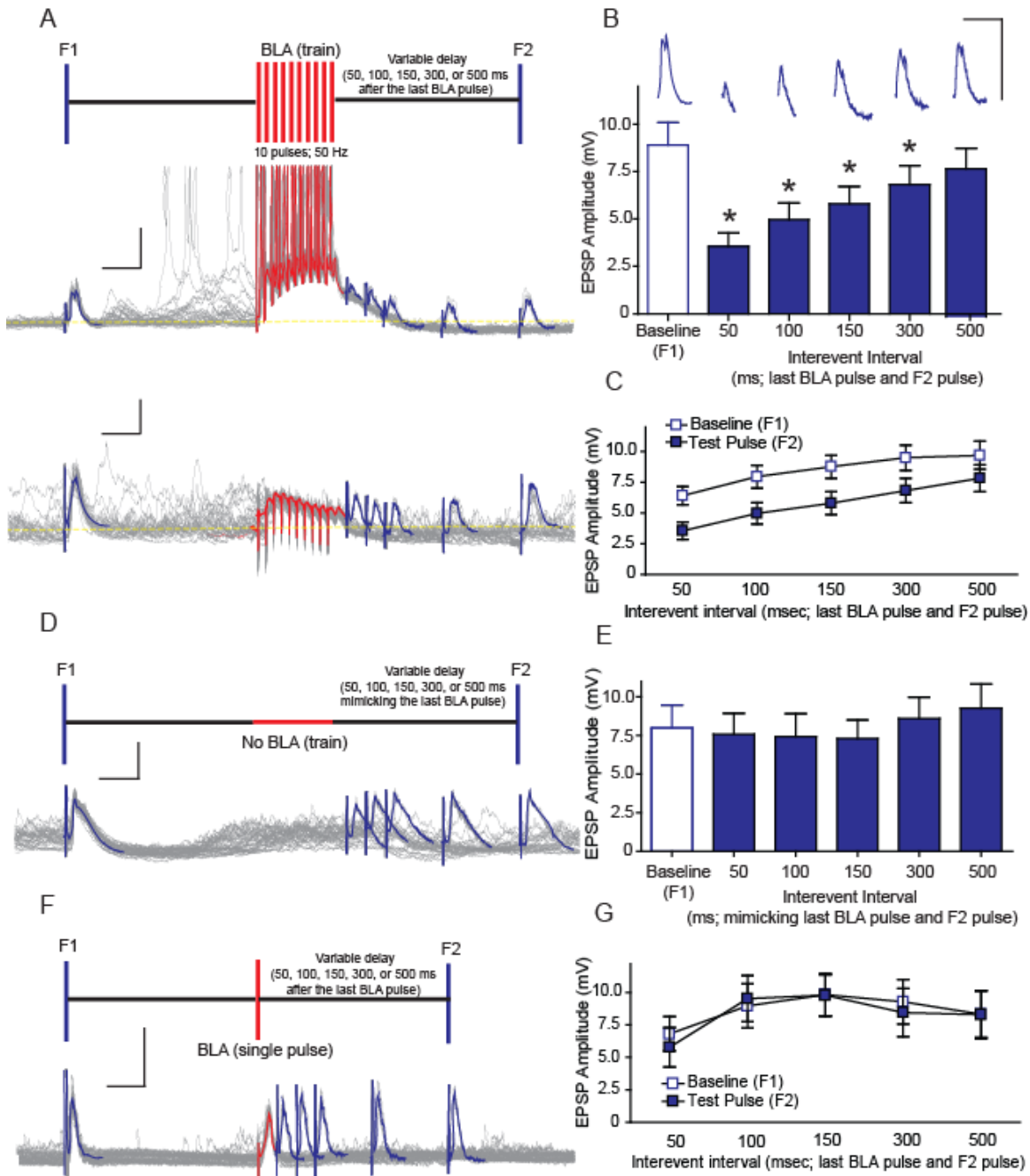


Figure 4.1: BLA train stimulation evokes heterosynaptic suppression of fornix-evoked synaptic responses in a time-dependent manner. **A, Top,** Diagram depicting stimulation protocol utilized to study heterosynaptic interactions between BLA and hippocampus inputs to the PFC. **Bottom,** Overlaid traces from consecutive sweeps of BLA-evoked heterosynaptic suppression of F2 synaptic responses relative to F1 in two representative PFC pyramidal neurons. Average baseline (F1) and test (F2) fornix-evoked responses are depicted in blue while the average response during BLA train stimulation is depicted in red. The yellow dashed line depicts the mean baseline Vm. **Top traces,** F2 responses are suppressed well beyond the BLA train and even at potentials more hyperpolarized than baseline. **Bottom traces,** BLA-evoked heterosynaptic suppression of F2 synaptic responses relative to F1 in a PFC pyramidal neuron where Vm during F1 was similar to Vm during F2. **B,** Fornix-evoked responses (EPSP amplitude) at baseline (F1) and the different test pulses (F2) at various delays (n=16 cells). (*) Reflects a significant difference from F1. Average F1 and F2 responses from neuron 1 of figure 1A are inset above their corresponding column. **C,** Fornix-evoked responses (EPSP amplitude) at baseline (F1) and the different test pulses (F2) at various delays when F1 and F2 responses were triggered at a similar Vm. **D, Top,** Diagram depicting stimulation protocol where the BLA train was omitted. **Bottom,** Overlaid traces from consecutive sweeps of showing that F1 synaptic responses were similar to F2 responses at all delays. **G,** Fornix-evoked responses (EPSP amplitude) at baseline (F1) and the different test pulses (F2) at various delays. **H, Top,** Diagram depicting stimulation protocol examining the effects of single pulse BLA stimulation on subsequent F2 responses. **Bottom,** Overlaid traces from consecutive sweeps demonstrating that single pulse BLA stimulation does not modify F2 synaptic responses relative to F1. **I,** Fornix-evoked responses (EPSP amplitude) at baseline (F1) and the different test pulses (F2) at various delays. Data represent mean \pm SEM. Scale bars: 100 ms, 10 mV.

BLA-evoked heterosynaptic suppression selectively inhibits hippocampal inputs

Electrical fornix stimulation could activate nearby nuclei (thalamus) or fibers of passage (i.e. corpus callosum). To determine the hippocampal specificity of BLA-evoked suppression of responses to fornix electrical stimulation, we used optogenetics. We injected a subset of rats with adeno-associated virus expressing channelrhodopsin-2 (ChR2)-YFP under control of the CaMKII promoter into the ventral hippocampus (Fig. 4.2A). ChR2 was expressed in the fornix at the same site that was targeted for electrical stimulation (Fig 4.2A). Optical stimulation with blue light at that site evoked EPSPs similar to those evoked by electrical stimulation. Optically-evoked fornix responses were suppressed by 50 Hz BLA train stimulation (Fig. 4.2C), even when taking into account differences in membrane potential between F1 and F2 (Fig. 4.2D). ANOVA revealed a main effect of pulse ($F_{(1,24)}=64.76$; $p<0.001$), no effect of delay ($F_{(4,24)}=1.867$; $p=0.149$), and a pulse x delay interaction ($F_{(4,24)}=2.789$; $p=0.049$). Thus, BLA-evoked heterosynaptic suppression of fornix-evoked responses in the mPFC is attributable to inhibition of information flow from the ventral hippocampus to the mPFC and not to non-specific activation of other inputs.

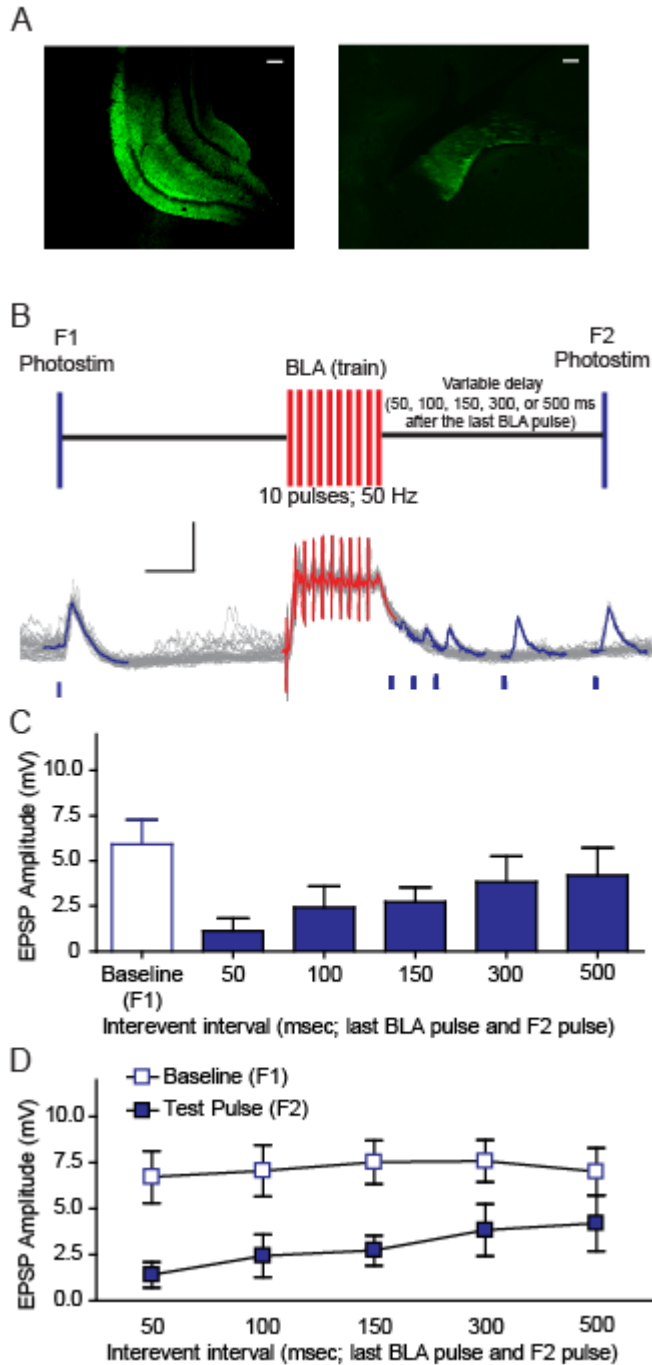


Figure 4.2: BLA-evoked heterosynaptic suppression of optically-evoked fornix responses. *A, Left*, Site of injection into the ventral HIPPOCAMPUS of AAV virus encoding ChR2-YFP. *Right*, Expression of ChR2 limited fornix. Note track from optical fiber placement above the fornix and the BLA stimulating electrode to the left *B. C, Top*, Stimulation protocol utilized to study heterosynaptic interactions between BLA and hippocampal inputs to the PFC above overlaid traces, *bottom*. Average baseline (F1) and test (F2) fornix-evoked responses are depicted in blue while the average response during BLA train stimulation is depicted in red. *D*, Mean EPSP amplitude of fornix-evoked responses at baseline (F1) and the different test pulses (F2) delivered at various delays ($n=7$ cells). Data represent mean \pm SEM. Scale bars: 100 ms, 10 mV.

BLA-evoked heterosynaptic suppression of hippocampal-evoked responses is frequency dependent

We next sought to determine whether the frequency of the BLA train determined the degree of heterosynaptic suppression of optically- and electrically-evoked fornix responses. Using the stimulation protocols described above, but with varied frequency of BLA pulse trains (10, 20, or 50 Hz; Fig. 4.3 A), we found that normalized BLA2 EPSP amplitude was temporally attenuated in a frequency-dependent manner. Repeated measures ANOVA revealed an effect of delay ($F_{(4,16)}=4.066$; $p=0.018$), a main effect of BLA frequency ($F_{(2,8)}=18.499$; $p=0.001$), and a delay x frequency interaction ($F_{(8,32)}=2.789$; $p=0.018$). These results demonstrate that with increasing BLA pulse train frequency heterosynaptic suppression becomes more robust and longer-lasting.

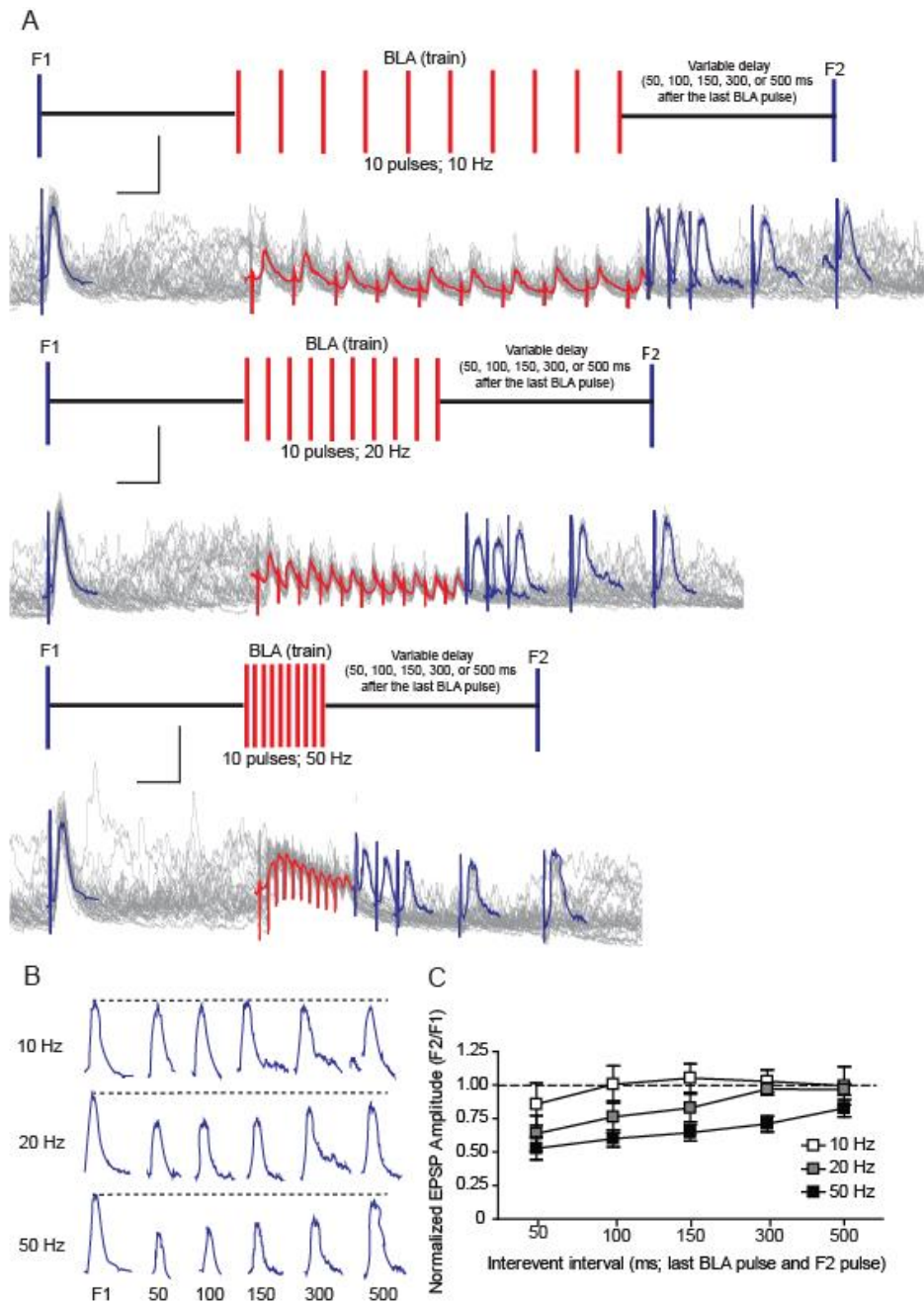


Figure 4.3: BLA-evoked heterosynaptic suppression of fornix-evoked responses is frequency dependent. *A*, Stimulation protocols depicted above overlaid traces of heterosynaptic suppression evoked by BLA train stimulation at variable frequencies (10, 20, 50 Hz) in a representative neuron. Average baseline (F1) and test (F2) fornix-evoked responses are depicted in blue while the average response during BLA train stimulation is depicted in red. *B*, Mean normalized EPSP amplitude (F2 amplitude/F1 amplitude) at various delays after the last pulse of a BLA train delivered at variable frequencies. Data represent mean \pm SEM. Scale bars: 100 ms, 10 mV.

BLA train stimulation produces heterosynaptic suppression of inputs from associative temporal cortex.

We then sought to determine whether BLA-evoked heterosynaptic suppression was a unique feature of hippocampus inputs. We determined the effects of BLA train stimulation on responses evoked by electrical stimulation of the amygdalopiriform transition area (AP), an associative temporal cortical region considered to be a part of the entorhinal cortex (Shammah-Lagnado and Santiago, 1999). Single pulse stimulation of the AP evoked short-latency monosynaptic EPSPs in mPFC neurons similar to those evoked by BLA and fornix stimulation, consistent with a direct innervation of mPFC neurons by the AP (Conde et al., 1995; Shammah-Lagnado and Santiago, 1999). BLA train stimulation decreased AP-evoked synaptic responses in a time-dependent manner (Fig. 4.4). ANOVA of AP1 and AP2 EPSP amplitudes revealed a main effect of delay ($F_{(1,9)}=14.849$; $p=0.004$), a main effect of pulse ($F_{(1,9)}=5.515$; $p=0.043$), and a delay x pulse interaction ($F_{(1,9)}=5.254$; $p=0.048$). Thus, BLA-mediated heterosynaptic suppression is not limited to hippocampal afferents to the mPFC, but is also present in mPFC afferents from other neocortical structures.

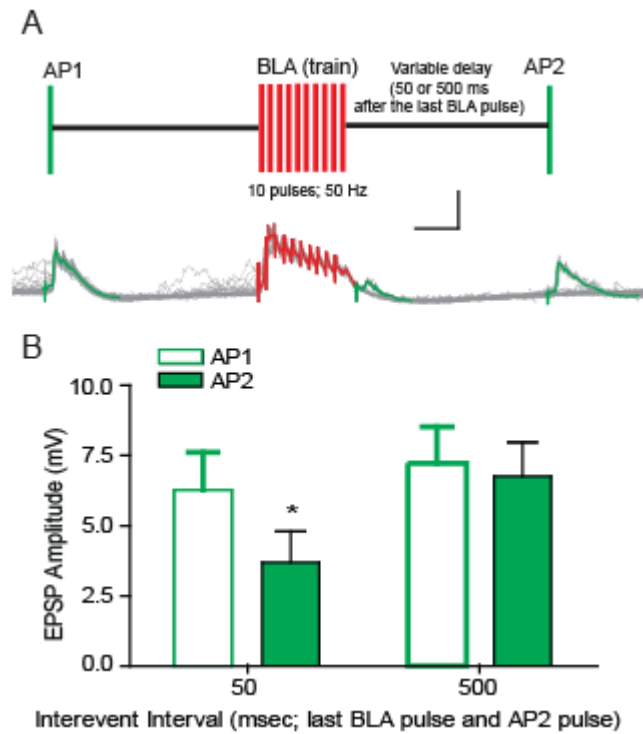


Figure 4.4: BLA-evoked heterosynaptic suppression is not unique to the hippocampal-mPFC pathway. **A**, Stimulation protocols depicted above overlaid traces of heterosynaptic suppression evoked by BLA train stimulation at variable frequencies in a representative neuron. Average baseline (APiri1) and test (APiri2) APiri-evoked responses are depicted in green while the average response during BLA train stimulation is depicted in red. **B**, Mean APiri-evoked EPSP amplitude at a short (50 ms) and long (500 ms) delay after the last pulse of a BLA train is delivered. Data represent mean \pm SEM. Scale bars: 100 ms, 10 mV.

Stimulation of hippocampal inputs to the mPFC does not modify BLA-evoked responses in the PFC

Hippocampus stimulation also produces hyperpolarizing responses and inhibits PFC pyramidal neuron activity (Degenetais et al., 2003; Ishikawa and Nakamura, 2003b; Tierney et al., 2004), and as such may be able to produce heterosynaptic suppression of the BLA-mPFC pathway. Stimulation protocols were conducted wherein baseline (BLA1) and test pulses (BLA2) were applied to the BLA while a conditioning pulse train stimulation was applied to the fornix (Fig. 4.5 A,B). Fornix train stimulation failed to produce heterosynaptic suppression of BLA 2 responses relative to BLA1 responses. Repeated measures ANOVA showed a main effect of delay when matching membrane potential ($F_{(4,7)}=8.832$; $p<0.001$). We failed to detect a main effect of pulse type ($F_{(1,28)}=1.931$; $p=0.207$) and a delay x pulse type interaction ($F_{(4,28)}=1.658$; $p=0.188$). Furthermore, single pulse fornix stimulation did not modify BLA2 EPSP amplitude relative to BLA1 at any delay tested (Fig 4.5 C,D). ANOVA revealed a main effect of delay ($F_{(4,28)}=5.394$; $p=0.002$) and no effect of pulse type ($F_{(1,7)}=1.132$; $p=0.323$) and delay x pulse type interaction ($F_{(1,7)}=0.657$; $p=0.627$). Although single pulse fornix stimulation produced hyperpolarizing and inhibitory responses in single unit activity, it did not attenuate BLA afferents to the PFC. It is possible that short term facilitation in BLA-evoked responses from repeated BLA stimulation could potentially be masking any heterosynaptic suppressive effects produced by fornix stimulation. However, BLA1 EPSP amplitude did not differ from that of BLA2 when BLA train stimulation was omitted (Fig 4.6 E,F; $F_{(5,40)}=1.270$; $p=0.296$). Collectively, these results suggest that heterosynaptic interactions between the BLA and hippocampus are unidirectional, with only BLA gating of hippocampal inputs, but not vice versa.

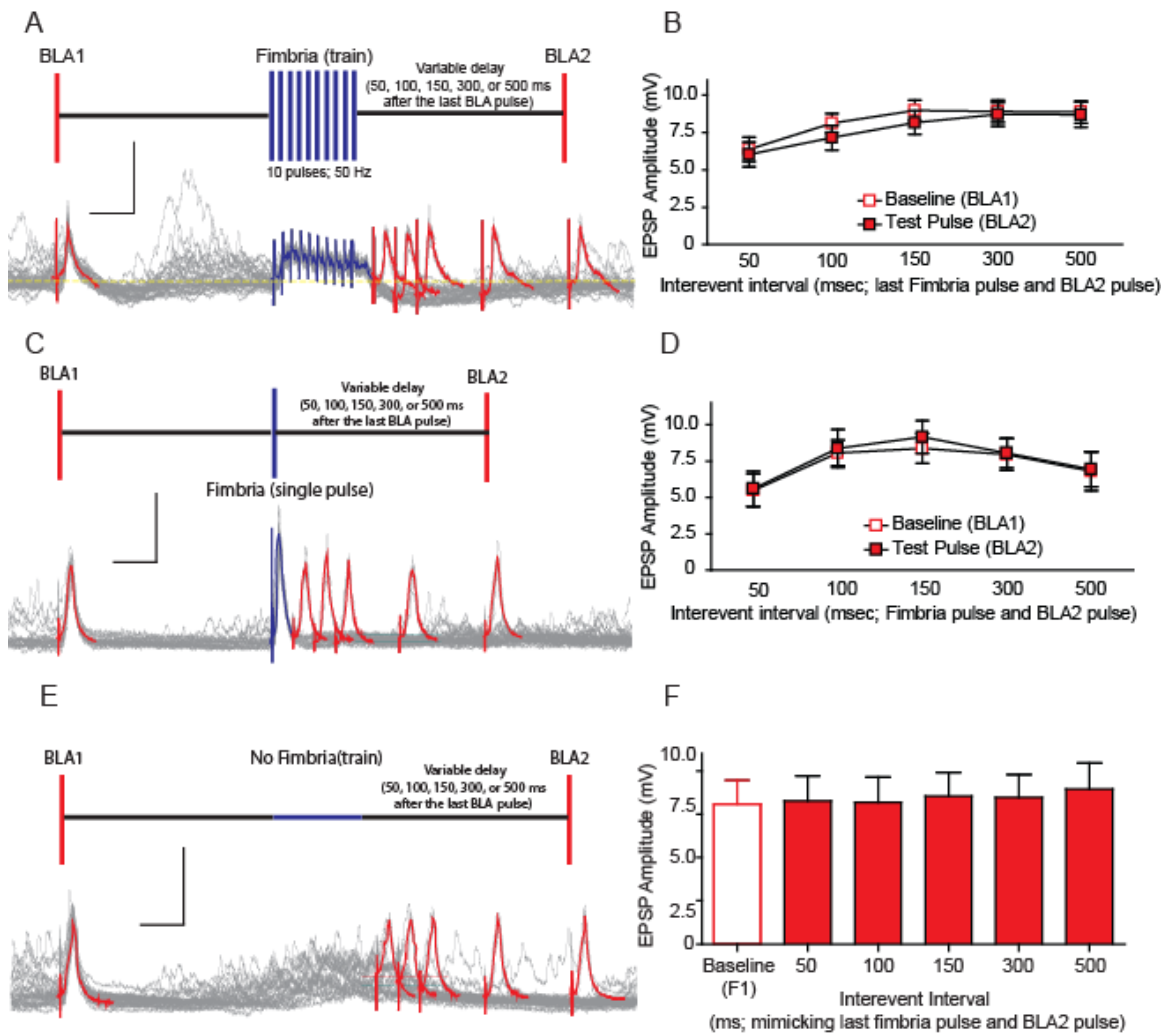


Figure 4.5: Fornix train stimulation does not induce heterosynaptic suppression of BLA-evoked synaptic responses. *A*, Top, Stimulation protocol above overlaid traces from consecutive sweeps showing the lack of effect of fornix train stimulation on BLA2 synaptic responses relative to BLA1 in a mPFC pyramidal neuron. Average baseline (BLA1) and test (BLA2) BLA-evoked responses are depicted in red while the average response during fornix train stimulation is depicted in blue. *B*, BLA-evoked responses (EPSP amplitude) at baseline (BLA1) and the different test pulses (BLA2) at various delays. *C*, Top, Stimulation protocol above overlaid traces from consecutive sweeps demonstrating that single pulse fornix stimulation does not modify BLA2 synaptic responses relative to BLA1. *D*, BLA-evoked responses (EPSP amplitude) at baseline (BLA1) and the different test pulses (BLA2) at various delays. *E*, Diagram depicting stimulation protocol above overlaid traces from consecutive sweeps of showing that BLA1 synaptic responses were similar to BLA2 responses at all delays when the BLA train was omitted. *F*, BLA-evoked responses (EPSP amplitude) at baseline (BLA1) and the different test pulses (BLA2) at various delays. Data represent mean \pm SEM. Scale bars: 100 ms, 10 mV.

KORs do not play a role in BLA-evoked heterosynaptic suppression of hippocampal inputs to the mPFC

As KORs have been shown to mediate heterosynaptic suppression in the mossy fiber pathway in the hippocampus (Weisskopf et al., 1993; Tunbridge et al., 2006), we determined the role of KORs in mediating heterosynaptic interactions between the BLA and hippocampus in the mPFC. Intra-mPFC nor-BNI pressure ejection failed to modify BLA-evoked heterosynaptic suppression of fornix-evoked fEPSPs (Fig. 4.6). Repeated measures ANOVA revealed a main effect of delay ($F_{(4,40)}=7.24$; $p=0.002$), no effect of drug ($F_{(1,40)}=2.29$; $p=0.16$), and no delay x drug interaction ($F_{(4,40)}=0.52$; $p=0.72$). This suggests that mPFC KORs do not play a role in heterosynaptic suppression. It is possible that mPFC KORs may play a role in heterosynaptic suppression, but pressure ejected nor-BNI is unable to reach mPFC KORs involved in this process. To address this issue, subsets of rats were pretreated with either saline or nor-BNI (10 mg/kg; sc) approximately 24 hrs prior to electrophysiological recordings (Fig. 4.6 C). Repeated measures ANOVA showed a main effect of delay ($F_{(4,120)}=11.84$; $p<0.0001$), no main effect of pre-treatment ($F_{(1,120)}=0.96$; $p=0.33$), and no delay x pre-treatment interaction ($F_{(4,120)}=1.59$; $p=0.19$). However, it should be noted that with both intra-mPFC and systemic nor-BNI administration a trend for decreased BLA-evoked heterosynaptic suppression was present. These results suggest that KORs do not play a major role in BLA-evoked heterosynaptic suppression of the hippocampus to mPFC pathway.

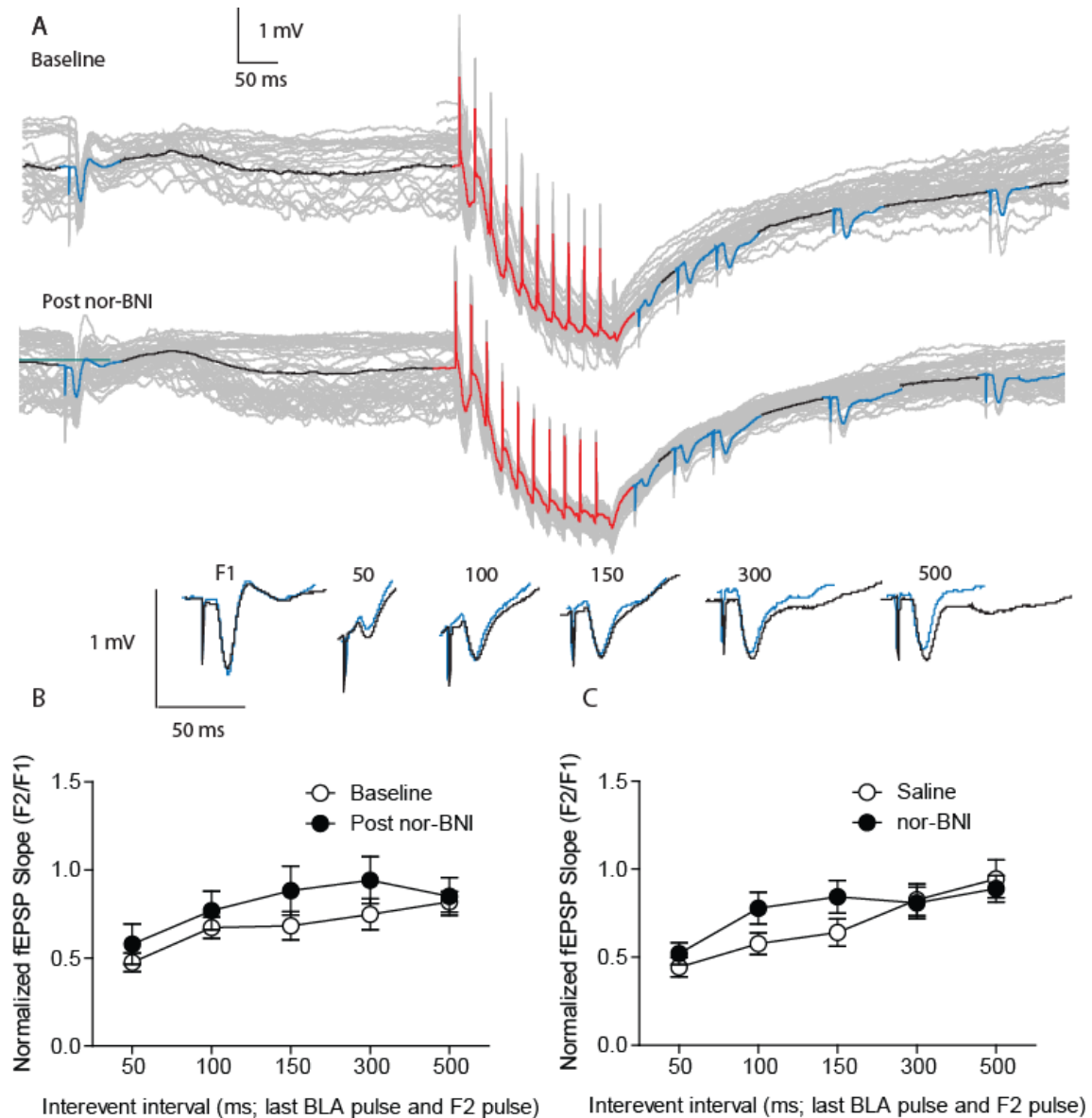


Figure 4.6: KORs do not play a major role in BLA-evoked heterosynaptic suppression of the hippocampus to mPFC pathway. **A**, *Top*, Representative example of BLA-evoked heterosynaptic suppression of fornix-evoked fEPSPs during baseline and after intra-mPFC nor-BNI pressure ejection. Average baseline (F1) and test (F2) fornix-evoked responses at baseline and after nor-BNI are depicted in blue while the average response during BLA train stimulation is depicted in red. *Bottom*, Overlaid mean F1 and F2 responses at baseline (black traces) and post nor-BNI pressure ejection (blue traces). Scale bars: 1.0 mV and 50 ms. **B**, Mean normalized fornix-evoked fEPSP slope (F2 normalized to F1) at different F2 delays after the last pulse in the BLA train at baseline (open circles) and after nor-BNI pressure ejection (filled circles). **C**, Mean normalized fornix-evoked fEPSP slope (F2 normalized to F1) at different F2 delays after the last pulse in the BLA train in rats pretreated with systemic saline (open circles) or systemic nor-BNI (filled circles). Data represent mean \pm SEM.

Chapter IV Summary

The findings from Specific Aim 3 suggest that BLA inputs to the mPFC produce heterosynaptic suppression of hippocampal inputs via a KOR-independent mechanism. Trains of stimuli to the BLA suppressed EPSPs evoked by fornix stimulation in a time-dependent manner. Such heterosynaptic suppression was strongest at shorter delays after the BLA train. BLA-evoked heterosynaptic suppression of hippocampus inputs was not due to membrane potential changes induced by the BLA train and required trains of stimuli, as it was not elicited by repeated fornix stimulation in the absence of a BLA pulse train or by single pulse BLA stimulation. Moreover, BLA-evoked heterosynaptic suppression was frequency-dependent, with more robust and longer-lasting suppression observed with higher BLA train frequencies. Heterosynaptic suppression of hippocampus-evoked responses was due to specific action on hippocampus afferents in the mPFC, as suppression of optically-evoked fornix EPSPs was present in animals expressing ChR2 in the ventral hippocampus. Since ChR2 was only expressed in excitatory projections from the ventral hippocampal formation, fibers of passage in the fornix would not be activated by blue light delivery. We also observed heterosynaptic suppression of AP inputs to the mPFC, suggesting that higher-order cortical signals are additionally shunted by BLA inputs. Heterosynaptic suppression was unidirectional and not evoked by fornix train stimulation, suggesting that BLA inputs are distinct from hippocampal inputs in their ability to produce heterosynaptic suppression. We suggest that BLA activity is able to deactivate the mPFC and interfere with communication between the hippocampus and mPFC. Moreover, we failed to find a significant role for KORs in BLA-evoked heterosynaptic suppression as intra-mPFC and systemic nor-BNI

administration did not alter this interaction. Taken together, our results are consistent with the notion BLA inputs to the mPFC produce heterosynaptic suppression of hippocampal inputs in a unidirectional manner via a KOR-independent mechanism.

Chapter V: Discussion

Chapter II Discussion

In Specific Aim 1, we provide evidence that mesocortical DA neurotransmission is directly and negatively modulated by mPFC KOR systems. This is in line with the hypothesis that mPFC KORs inhibit synaptic inputs from the VTA. In Specific Aim 1, we initially predicted that KOR would play an inhibitory role on mesocortical DA transmission. Our findings that systemic U69,593 inhibited extracellular DA levels in the PFC was consistent with our hypothesis. However, mPFC-projecting VTA DA neurons are hyperpolarized by U69,593, resulting in decreased mPFC DA levels (Margolis et al., 2006). As KORs are also found on axonal varicosities in mPFC (Svingos and Colago, 2002), we determined the role of KORs therein in modulating local DA levels. We then predicted that KOR was located on DA varicosities in the mPFC and activation of this receptor inhibits DA release. mPFC extracellular DA levels were reduced by reverse dialysis administration of U69,593, suggesting that KOR in the mPFC has an inhibitory role in mPFC DA neurotransmission. This is consistent with findings that KOR activation decreases DA efflux in frontal cortex synaptosomes and slices (Heijna et al., 1990; Grilli et al., 2009). In the NAcc, KORs have been shown to tonically inhibit DA levels (Spanagel et al., 1992; Chefer et al., 2005). We predicted that KOR in the mPFC tonically inhibits DA overflow. Reverse dialysis of the KOR antagonist nor-BNI enhanced mPFC DA levels, demonstrating that KOR signaling constitutively inhibits extracellular DA in the mPFC. Taken together these results suggest that in addition to inhibiting mesocortical DA systems by acting in the VTA, KORs in the mPFC are also able to inhibit this pathway.

Medial PFC KOR activation may alter local DA tone indirectly by modulating other systems regulating DA terminals and/or DA neuron activity. To elucidate if KORs on DA terminals mediate inhibition of DA by intra-mPFC U69,593, we determined whether intra-mPFC U69,593-induced decreases in local DA levels were altered in DAT-KOR KO mice. U69,593 failed to inhibit mPFC DA levels in DAT-KOR KO mice, while the inhibitory effects of U69,593 on DA levels were still present in control mice. As KOR-expression is spared in KOR-expressing non-DA terminals or cell body in the mPFC, then the effects of U69,593 on DA levels was not due to action at these non-DAergic sites. Recently, Dr. William Carlezon's laboratory at Harvard generated mice lacking KOR in DAT-expressing neurons. However, the lox-P sites flanked exon 3 in the Harvard DAT-KOR KO KOR^{loxP/loxP} mice, whereas the NIDA KOR^{loxP/loxP} mice had exon 2 flanked. In that study, U69,593 binding in the mPFC was decreased, albeit not robustly, after deletion of KOR in DA neurons (Van't Veer et al., 2013). This suggests that membrane KOR in DA terminals make up a small portion of the total KOR present or that particular approach did not result in ablation of KOR in a large proportion of mesocortical DA neurons. Regardless of how much mesocortical DA neurons contribute to the total KOR population available, this is consistent with our finding that functional KOR is localized to DA varicosities in the mPFC. Strategic localization of KOR on DA varicosities would allow dynorphin signaling through the KOR to locally modulate the output of DA neurons, without modification of DA neuron activity. As mesocortical DA neurons express low levels of D2 receptors, which normally act as autoreceptors, KORs may provide presynaptic control of DA to tune down on-going DA release. Moreover, if KORs are tonically active, then enhancements or decrements in dynorphin tone in

response to environmental or interoceptive stimuli would be predicted to bidirectionally control DA efflux. Collectively, our results are consistent with the hypothesis that KORs negatively regulate DA inputs from the VTA by directly acting on mPFC DA varicosities.

The inhibitory effects of KOR agonists on extracellular DA levels are attributed to KOR-mediated inhibition of DA release (Spanagel et al., 1992). However, work from our laboratory has demonstrated that KOR signaling in the NAcc also upregulates DAT function *in vivo* (Thompson et al., 2000; Chefer et al., 2005). Our collaborator, Dr. Sammanda Ramamoorthy at Virginia Commonwealth University demonstrated that U69,593 failed to modify total DA uptake, DAT-, and NET-mediated DA uptake in mPFC synaptosomes using conditions that reliably increase DA uptake in NAcc synaptosomes (Tejeda et al., 2013). Collectively, these results suggest that KORs on DA terminals in the mPFC tonically decrease extracellular DA levels by decreasing DA release. Since there is no difference in extracellular DA levels and uptake in controls and DAT-KOR KO mice, it is likely that release is unaltered. These findings suggests that KORs may not provide tonic inhibition in the mPFC or that species differences exist between mouse and rat. It is also possible that developmental compensation in mPFC DA dynamics occurs in absence of a functioning mesocortical KOR system. Our finding that nor-BNI effects on DA “washed-out” would argue that DA systems undergo homeostatic adaptations in response to KOR antagonism (i.e. VTA DA neuron activity down-regulation) as nor-BNI-mediated KOR antagonism persists for weeks. As mPFC KORs do not appear to modulate clearance of extracellular DA, their inhibitory regulation of extracellular DA is likely via inhibition of release.

Although KOR systems appear to generally have an inhibitory role in DA systems, marked differences exist in KOR regulation between pathways. Activation of VTA KORs reduces mPFC (Margolis et al., 2006), but not NAcc (Spanagel et al., 1992; Devine et al., 1993; Margolis et al., 2006), DA levels. Furthermore, KOR agonists directly hyperpolarize mesocortical DA neurons, but not mesoaccumbal neurons in slices containing the VTA (Margolis et al., 2006). Therefore, KOR has two levels of control over mesocortical DA neurotransmission, at the level of the cell body in the VTA and DA varicosities in the mPFC. Mesoaccumbal DA output appears to only be modulated by KOR in the NAcc. Here we find that KORs in the mPFC tonically inhibit extracellular DA levels, whereas intra-VTA nor-BNI does not modify mPFC DA levels (Margolis et al., 2006). This also suggests that there are differences in the level of KOR regulation even within the same DA pathway. Similarities and differences between mPFC and NAcc KORs in regulating terminal DA levels also exist. Our finding that KORs in mPFC terminals tonically inhibit DA levels is similar to the tonic inhibition of NAcc DA by local KORs (Spanagel et al., 1992). In the NAcc, KORs decrease DA tone by inhibiting DA release and increasing DA uptake (Thompson et al., 2000; Chefer et al., 2005), whereas mPFC KORs do not alter DA uptake. KOR system recruitment may differentially shape mesocortical and mesoaccumbal DA responses, depending on where dynorphins are released (i.e. cell bodies vs. terminal regions) or whether tonic KOR activity exists (i.e. in DA terminals).

MOR and KOR have opposing control of DA output, a relationship often compared to yin and yang symbolism. As mentioned above, KORs negatively regulate mesoaccumbal DA output by controlling DA terminals and decrease the activity of

subsets of DA neurons by directly hyperpolarizing neurons. MOR receptor activation results in enhancements in mesoaccumbal DA output predominantly by enhancing DA neuron activity in the VTA via inhibition of GABAergic control (Johnson and North, 1992). This yin/yang relationship appears to occur within the mPFC as well. Reverse dialysis of DAMGO (using a dose our laboratory found to be ineffective in MOR KO mice) enhanced extracellular DA levels. To our knowledge, this is the first report demonstrating that mPFC MORs positively modulate local DA tone. Both MOR and KOR are Gi/o-coupled GPCRs with inhibitory presynaptic and post-synaptic effects. Thus is not likely that the MOR mechanism of action is directly on mesocortical DA neurons, but rather via a mechanism that alleviates inhibitory control of DA terminals or midbrain DA neuron activity. MOR is localized on FSI in the mPFC, thus DAMGO may disinhibit mPFC pyramidal neurons that project to DA neurons in the VTA (Sesack and Carr, 2002). In preliminary experiments I conducted at NIDA, I found that the ability of intra-mPFC DAMGO to elevate local DA was completely blocked by pretreatment with intra-mPFC CNQX (n=4; data not shown). This effect was significant even with this low sample size. This would suggest that by altering excitation/inhibition balance in the mPFC MOR can control local DA levels, while mPFC KOR would play an inhibitory role by directly controlling DA varicosities. Thus, the opposing influence of MORs and KORs on extracellular DA levels is also present in the mPFC.

Chapter III Discussion

In Specific Aim II, we found evidence consistent with the predictions that mPFC KOR systems negatively modulate extracellular glutamate levels and inhibit BLA inputs to the mPFC. We found that KOR activation inhibits presynaptic glutamatergic function

in vitro and in vivo. Basal, dialysate mPFC glutamate levels, which are not TTX-sensitive or Ca^{2+} channel-sensitive (Melendez et al., 2005), were unaltered by U69,593 and nor-BNI. However, U69,593 attenuated tPDC-evoked elevations in extracellular glutamate. tPDC-evoked elevations in extracellular glutamate have TTX-sensitive and Ca^{2+} -dependent components (Herrera-Marschitz et al., 1996; Rawls and McGinty, 1997; Kreuter et al., 2004), suggesting tPDC reveals synaptic sources of extracellular glutamate. tPDC has been utilized to unmask the inhibitory effects of U69,593 on striatal glutamate (Rawls and McGinty, 1998). The ability of U69,593 to inhibit striatal glutamate was dependent on extracellular Ca^{2+} . An inhibitory role of mPFC KOR via presynaptic regulation of glutamate transmission was demonstrated in collaboration with Dr. Danielle Counotte, a post-doctoral fellow in the O'Donnell lab, utilizing whole-cell recordings from layer V pyramidal neurons in mPFC slices. U69,593 produced a decrease in mEPSP frequency, but not amplitude, in a nor-BNI-sensitive manner. This suggests KORs inhibit glutamate release via a presynaptic site of action and is consistent with previous findings. KOR-immunoreactivity has been localized to presynaptic terminals of excitatory synapses (Svingos and Colago, 2002). KOR activation inhibits K^{+} -stimulated glutamate release from mPFC synaptosomes (Sbrenna et al., 1999). Dynorphin either enhances or decreases electrically-evoked EPSPs, but not EPSPs evoked by iontophoretically-applied glutamate, which bypasses the presynaptic terminal (Sutor and Zieglansberger, 1984). If KOR modulate EPSPs via a post-synaptic effect, dynorphin would be expected to similarly modulate EPSPs evoked by exogenous glutamate. KOR signaling in the mPFC may serve to decrease local glutamatergic synaptic transmission via a presynaptic site of action.

Prefrontal pyramidal neurons and interneurons are innervated by limbic, thalamic, and cortical glutamatergic afferents. However, it is not clear which glutamatergic mPFC inputs are inhibited by KORs, as limbic and thalamic efferent regions are rich in KOR mRNA (Meng et al., 1993). We found that KOR decreased the synaptic efficacy of BLA to mPFC glutamatergic synapses, as assessed by the slope of the fEPSP. This was observed using both electrical stimulation to activate fibers in the BLA, which includes fibers of passage and antidromically-activated mPFC pyramidal neuron terminals. It is possible Intra-mPFC activation similarly inhibited fEPSPs evoked by photoactivation of ChR2-expressing BLA terminals in the mPFC, suggesting that mPFC KORs do specifically inhibit the glutamatergic transmission in the BLA to mPFC pathway. KOR-immunoreactivity appears to be primarily localized to presynaptic sites of excitatory synapses (Svingos and Colago, 2002), decreases mEPSP frequency (Tejeda et al., 2013), and inhibits evoked glutamate responses in mPFC synaptosomes (Sbrenna et al., 1999). Therefore, KOR likely inhibits BLA to mPFC synaptic efficacy by inhibiting BLA terminals in the mPFC. Future experiments using whole-cell recordings in mPFC slices with optogenetic activation of BLA afferents in the mPFC and anatomical studies will allow us to directly assess whether KOR is inhibiting the BLA to mPFC pathway via a presynaptic site of action.

KORs seem to differentially gate BLA and hippocampal inputs to the mPFC. To date, research demonstrating the presence of mPFC KORs in terminals from limbic afferents is lacking. KOR-immunoreactivity is present in asymmetric synapses (Svingos and Colago, 2002), which are indicative of excitatory synapses. However, it is not clear which terminals (i.e. limbic afferents) are modulated by KORs. KOR mRNA can be

trafficked to axonal terminals to be locally translated to KOR receptors in axon terminals in response to depolarization (Bi et al., 2006). In order to express functional KOR at the terminal, it is assumed that a neuron must express mRNA in the soma at some point whether KOR translation occurs in the soma, axon terminals, or en-route from the soma to the terminal. Therefore the prediction can be made that afferent structures that do not express or express low levels of KOR mRNA would not be likely be regulated by KOR. In the present study, U69,593 inhibited BLA-, but not fornix-evoked fEPSPs. This suggests that KORs negatively regulate BLA-mPFC glutamatergic transmission, but not hippocampus afferents into the mPFC. This is consistent with high levels of KOR mRNA expression in the BLA, but not the output regions of the ventral hippocampus: the ventral subiculum or CA1 region of the hippocampus (Meng et al. 1993; Mansour et al. 1994; George et al. 1994). A lack of homogenous regulation of all glutamatergic synapses explains our previous results where U69,593 produces a modest, nor-BNI-sensitive decrease in the frequency of mEPSPs, which are spontaneous events from both KOR-sensitive and KOR-insensitive terminals (Tejeda et al. 2013). It is also possible that KOR also modulates other glutamatergic projections, such as the thalamus. Furthermore, as a proportion of DA neurons co-release glutamate (Yamaguchi et al., 2011) and as KORs directly inhibit DA varicosities, KORs may be inhibiting these sources of glutamate. Collectively, this is consistent with the hypothesis that BLA afferents express functional KORs on their terminals, while KORs on hippocampal terminals in the PFC, if even present, may not be functional under our recording conditions.

Experiments examining the effects of mPFC KOR activation on stimulation/response curves revealed that inhibition was not observed at low intensities,

but was more pronounced at high intensities. The amount of fibers recruited by electrical stimulation is proportional to stimulation intensity. It is likely that not all BLA to mPFC neurons express KOR. Therefore, with increasing stimulation intensity more KOR-expressing fibers may be expected to be recruited. This is consistent with our results where KOR inhibition was more pronounced at higher intensities. Thus, not all recruited fibers may express KORs. This finding raises questions about KOR regulation of the BLA-mPFC pathway and can serve as a basis for future work. It is currently not clear how activity in the BLA-mPFC pathway would impact the expression of KOR in different populations of BLA neurons. Would more BLA neurons express KOR after stimuli that engage the system, such as stress? Would the amount of functional KOR expression rise in all neurons that express KOR, and would it be uniform in all terminals within a given neuron?

The inhibitory effects of U69,593 on BLA-evoked synaptic responses were not modified by pulse train stimulation. When examining the level of inhibition from baseline at every pulse in mPFC responses evoked by 20 or 40 Hz BLA train stimulation, we find that the level of inhibition is not modified by either train frequency. This would suggest that KOR inhibition of BLA-evoked responses would not be overcome by burst firing of BLA neurons. In the NAcc, KOR inhibition of evoked DA release is not overcome by high frequency stimulation (Britt and McGehee, 2008). This is in stark contrast to other neuromodulators such as nicotinic acetylcholine receptors or MORs. Thus, KORs may serve to reduce the overall “gain” of the BLA to mPFC pathway by uniformly inhibiting responses during transient or burst like activity in this pathway.

In the BLA to mPFC pathway, the predominant effect was facilitation of synaptic efficacy within 20 and 40 Hz BLA train stimulations. Synaptic facilitation within a 40 Hz train was larger in magnitude and longer-lasting for the 40 Hz train than the 20 Hz train. This facilitation may not be a universal feature of limbic inputs to the mPFC as depression is observed within responses evoked by ventral hippocampus 20 and 40 Hz train stimulation. Studies examining plasticity in the BLA to mPFC pathway has been limited. BLA theta burst stimulation (100 Hz trains in theta cycles) induces LTP in the BLA to mPFC pathway (Maroun and Richter-Levin, 2003). Thus, strengthening of synaptic efficacy in the mPFC in response to strong BLA activation may be one mechanism by which information transfer between these two brain regions can be coordinated during behavior.

Regulation of short-term synaptic plasticity in the BLA to mPFC pathway by mPFC KORs appears to be frequency-dependent. Short-term facilitation (i.e. paired-pulse) is enhanced by GPCRs that inhibit presynaptic glutamate release via actions on presynaptic Ca^{2+} , which mediates transmitter release. U69,593 did not modify plasticity of synaptic efficacy during a 20 Hz BLA pulse train. However, short-term facilitation evoked by a 40 Hz BLA train was only slightly, but significantly, enhanced by U69,593. It is not clear why enhancement of facilitation was frequency-dependent, though the amount of inhibition was similar at both frequencies. One possible explanation is that mPFC KORs negatively regulate BLA-mPFC transmission largely via a Ca^{2+} -independent manner. KOR activation of K^{+} channels in the presynaptic terminal would be expected to shunt incoming action potentials. Under such a scenario, inhibition would be expected to be similar at each pulse without largely altering presynaptic plasticity.

Future work should be aimed at elucidating the mechanism by which KORs inhibit glutamatergic transmission. Another possible explanation is that KOR modulation at BLA terminals in the mPFC is constant throughout the train, while mPFC KOR signaling may be inhibiting a negative modulator of short-term synaptic plasticity. We have previously demonstrated that BLA activation drives FSI activity in the mPFC, resulting in feedforward inhibition (Dilgen et al., 2013). Our observations that U69,593 inhibits tPDC-evoked elevations in extracellular GABA suggest that KORs may inhibit recruitment of GABAergic transmission in the mPFC. Thus, KORs may be modifying the ability of the BLA to recruit mPFC networks (glutamate-GABA interactions) in a frequency-dependent manner. These provides a framework whereby short-term synaptic plasticity may be slightly enhanced even in the presence of persistent U69,593-evoked inhibition.

Chapter IV Discussion

In Specific Aim III, we characterized heterosynaptic interactions between the BLA and hippocampus in the mPFC using *in vivo* intracellular recordings and determined whether KOR signaling is involved in these interactions. We found that BLA train stimulation suppressed synaptic responses in the mPFC evoked by hippocampal and temporal cortical inputs. BLA-evoked heterosynaptic suppression may be most effective during a short window when strong BLA activity is closely followed by hippocampus or cortical inputs. The ability of the BLA to inhibit hippocampal inputs to mPFC appears to be dependent on the intensity of activation. This interaction is consistent with what is observed during anxiety and stress (Kim et al., 2011a; Townsend and Altshuler, 2012; Veer et al., 2012). Heterosynaptic suppression was not observed after BLA single pulse

stimulation, suggesting that strong BLA activation is required for heterosynaptic suppression at the delays tested. BLA and hippocampus interactions have previously been investigated utilizing extracellular single unit activity utilizing shorter intervals than those in the present study. Single pulse BLA stimulation was shown to facilitate hippocampus-evoked spiking at short windows after the BLA pulse (< 20 ms) and/or inhibit hippocampus-evoked orthodromic spikes in the mPFC and at intervals after 20 ms (Ishikawa and Nakamura, 2003b). However, a recent report suggested the opposite. BLA single pulse stimulation inhibited hippocampus-evoked mPFC neuron spiking briefly (< 20 ms), but enhanced hippocampus-evoked firing at intervals longer than 40 ms (Esmaili and Grace, 2013). Furthermore, our findings that heterosynaptic suppression requires trains of pulses to the BLA and is dependent on the frequency of BLA train stimulation suggest that BLA activity may be able to inversely scale mPFC responsiveness to hippocampus and cortical inputs that drive mPFC network activity. Thus, the BLA would most effectively gate hippocampal information flow into the mPFC in situations where the BLA would be strongly activated. By inhibiting synaptic hippocampus responses in the mPFC, the BLA may also influence hippocampal entrainment of theta oscillations in the mPFC, which are associated with optimal mnemonic processing (Gordon, 2011). Heterosynaptic suppression of hippocampal and cortical afferents is one mechanism by which the BLA is able to decrease mPFC activation and may provide a basis for “bottom-up” inhibition of mPFC activity by the BLA.

Heterosynaptic suppression may serve to restrict activated mPFC neural ensembles to those relevant to the behavioral state driving BLA activity. Under

conditions of BLA-evoked mPFC deactivation, activity of BLA-driven mPFC neuronal ensembles would be sharpened in part by the decrease in “noise” by heterosynaptic suppression of weak inputs, allowing the BLA to guide mPFC output. Dampening hippocampus and other inputs would allow only the strongest hippocampus and cortical inputs, capable to override heterosynaptic suppression, to influence mPFC neural ensembles. BLA activity is necessary for conditioned fear-induced increases in mPFC neuron firing (Laviolette et al., 2005; Sotres-Bayon et al., 2012), but BLA activity suppresses the majority of mPFC neurons during expression of conditioned fear (Garcia et al., 1999). These findings are consistent with the hypothesis that BLA-mediated mPFC inhibition and heterosynaptic suppression may highlight the activity of behaviorally relevant mPFC ensembles. Control of mPFC ensembles by the BLA via heterosynaptic suppression would mean that mPFC-dependent decision-making and cognition would be largely influenced by information being processed in the BLA, such as emotional valence. Moreover, in the proper context strong hippocampus activity may be able to overcome BLA-mediated heterosynaptic suppression allowing for integration of contextual and emotional information within mPFC neuronal assemblies.

BLA electrical stimulation is not only activating BLA neurons that project to the mPFC, but also BLA neurons that project to the NAcc and other BLA efferents, which may lead to heterosynaptic suppression via indirect circuit effects. This is unlikely as the NAcc does not project to the mPFC, not allowing heterosynaptic suppression to be present at the 50 ms delay. *In vitro* whole cell electrophysiological experiments utilizing slices containing the mPFC in rats with the red-shifted opsin C1V1 in the hippocampus and the fast-kinetic form of ChR2 (ChETA) in BLA will allow us to determine whether

BLA terminals in the mPFC, and not elsewhere, are responsible for heterosynaptic suppression hippocampal afferents.

The mechanisms responsible of heterosynaptic suppression of hippocampus inputs to the mPFC are not yet clear. KORs do play a role in heterosynaptic suppression within the mossy fiber pathway of the hippocampus (Weisskopf et al., 1993). Here we found that BLA-evoked heterosynaptic suppression was independent of KOR signaling. Given that KORs do not modify hippocampal inputs to the mPFC, it was already unlikely that KORs would play a role in BLA-evoked heterosynaptic suppression by directly inhibiting hippocampal terminals. However, as KORs appear to affect other systems in the mPFC (i.e. DA) and glutamate-GABA interactions, we pursued KOR as a potential mechanism for BLA-evoked heterosynaptic suppression of hippocampal inputs. Future work will be aimed at determining the mechanism mediating interactions between the BLA and hippocampus in the mPFC, such as GABA. BLA stimulation inhibits the majority of mPFC pyramidal neurons (Perez-Jaranay and Vives, 1991; Ishikawa and Nakamura, 2003a; Floresco and Tse, 2007; Dilgen et al., 2013) via recruitment of local-circuit fast-spiking interneurons (Dilgen et al., 2013). Therefore, it is possible that sustained recruitment of mPFC interneurons by BLA train stimulation may play a role in shunting incoming hippocampus EPSPs. We previously demonstrated that GABA-A receptor signaling mediates a short-latency inhibitory response on single unit activity (lasting approx. 50 ms) to single pulse BLA stimulation, while longer-lasting hyperpolarizing synaptic responses and inhibition of spiking (lasting approx. 200 ms) are not modified by GABA-A receptor blockade in mPFC single units (Dilgen et al., 2013). It is possible that BLA-evoked heterosynaptic suppression is mediated by multiple

mechanisms. For-example, short-delay heterosynaptic suppression may be mediated by GABA-A shunting of hippocampus synaptic responses while heterosynaptic suppression expressed at long delays may be mediated by a slower inhibitory process, such as endocannabinoid or GABA-B signaling.

Hippocampus afferents do not appear to gate integration of BLA-evoked synaptic responses in the mPFC. Fornix train stimulation failed to produce heterosynaptic suppression of BLA-evoked responses, suggesting heterosynaptic suppression by the BLA is unidirectional. This is consistent with a recent study demonstrating that single pulse hippocampus stimulation fails to modify BLA-evoked spiking of mPFC neurons (Esmaili and Grace, 2013). Inhibition of BLA-evoked spiking by a priming hippocampus pulse has also been previously reported, but this effect lasted less than 80 ms (Ishikawa and Nakamura, 2003b). The hippocampus may play a limited role in gating BLA inputs to the mPFC. It is not clear why hippocampal inputs would not be able to suppress BLA inputs, as hippocampal inputs increase mPFC interneuron activity and inhibit the activity of some mPFC neurons (Degenetais et al., 2003; Tierney et al., 2004). Hippocampus train stimulation at frequencies similar to those employed in the present study recruit inhibitory components involving GABA-A receptors (Thomases et al., 2013). Therefore, it is likely that the unidirectional gating we observed is not due to differences in the ability of BLA and hippocampus afferents to recruit inhibitory mPFC networks. It is possible that hippocampus-mediated heterosynaptic suppression exists, but it may be dependent on the state of the network. Hippocampus inactivation did not influence encoding of fear in the mPFC in fear-conditioned rats, but did in rats that had undergone extinction (Sotres-Bayon et al., 2012). Moreover, in a developmental animal

model of schizophrenia, BLA-evoked heterosynaptic suppression is lost, while hippocampus inhibition of BLA-evoked orthodromic spikes is gained (Esmaeili and Grace, 2013). Under normal resting states, unidirectional heterosynaptic suppression may be the default mode interaction, only overcome by strong hippocampus activity.

PFC dysfunction has been implicated in several psychiatric disorders (O'Donnell, 2011; Myers-Schulz and Koenigs, 2012; Townsend and Altshuler, 2012; Chen et al., 2013). Anxiety and stress, which potentiate amygdala responses to emotionally-relevant features, is associated with increased negative coupling between the amygdala and PFC (Kim et al., 2011a; Veer et al., 2012). This is of relevance given that stress triggers and/or exacerbates emotional, motivational, and cognitive alterations in psychiatric disorders. Increases in amygdala activity in response to stress or anxiogenic stimuli may produce a subsequent decrease in general PFC function. Indeed, in bipolar disorder the amygdala shows hyperactivation while the PFC shows hypoactivation (Townsend and Altshuler, 2012). Furthermore, hyperactivity in the BLA has also been suggested to decrease mPFC function in animal model of pain (Ji et al., 2010). Excessive heterosynaptic suppression as a result of hyperactivity in the BLA to PFC pathway and the subsequent “bottom up” inhibition of PFC neural activity may impair synchronization of the PFC with upstream structures such as the hippocampus. The BLA may therefore play a critical role in gating information flow into the PFC from other temporal cortical structures. BLA-driven heterosynaptic suppression of hippocampus inputs to the mPFC bridges two hypotheses in psychiatric disease whereby the antagonistic relationship between the BLA and PFC could result in dysfunction in hippocampus to PFC information flow.

Implications

Although KORs can inhibit mPFC DA, glutamate, and glutamate-driven GABA enhancements, it is currently not clear how KORs ultimately modulate cortical network activity. DA bi-directionally modulates GABAergic and glutamatergic amino acid transmission (Seamans and Yang, 2004). The whole-cell experiments examining the effects of U69,593 on mEPSP frequency and ultrastructural localization of KOR to asymmetric synapses suggest that KOR action is directly on glutamatergic terminals. However, it is not clear how much the ability of KORs to regulate glutamate transmission is influenced by its inhibitory effects on DA and potential inhibitory effects on GABA neurotransmission. It is likely that KOR regulation of DA is completely independent of its effects on glutamate as U69,593-mediated inhibition of mPFC DA is not present after selective ablation of KORs in DAT-expressing neurons.

KORs may have complex effects on information processing in cortical circuits by altering GABA/glutamate interactions and decreasing DA modulation. This is evident in EEG recordings in humans and rats where systemic KOR agonists decrease spectral power in overall oscillations, but enhance power in the theta band (Young and Khazan, 1984; Ranganathan et al., 2012). Understanding the role of mPFC dynorphin/KOR signaling in information processing in cortical networks may reveal an understanding of processes underlying behavioral effects of KOR ligands.

It is unclear which neurons release dynorphin in the mPFC and the stimuli that cause dynorphin release in the mPFC. Dynorphin tone can be increased in response to strong depolarization, synaptic inputs, ethanol, and DA modulation elsewhere in the brain (You et al., 1994; Marinelli et al., 2006; Iremonger and Bains, 2009). Therefore, it is

likely that mPFC dynorphin release may result from strong activation and/or metabotropic modulation of the sparse population of dynorphin-expressing mPFC neurons or dynorphin-releasing mPFC afferents. A means to selectively decrease dynorphin turnover, and hence increase dynorphin tone, is not possible as peptidases involved in dynorphin metabolism also metabolize other neuropeptides, which has limited our ability to understand of dynorphin signaling in the nervous system. However, the recent development of photo-activatable dynorphin and mice that express Cre-recombinase under the prodynorphin promoter coupled with optogenetic or DREADD-based drivers or inhibitors of neural activity will facilitate our understanding of dynorphin action in the mPFC and elsewhere. These approaches will also allow us to map and activate dynorphin-containing pathways in the brain and probe their role in behavior.

Although not part of our hypothesis, we sought to determine the role of mPFC KOR signaling in behavioral effects produced by a systemically-administered KOR agonist. Intra-mPFC KOR activation is sufficient to produce CPA (Bals-Kubik et al., 1993). As systemically-administered KOR agonists act at various neural loci to produce CPA, we assessed whether mPFC KOR activation was necessary for KOR-mediated aversion. mPFC nor-BNI microinjection blocked CPA produced by systemically-administered U69,593. Since mPFC KOR signaling is necessary for KOR-mediated aversion, KOR signaling in the mPFC may encode alterations in affect, stress/anxiety-like behavior, and/or brain reward function. Infralimbic U69,593 or nor-BNI microinjections produce anxiolytic and anxiogenic effects in mice, respectively (Wall and Messier, 2000, 2002). Considering prelimbic and infralimbic cortices have divergent roles in reward-seeking behavior, fear, and drug relapse (Peters et al., 2009), it is

plausible that prelimbic KOR signaling encodes opposite behavioral effects as infralimbic. This is consistent with the present study where dorsal mPFC microinjections of nor-BNI focused on the prelimbic/ventral anterior cingulate cortex blocked KOR-mediated CPA. The question remains whether KOR modulation of mPFC excitation/inhibition, and not DA, mediates this aversive effect, since mesocortical DA denervation does not modify KOR-mediated aversion (Shippenberg et al., 1993).

There is evidence of upregulation of the DYN/KOR system in the PFC of post-mortem alcoholics relative to controls (Bazov et al., 2013). Increased KOR signaling in PFC regions of alcoholics would be expected to result in a hypo-DAergic cortical state and alterations in glutamate synaptic transmission. It is feasible that PFC KOR upregulation may contribute to deficits in PFC-dependent decision-making and cognition in alcoholics. Furthermore, our results that mPFC KOR signaling is sufficient to produce aversion and necessary for KOR-mediated aversion, suggest that heightened PFC KOR signaling could contribute to a state of malaise or anhedonia during abstinence. This is of importance as negative affective states are powerful mediators of negative reinforcement processes that drive alcohol or opiate addiction.

KOR agonists produce psychotomimetic and anxiogenic effects in humans (Pfeiffer et al., 1986). The psychoactive ingredient in *Salvia divinorum*, a hallucinogenic mint plant, is salvinorin-A, an extremely potent and selective KOR agonist (Roth et al., 2002). Our findings that mPFC KORs alter DA and glutamatergic neurotransmission in the mPFC suggests that psychoactive effects produced by KOR agonists in man and animal models may be due in part to KOR signaling in the mPFC. This is substantiated by our finding the mPFC KOR antagonism blocked the ability of systemically-

administered U69,593 to produce aversion. Therefore, KOR may provide a therapeutic target aimed at ameliorating symptoms in psychiatric disorders associated with aberrant PFC neurotransmission. Dysregulation of dynorphin/KOR signaling in PFC regions need not occur for KOR ligands to be utilized as a treatment. For instance, KOR antagonism may be beneficial to ameliorate hypodopaminergic states in PFC regions that contribute to cognitive deficits. Thus, future research aimed at understanding how mPFC control local network activity and influence downstream structures may aid in the development of such therapeutics.

Conclusions

Here we tested the hypothesis that *KORs negatively regulate synaptic inputs from afferents structures into the mPFC* (See Fig. 5.1 for my working model). Our results demonstrate that mPFC KORs negatively regulate the mesocortical DA pathway by directly acting on DA varicosities. KORs in the mPFC also negatively modulate glutamatergic inputs to the mPFC in a pathway-specific manner, with BLA but not hippocampus afferents being inhibited by local KORs. Furthermore, we provide evidence of an inhibitory influence of the BLA on hippocampus to mPFC information flow, a process that does not require KOR signaling. These findings extend our basic understanding of KOR regulation of mPFC neurotransmission and interactions of limbic afferents therein. This is of importance as the mPFC is an integral brain region involved in motivation, decision-making, cognition under normal conditions that can be pathologically altered in psychiatric conditions.

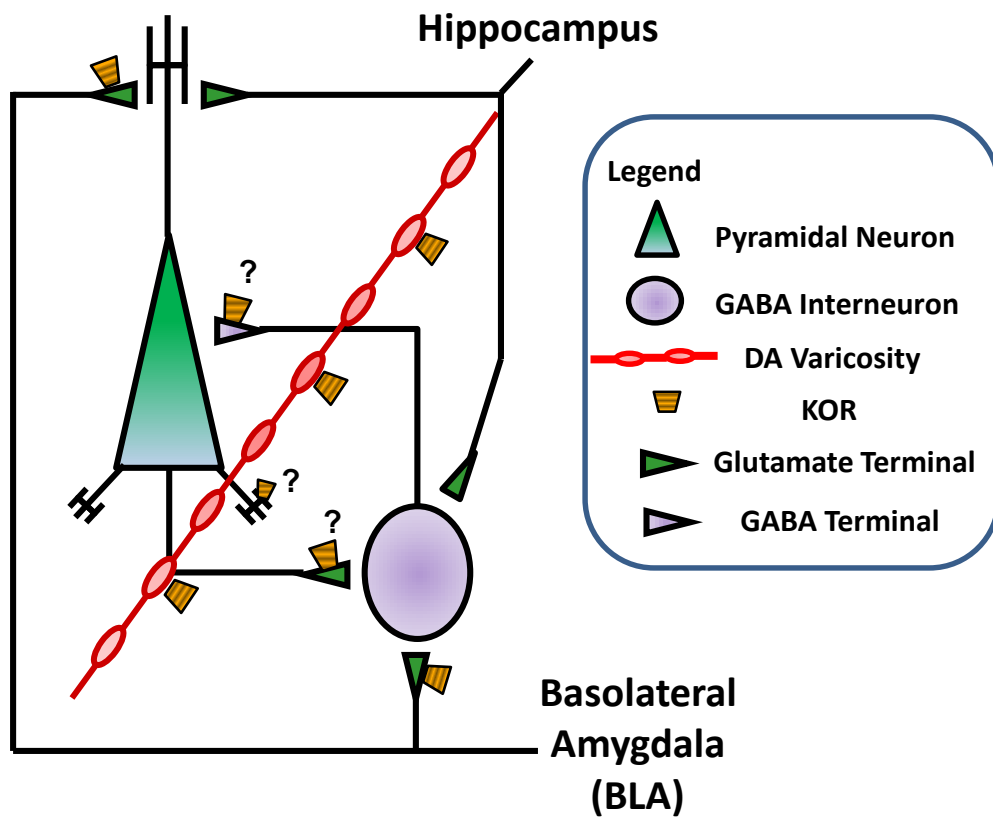


Figure 5.1: Working model of mPFC KOR regulation of mesocortical and limbic inputs to the mPFC. Highly simplified mPFC microcircuit and afferents from VTA DA, BLA, and hippocampal to local circuit interneurons and pyramidal neurons.

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