

2015

Molecular Mechanisms Underlying Environmental Enrichment-Induced Neuroprotection in Vulnerability to Nicotine Addiction

Adrian Michael Gomez
University of South Carolina - Columbia

Follow this and additional works at: <https://scholarcommons.sc.edu/etd>



Part of the [Other Pharmacy and Pharmaceutical Sciences Commons](#)

Recommended Citation

Gomez, A. M.(2015). *Molecular Mechanisms Underlying Environmental Enrichment-Induced Neuroprotection in Vulnerability to Nicotine Addiction*. (Doctoral dissertation). Retrieved from <https://scholarcommons.sc.edu/etd/3174>

This Open Access Dissertation is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact digres@mailbox.sc.edu.

MOLECULAR MECHANISMS UNDERLYING ENVIRONMENTAL ENRICHMENT-
INDUCED NEUROPROTECTION IN VULNERABILITY TO NICOTINE ADDICTION

by

Adrian Michael Gomez

Bachelor of Science
College of Charleston, 2008

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Pharmaceutical Sciences

College of Pharmacy

University of South Carolina

2015

Accepted by:

Jun Zhu, Major Professor

Kim Creek, Committee Member

Steven Harrod, Committee Member

Douglas Pittman, Committee Member

Kennerly Patrick, Committee Member

Lacy Ford, Vice Provost and Dean of Graduate Studies

© Copyright by Adrian Michael Gomez, 2015
All Rights Reserved

ACKNOWLEDGEMENTS

First and foremost I would like to thank my mentor, Dr. Jun Zhu. Your guidance, patience, and care have helped shape my initial career as a scientist. My experience in your laboratory is an unforgettable one and will always be cherished more than words can even express.

I would also like to thank my research family (in no particular order): Narasimha Midde, Dr. Wei-Lun Sun, Pamela Quizon, Ivory Chen, Richard McCain, Dr. Steven Harrod, Dr. Ryan Lacy, Dr. Rosemary Booze, Dr. Charles Mactutus, Dr. Kim Creek, Dr. Diego Altomare, Dr. Michael Shtutman, Hao Ji (Emily), Dr. Douglas Pittman, Dr. Kennerly Patrick, Dr. Jill Turner, Dr. Lawrence Reagan, and Dr. Jim Fadel.

In addition, I would like to thank my non-research family (in no particular order): Dr. Lauren Richmond, Gabriel Gomez, Richard Gomez, Lynn Gomez, Donte Stewart, Victor and Liz Sahijram, Daris and Claire Brown, David Elliott, Dr. Emily Stanley, Dr. Brittany Law, Denise Grosenbaugh, and Sarah Bertrand.

Everyone listed above will never realize the enormous impact they have had on me in some shape or fashion. A thank you will never be enough to express my gratitude to each and every one of you.

ABSTRACT

Tobacco use is the number one preventable cause of death in the world. Nicotine is the principal, addictive component in tobacco responsible for its reinforcing properties that drive the behavioral manifestations of nicotine addiction. Environment is becoming increasingly implicated in nicotine susceptibility. However, the molecular mechanisms that underlie susceptibility to nicotine addiction remain unknown. An ideal animal model that addresses environmental factors uses rats raised in an enriched condition (EC), a standard condition (SC), and an impoverished condition (IC); which differ in novelty, social cohorts, handling, and physical activity. EC rats exhibit a neuroprotective-like phenotype in the behavioral resistance to drugs of abuse; however, the impact of enrichment on nicotine-mediated behaviors and the subsequent molecular mechanisms underlying these behavioral adaptations remain unidentified.

EC rats were found to have increased behavioral sensitization, an indirect measure of drug-mediated motivation, in response to repeated experimenter-delivered nicotine in comparison to IC and SC rats. Additionally, EC rats have decreased nicotine-maintained responding compared to IC rats in a self-administration paradigm, the most reliable experimental model to evaluate the reinforcing properties of drugs of abuse. One commonality we observed in both behavioral paradigms was that phosphorylated extracellular signal-regulated kinase1/2 (pERK1/2), an intracellular signaling protein kinase involved in drug-induced neuroplasticity, levels in the prefrontal cortex (PFC)

were significantly increased in response to nicotine in IC rats, whereas nicotine-mediated increases in pERK1/2 activity were attenuated in EC rats. MicroRNAs (miRs) are post-transcriptional regulators of gene expression that have recently been implicated in drug-mediated neuroadaptations. MiR-221 was found to be highly enriched strictly in the PFC of EC rats in response to repeated nicotine administration. Lentiviral overexpression of miR-221 in the PFC of IC rats enhanced nicotine-mediated locomotor sensitivity while attenuating nicotine-mediated increases in pERK1/2 activity. However, enrichment-induced decreases in nicotine-intake were associated with an attenuation in nicotine-mediated orexin receptor-1 (OX1R) upregulation within the PFC. Additionally, although not as robustly as in the repeated model of repeated nicotine administration, miR-221 was increased significantly within the PFC of EC rats that underwent nicotine self-administration. Collectively, these studies implicate miR-221-dependent regulation of ERK1/2 within the PFC in response to nicotine in mediating the increased behavioral sensitivity observed in EC rats, as well as an OXR1-dependent regulation of ERK1/2 within the PFC in decreasing nicotine-intake in EC rats. Moreover, these studies suggest that miR-221 may be a universal mediator of the enrichment-induced protective-like phenotype in response to nicotine exposure. Future studies examining the upstream and downstream mechanisms in which miR-221 is mediating its effects will better clarify the exact mechanism of miR-221 and lead to potential therapeutic targets for nicotine addiction.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT	iv
LIST OF FIGURES	viii
LIST OF SYMBOLS	x
LIST OF ABBREVIATIONS.....	xi
CHAPTER 1. GENERAL INTRODUCTION.....	1
1.1 SIGNIFICANCE.....	1
1.2 NICOTINE ADDICTION.....	2
1.3 DRUG-INDUCED LOCOMOTOR SENSITIZATION.....	3
1.4 DRUG-INDUCED CONDITIONED PLACE PREFERENCE.....	4
1.5 DRUG SELF-ADMINISTRATION.....	5
1.6 MESOCORTICOLIMBIC DOPAMINERGIC SYSTEM	6
1.7 NEUROCHEMICAL SUBSTRATES OF NICOTINE ADDICTION	7
1.8 MICRORNAs.....	10
1.9 MICRORNAs AND ADDICTION.....	11
1.10 ENVIRONMENTAL ENRICHMENT	13
1.11 ENRICHMENT AND DRUG-MEDIATED BEHAVIORS.....	14
1.12 PREFRONTAL CORTEX: KEY AREA FOR ENRICHMENT-INDUCED BASAL ADAPTATIONS.....	17
1.13 PREFRONTAL CORTEX: POTENTIAL KEY AREA FOR ENRICHMENT-MEDIATED PROTECTION TO DRUG-INDUCED NEUROADAPTATIONS	21

1.14 SIGNIFICANCE REVISITED	23
CHAPTER 2. EFFECTS OF ENRICHMENT ON NICOTINE-MEDIATED SENSITIZATION.....	26
2.1 INTRODUCTION.....	26
2.2 METHODS	29
2.3 RESULTS.....	36
2.4 DISCUSSION	48
CHAPTER 3.ROLE OF MICRORNAs IN ENRICHMENT-INDUCED ALTERATIONS IN NICOTINE-MEDIATED SENSITIZATION.....	72
3.1 INTRODUCTION.....	72
3.2 METHODS	75
3.3 RESULTS.....	85
3.4 DISCUSSION	91
CHAPTER 4. EFFECTS OF ENRICHMENT ON NICOTINE SELF-ADMINISTRATION.....	108
4.1 INTRODUCTION.....	108
4.2 METHODS	110
4.3 RESULTS.....	117
4.4 DISCUSSION	120
CHAPTER 5. GENERAL DISCUSSION.....	133
5.1 SUMMARY	133
5.2 CONCLUSIONS	135
5.3 FUTURE DIRECTIONS	137
REFERENCES	141

LIST OF FIGURES

Figure 1.1 Diagram of nicotine signaling within the mesocorticolimbic circuitry.....	24
Figure 1.2 Integrated model of nicotine-mediated intracellular signaling cascades.....	25
Figure 2.1 Timeline for nicotine sensitization experiments.....	61
Figure 2.2 Baseline activity in EC, IC, and SC rats.....	62
Figure 2.3 Effect of repeated nicotine across sessions on locomotor sensitization in EC, IC, and SC rats	63
Figure 2.4 Effect of repeated nicotine within session on locomotor sensitization in EC, IC, and SC rats	64
Figure 2.5 Basal and repeated nicotine-mediated pDARPP-32 activity within the mesocorticolimbic circuitry in EC, IC, and SC rats.....	66
Figure 2.6 Basal and repeated nicotine-mediated pCREB activity within the mesocorticolimbic circuitry in EC, IC, and SC rats.....	67
Figure 2.7 Basal and repeated nicotine-mediated pERK1/2 activity within the PFC of EC, IC, and SC rats	68
Figure 2.8 Correlations of pDARPP-32 and pERK1/2 expression with basal locomotor levels of EC, IC, and SC rats	69
Figure 2.9 Effect of SL327 on acute nicotine-mediated locomotor levels in EC, IC, and SC rats.....	70
Figure 2.10 Effect of SL327 on acute nicotine-mediated pERK1/2 levels in EC, IC, and SC rats.....	71
Figure 3.1 Timeline for identification of prefrontal miR expression in EC, IC, and SC rats in response to repeated nicotine	100
Figure 3.2 Profile of prefrontal miR expression in EC, IC, and SC rats in response to repeated nicotine	101
Figure 3.3 Effect of <i>in vitro</i> miR-221 overexpression on nicotine-mediated pERK1/2	

activity.....	103
Figure 3.4 Timeline for nicotine sensitization experiments involving <i>in vivo</i> miR-221 overexpression	104
Figure 3.5 Validation of <i>in vivo</i> miR-221 overexpression in the PFC.....	105
Figure 3.6 Effect of <i>in vivo</i> miR-221 overexpression in the mPFC of EC, IC, and SC rats . on nicotine-mediated locomotor sensitization	106
Figure 3.7 Effect of <i>in vivo</i> miR-221 overexpression on nicotine-mediated pERK1/2 activity in the mPFC of EC, IC, and SC rats	107
Figure 4.1 Timeline for nicotine self-administration experiments	129
Figure 4.2 Nicotine self-administration behavior in EC and IC rats	130
Figure 4.3 Effect of nicotine self-administration on pERK1/2 activity within the PFC of EC and IC rats	131
Figure 4.4 Effect of nicotine self-administration on OXR1 expression within the PFC of EC and IC rats	132
Figure 5.1 Pathway analysis for differential prefrontal mRNA expression in EC and IC rats in response to repeated nicotine administration	138
Figure 5.2 Pathway analysis for differential prefrontal mRNA expression in EC and IC rats in response to nicotine self-administration	139
Figure 5.3 Prefrontal miR-221 expression profile for EC and IC rats in response to various nicotine-mediated paradigms	140

LIST OF SYMBOLS

\pm	Plus/minus
μ	Micro
Δ	Delta
$^{\circ}$	Degree
n	Number of samples / subjects
$\Delta\Delta$	Delta delta
V	Volts
C	Celsius
h	Hour (time)
s	Second (time)
min	Minutes (time)
mg	Milligram
g	Gram
kg	Kilogram
μl	Microliter
ml	Milliliter

LIST OF ABBREVIATIONS

AcH.....	Acetylcholine
ANOVA	Analysis of Variance
CAMKII.....	Calcium/Calmodulin-dependent Protein Kinase II
cAMP	Cyclic Adenosine Monosphosphate
cDNA	Complementary DNA
CPP	Conditioned Place Preference
CREB	cAMP response-element binding protein
D1.....	Dopamine receptor D1
D2.....	Dopamine receptor D2
DA.....	Dopamine
DARPP-32	Dopamine- and cAMP-regulated Phosphoprotein
DAT	Dopamine Transporter
DOPAC.....	3,4-Dihydroxyphenylacetic Acid
EC	Enriched Condition
ELK-1	Ets-like protein-1
ERK1/2	Extracellular signal-regulated kinase 1/2
FR.....	Fixed Ratio
GFP	Green Fluorescent Protein
i.p.	Intraperitoneal
IC.....	Impoverished Condition
LV	Lentivirus
MAPK.....	Mitogen-Activated Protein Kinase

miR.....	MicroRNA
mPFC	Medial Prefrontal Cortex
NAc.....	Nucleus Accumbens
Nic.....	Nicotine
NMDA	N-methyl-D-aspartate
OXR1	Orexin Receptor-1
PBS	Phosphate Buffer Saline
PC12.....	Pheochromocytoma cell line
pCREB	Phosphorylated CREB
pDARPP-32 Thr34.....	Phosphorylated DARPP-32 at Threonine 34
pDARPP-32 Thr75.....	Phosphorylated DARPP-32 at Threonine 75
pERK1/2	Phosphorylated ERK1/2
PFC	Prefrontal Cortex
PKA.....	Protein Kinase A
PND.....	Postnatal Day
PP-1.....	Protein phosphatase-1
PR.....	Progressive Ratio
qPCR.....	Quantitative Reverse-Transcriptase PCR
s.c.	Subcutaneous
S.E.M.	Standard Error of the Mean
Sal	Saline
STR	Striatum
VTA	Ventral Tegmental Area

CHAPTER 1

GENERAL INTRODUCTION

1.1 SIGNIFICANCE

Tobacco use is the number one preventable cause of death (Benowitz, 2010). Yet, tobacco use is linked to 1 in 5 deaths (~440,000/year) in the United States every year and tobacco-related disease is estimated to cost the United States \$160 billion annually (Benowitz, 2008, Mathers and Loncar, 2006). These staggering numbers contribute to the grim statistic that globally, 5.4 million people die each year from tobacco use, and this statistic is estimated to rise to 8 million deaths per year in the next 30 years. (Mathers and Loncar, 2006, Benowitz, 2008, Ezzati and Lopez, 2003). Nicotine is the primary psychoactive, addictive component responsible for the reinforcing properties in tobacco products (Stolerman and Jarvis, 1995). Approximately 1/3 of the world's adult population uses a form of tobacco, thus making nicotine the most widely used, addictive drug of abuse (Peto et al., 1996). Notably, of the 60-70% of people who try tobacco products, only 20-25% progress to become daily dependent users (Benowitz, 2010). As a result, there is a critical need to identify the mechanism(s) underlying the initiation and development of nicotine addiction.

Gene-environment interactions contribute to nicotine abuse vulnerability (i.e. whether a person becomes addicted or not). In particular, previous research has determined that vulnerability to drug abuse is highly dependent on environmental factors

(Leshner, 2000, Rhee et al., 2003, Compton et al., 2005). Additionally, shared genetic factors involved in addiction vulnerability can be influenced by environmental conditions (Hopfer et al., 2003, McGue et al., 1996). Therefore, one key mechanism to unlock what underlies nicotine addiction is to understand how environmental factors shape individual vulnerability to nicotine addiction.

1.2 NICOTINE ADDICTION

Nicotine addiction is characterized as a relapse disorder consisting of a vicious cycle of: intoxication (initial drug intake), bingeing (loss of control in drug intake), withdrawal and emergence of a negative emotional state, craving of the drug, and then relapse of the drug (Koob and Volkow, 2010, Koob and Le Moal, 1997). There also appears to be two shifts in the progression of addiction. First, there is a shift from impulsive to compulsive use of the drug. And second, there is a shift from positive reinforcement (i.e. engage in the drug due to the euphoric-like effects) to negative reinforcement (i.e. engage in the drug to relieve the unpleasant withdrawal-like effects) (Koob et al., 2004, Koob, 2004). Thus, nicotine addiction within an individual is a constant struggle to maintain an equilibrium in receiving the euphoric-like properties of the drug, yet avoid the withdrawal-like symptoms of the drug. However, the neurobiological mechanisms underlying each component of the nicotine addiction cycle remains infantile at best, and future research is desperately needed to aid in therapeutic strategies for prevention in the initiation, maintenance, and relapse of nicotine addiction. The molecular adaptations in response to drug exposure serve as a gateway to the drug-induced behavioral adaptations. Thus, drug addiction is a behavioral manifestation and

development of rodent behavioral models of addiction has served as a preclinical method for evaluating addiction in humans.

1.3 DRUG-INDUCED LOCOMOTOR SENSITIZATION

One common behavioral adaptation in response to repeated administration of addictive drugs is a sensitized behavioral or locomotor response. Following repeated injections of a drug, rodents will display hyperlocomotor activity upon each subsequent injection. Although locomotor sensitization does not measure reward or reinforcement directly per se, this behavioral paradigm can enhance aspects of both conditioned place preference (CPP) and drug self-administration. Because locomotor sensitization reflects the sensitized dopaminergic activity within the motivational circuitry, this paradigm represents an indirect measure of addiction in rodents with an emphasis on measuring the incentive salience or wanting of the drug, and the subsequent drug-related stimuli (Robinson and Berridge, 1993, Robinson and Berridge, 2008). Additionally, many of the neural correlates of the locomotor sensitization paradigm overlap with the neural correlates of CPP and SA. Therefore, locomotor sensitization is a more “simpler” tool for dissecting how repeated exposures of a drug influence neuroadaptations. Notably, locomotor sensitization persists for months and potentially years, even in the absence of the drug during this timeframe (Robinson and Becker, 1986, Paulson et al., 1991). Moreover, there is evidence that sensitization does indeed occur in humans (Strakowski et al., 1996, Strakowski et al., 2001, Boileau et al., 2006). But due to the indirect nature of this behavioral measurement there are limitations surrounding behavioral sensitization. Firstly, a few drug exposures appear to be adequate to induce behavioral sensitization

supporting the notion that the sensitization may be a critical process in initiating addiction, whereby drug addiction seems to involve a more long-term drug exposure. Secondly, experimenter-delivered drug appears to increase the wanting of non-drug reinforcers (i.e. food and sexual stimuli) (Nocjar and Panksepp, 2002, Nocjar and Panksepp, 2007). However, this model is very useful in examining molecular changes in response to drugs of abuse as all animals receive the same amount of the drug. To conclude, locomotor sensitization may not include all aspects that encompass addiction, but this behavioral paradigm has amassed immense validation in dissecting the neuroadaptations accompanying exposure to drugs of abuse.

1.4 DRUG-INDUCED CONDITIONED PLACE PREFERENCE (CPP)

CPP reflects the rewarding properties of drugs of abuse. Although CPP experiments were not performed directly in the studies outline here, CPP will be referred to throughout this document, and is needed to understand some of the mentioned behavioral effects to drugs of abuse. As with locomotor sensitization, CPP does not reflect the full spectrum of addiction as both paradigms employ experimenter-delivered drugs. Animals are injected with the drug in one-context specific chamber, and also injected with the control/vehicle in a separate context-specific chamber. After multiple injection-context pairings, animals learn to associate the euphoric-like properties of the drug within a specific context. When post-tested in an injection-free environment, where animals can opt to stay in the drug-paired chamber (animals display CPP) or the vehicle-paired chamber (animals display conditioned place aversion), animals will spend more time in the drug-paired chamber due to the association of the context with the rewarding

effects of the drug. Hence, CPP is thought of as an indirect measure of addiction by characterizing the reward-like properties of addictive drugs.

1.5 DRUG SELF-ADMINISTRATION

Self-administration measures the reinforcing properties of drugs of abuse. Unlike, locomotor sensitization or CPP which involves experimenter-delivered drugs, the self-administration paradigm allows the animals to control one's own drug intake. For this reason, the drug self-administration procedure is the best preclinical model of addiction and is considered the most reliable and predictive experimental model for evaluating the reinforcing effects of drugs in animals (Panlilio et al., 2008). Indwelling intra-jugular catheters are surgically placed in the animals. Animals are then placed in an operant box and learn to perform an instrumental action (lever press or nose poke) to receive a drug infusion via the intravenous catheter. This allows researchers to assess the reinforcing effects of drugs of abuse through a variety of different constructs by altering schedules of reinforcement and doses. Fixed-ratio (FR) schedules require animals to make a set number of responses to receive the drug (i.e. FR-5, animal has to make 5 lever presses to receive drug) and at its simplest, measures drug-intake behavior (Koob, 1992).

Additionally, a progressive-ratio (PR) schedule reflects how hard an animal is willing to work for the drug (motivation) by exponentially increasing the number of actions required to obtain the drug after each successful drug infusion (Richardson and Roberts, 1996). Moreover, experimenters can emulate the consumption of large quantities of the drug seen in humans via the escalation model (Ahmed and Koob, 1998). Here, animals are allowed to self-administer the drug for a short or prolonged duration. Animals in the

prolonged group will gradually begin to increase drug responding, therefore representing the binge intake reflected in human drug addicts. For these reasons, self-administration appears to represent the most advantageous pre-clinical model for studying drug addiction.

1.6 MESOCORTICOLIMBIC DOPAMINERGIC SYSTEM

The mesocorticolimbic dopaminergic (DAergic) system, which is responsible for the transmission of the catecholaminergic neurotransmitter dopamine (DA), consists of initial projections from the ventral tegmental area (VTA) that innervate the nucleus accumbens (NAc) as well as the prefrontal cortex (PFC) (Tzschentke, 2001). The mesocorticolimbic DAergic system participates in a variety of processes including motivational salience, motor functions for initiating goal-directed behaviors, attention/cognition, and encoding the cues and values that regulate the rewarding and reinforcing-like properties of external stimuli (Horvitz, 2000, Tzschentke, 2001, Salamone and Correa, 2012). However, recent research has also found that this rewarding system is intertwined with the aversive-like nature of events (Salamone and Correa, 2012, Volman et al., 2013, Brooks and Berns, 2013). Thus, for all intents and purposes, this DAergic circuit maximizes the ability to assign “value” or update the current “value” to both appetitive and aversive stimuli for future actions.

The basal activity of DAergic neuronal firing within this circuitry consist of a tonic, steady state, however, when salient or aversive stimuli is encountered (i.e. food, sex, etc.) DAergic neuronal firing is altered to strengthen the behaviorally-relevant stimuli to further promote goal-directed actions (Grace, 2000). In respect to the current

topic, drugs of abuse prey on this circuitry to mediate the rewarding and reinforcing properties of drugs of abuse that underlie addiction.

1.7 NEUROCHEMICAL SUBSTRATES OF NICOTINE ADDICTION

For the purpose of the present topic, the review of the neurochemical substrates of nicotine addiction will focus on the pharmacology of nicotine's actions and the initial neurochemical events leading up to the neuroadaptations underlying addiction. Nicotine initially acts on nicotinic acetylcholine receptors (nAChRs) found abundantly throughout the brain. NACHRs are pentameric receptor complexes consisting of a heteromeric or homomeric assortment of 12 subunits ($\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$) that differ in pharmacokinetic properties (McGehee and Role, 1995, Role and Berg, 1996). These receptors are distributed throughout the entire brain, and thus determining the exact mechanism that causes nicotine addiction is very difficult to examine. Nicotine binds ligand-gated ion channels allowing for the entry of sodium or calcium, and which mediate subsequent presynaptic- and postsynaptic-dependent neurotransmitter release (Dajas-Bailador and Wonnacott, 2004). Corresponding with all psychostimulants, nicotine's rewarding and reinforcing properties are due to nicotine's actions within the mesocorticolimbic DAergic circuitry (Berridge and Robinson, 1998, Laviolette and van der Kooy, 2004). The basal activity of DAergic neuronal firing within this circuitry consist of a tonic, steady state; however when salient-stimuli is encountered (i.e. food, sex, etc.) DAergic neuronal firing shifts to a more rapid, phasic/burst state to shape and strengthen the reward-associated stimuli to further promote goal-directed actions (Grace, 2000). Nicotine "hijacks" this circuitry by initially binding to high affinity, fast desensitizing $\alpha 4\beta 2$ receptors on

GABAergic cell bodies that inhibit VTA DAergic neuronal firing. These nAChRs are highly concentrated in VTA cell bodies, and allow GABAergic neurons to quickly desensitize allowing for the disinhibition and increased firing of VTA DAergic neurons (Mansvelder and McGehee, 2002, Mansvelder et al., 2002). These DA increases are compounded by the fact that nicotine also binds to low affinity, slow desensitizing $\alpha 7$ -containing nAChRs on glutamatergic-containing neurons that are highly concentrated within the PFC (McGehee et al., 1995). Besides receiving dense dopaminergic projections from the VTA and NAc, the PFC has reciprocal glutamatergic connections to these same brain regions (Grace et al., 2007). This allows nicotine to induce glutamate, the main excitatory neurotransmitter in the brain, release onto NAc and VTA dopaminergic cell bodies and allowing for increased DAergic excitation (Girod et al., 2000). Thus, the PFC modulates the activity of the NAc and VTA in response to nicotine (see figure 1.1). These nicotine-dependent DAergic and glutamatergic alterations appear critical in shaping the neuroadaptations responsible for the initial addiction process. Further, these neuroadaptations are thought to be dependent upon the activity of complex intracellular signaling systems between the cell surface and the nucleus (Dajas-Bailador and Wonnacott, 2004) (see figure 1.2). Thus, nAChRs may not directly affect many of the implicated intracellular signaling systems such as DA- and cAMP-regulated phosphoprotein-32 (DARPP-32), extracellular signal-regulated kinase1/2 (ERK1/2, two isoforms with overlapping structure and function), and cAMP-response element-binding protein (CREB), but rather indirectly through nAChR-mediated dopamine and glutamate release.

Activation of the DA/D1 receptor/cAMP/protein kinase A (PKA) pathway increases phosphorylation of DARPP-32 at the site Threonine 34 (pDARPP-32 Thr34), but decreases phosphorylation of DARPP-32 at Threonine 75 (pDARPP-32 Thr75) (Nishi et al., 2000). In contrast, phosphorylation of DARPP-32 at Thr75 by cyclin-dependent kinase 5 has a major inhibitory effect on pDARPP-32 Thr34, thereby reducing D1 DA signaling through the DARPP-32/ protein phosphatase-1 (PP-1) cascade (Nishi et al., 2000, Bibb et al., 1999). In addition, activation of this PKA pathway enhances phosphorylation of the transcription factor CREB at serine 133, (Nairn et al., 2004, Svenningsson et al., 2005, Dash et al., 1991). In an opposing fashion, activation of Ca²⁺-dependent calcineurin by D2 receptors and glutamate-activated N-methyl-D-aspartate (NMDA) receptors results in dephosphorylation of pDARPP-32 Thr34 (Lindskog et al., 1999). ERK1/2, a member of the mitogen-activated protein kinase (MAPK) signaling pathway also signals to CREB at Ser133 via glutamate-dependent NMDA receptor activation of calcium-mediated calcium/calmodulin-dependent protein kinase II (CAMKII) activation, in addition to DARPP-32 being able to control ERK activation via PP-1 (Valjent et al., 2000, Valjent et al., 2005). Consequently, these intracellular signaling proteins are essential for neuronal plasticity in response to repeated nicotine exposure. Therefore, long-term use of nicotine mediates signaling-induced neuroadaptations responsible for the manifestation of the addicted phenotype. However, research into nicotine's regulatory processes on intracellular signaling-induced gene expression has been largely unexplored.

1.8 MICRORNAs

MicroRNAs (miRs) are small (~22 nucleotides in length) non-coding RNAs encoded by the genome that function as gene regulators (Bartel, 2004). Biogenesis of mature miRs are first derived from nuclear transcription by RNA polymerase II/III to form hairpin structures known as primary miRs (pri-miR); which are then further cleaved by the Drosha RNase III endonuclease to give rise to a smaller hairpin structure known as precursor miR (pre-miR) (Lee et al., 2002, Lee et al., 2003). The pre-miR is then exported via exportin-5 into the cytoplasm, where another RNase III endonuclease (Dicer) further cleaves the pre-miR into a miR duplex consisting of a “guide” strand (miR) and a “passenger” strand (miR*) (Lee et al., 2003, Yi et al., 2003). The mature miR or miR* strand is then translocated to the RNA-induced silencing complex (RISC), where the miR-RISC complex is guided to the target mRNA transcript to facilitate either mRNA degradation or mRNA silencing depending on the seed region (nucleotides 2-7 on the 5' region) of the miR (Sempere et al., 2004, Bartel, 2004, He and Hannon, 2004). Although both methods serve a similar outcome by preventing translation, perfect complementarity in base-pairing interactions between the miR and the target mRNA results in mRNA degradation, whereas imperfect complementarity in base-pairing interactions between the miR and the target mRNA results in mRNA silencing. After translational disruption, miRs can actually take place in multiple rounds of cleavage, thereby allowing for the effects of miRs to be amplified. Adding to the complexity of miRs, is the fact that multiple miRs can target the same mRNA transcript, and a single miR may target multiple mRNA transcripts (Esteller, 2011). Additionally, the non-seed region of the miR may be involved in the pairing and translational repression of the target

mRNA (Orom et al., 2008, Elcheva et al., 2009). Since their discovery in 1993 (Lee et al., 1993), the complex nature of miRs seems to be ever-growing. However, due to the ability of miRs to regulate gene expression, miRs are rapidly being implicated in the neuroadaptations involved in neurological diseases, where miRs are now being touted as potential therapeutic targets (Kosik, 2006).

1.9 MICRORNAS AND DRUG ADDICTION

Due to the nature of drug addiction, it should come as no surprise that miRs are now implicated in the pathological process of addiction. The root of drug addiction is the ability of drugs of abuse to impinge on the brain's gene expression networks to produce subsequent neuroplastic alterations. In fact, very recently, there have been several seminal studies implicating miRs as regulators of not only drug-induced plasticity within the reward circuitry, but of drug-induced behaviors as well. A study from the Kenny laboratory (Hollander et al., 2010, Im et al., 2010), recently found that miR-212 was drastically upregulated in the striatal regions of rats undergoing a cocaine-escalation model. Interestingly, overexpressing exogenous miR-212 in the striatum decreased cocaine-intake, whereas blocking endogenous miR-212 expression increased cocaine-intake; suggesting a regulatory switch of miR-212 in controlling the motivational properties of cocaine. This behavioral manifestation was later found to be due to homeostatic interactions of miR-212 with MeCP2 to regulate cocaine-induced of brain-derived neurotrophic factor (BDNF) expression within the striatum. Chandrasekar and Dreyer (Chandrasekar and Dreyer, 2009, Chandrasekar and Dreyer, 2011) also demonstrated that chronic cocaine injections upregulates miR-181a, and downregulates

miR-124 and let-7d within the NAc of rats. Subsequent overexpression of miR-124 and let-7d into the NAc attenuated cocaine-induced CPP, whereas overexpression of miR-181a within the NAc enhanced cocaine-induced CPP. Although not as specific as the previously mentioned study in determining the mRNA targets of the identified miRs, the cocaine-induced CPP alterations depended on the ability of the three miRs to differentially target multiple mRNA plasticity genes that have been implicated in cocaine addiction including, but not limited to, DAT, FosB, D2 receptor, BDNF, MeCP2, and pCREB levels. However, the actions of miRs are not exclusive to cocaine exposure. In a study by Tapocik and colleagues (Tapocik et al., 2014), miR-206 was found to be upregulated in the mPFC of rats after long-term exposure to alcohol vapor. When miR-206 was overexpressed into the mPFC of drug-naïve rats undergoing alcohol self-administration, rats that received exogenous miR-206 were found to significantly consume more alcohol and induce escalation within these rats. Further analyses determined miR-206 inhibits BDNF expression within the mPFC, thereby preventing BDNF-induced neuroadaptations within the mPFC that may underlie the increased alcohol consumption. Relevant to the current studies outlined here, nicotine exposure has been recognized to differentially regulate a multitude of miRs depending on *in vitro* or *in vivo* models, dose, and length of exposure (Huang and Li, 2009, Lippi et al., 2011, Taki et al., 2014); however, the involvement of these nicotine activated miRs in mediating nicotine-induced neuroplasticity and behavior remain unexplored. MiRs, therefore represent critical mediators in regulating the behavioral consequences of drugs of abuse that correspond to the homeostatic mechanisms involved in synaptic signaling and plasticity.

1.10 ENVIRONMENTAL ENRICHMENT

An enriched environment is “a combination of complex inanimate and social stimulation” (Rosenzweig et al., 1978) typically employed to dissect the role of environmental factors in inbred animals within a given experimental paradigm. By and large, an experimental setting consists of an enriched condition (EC) consisting of novel objects, social cohorts in a large-scale cage, and an enhancement of physical activity. This EC condition is subjected to comparison to either both or one of the controlled environments; an impoverished condition (IC) consisting of no novelty and no social cohorts in a small-scale cage, and a standard condition (SC) consisting of social cohorts with no novelty in an NIH-standard cage. These housing conditions are typically utilized during the animals’ adolescence period before experimental testing to prevent an experimental outcome, or utilized after an experimental paradigm to attenuate or reverse the results of an experimental outcome. The complexity of the enriched environment paradigm can have its limitations as this model comprises of multiple components: a large space, physical exercise, novel objects, and social cohorts. Although it appears that each factor may contribute to the enrichment-induced protective effect in response to drugs of abuse (Kanarek et al., 1995, Meeusen and De Meirleir, 1995, Lynch et al., 2010, Solinas et al., 2008, Gipson et al., 2011); isolating and determining which factor contributes to the neuroprotective-like phenotype is necessary for further understanding the role of environmental factors in nicotine abuse vulnerability. In fact, due to the variety of factors involved in an enriched condition, some experimenters have adopted even more groups to control for these factors such as including a novelty condition where

impoverished rats are housed with novelty, and a social condition consisting of social cohorts with no novelty in a large-scale cage (Gipson et al., 2011). However, these may not prove feasible in every laboratory as this drastically increases the number of animals and groups needed for the experiment. Nevertheless, the environmental enrichment paradigm has been critical in establishing how environment influences behavioral and neurochemical adaptations across multifaceted research fields.

1.11 ENRICHMENT AND DRUG-MEDIATED BEHAVIORS

Because both genetic and environmental factors mediate whether or not an individual progresses from a habitual drug user into an addict, it is not surprising that differential rearing conditions in genetically identical rodents affect subsequent addiction-related behaviors. There has been a vast amount of research into multiple preclinical paradigms demonstrating that rodents raised in an enriched environment display a neuroprotective-like phenotype to drug-induced behaviors in comparison to rats raised in impoverished or standard conditions, with the majority of studies focusing on psychostimulants.

In comparison to IC and SC rats, rodents raised in an enriched environment appear to have increased sensitivity to the locomotor-activating effects of acute amphetamine and cocaine administration (Bowling et al., 1993, Smith et al., 1997), however, enrichment decreases the locomotor response to acute nicotine (Green et al., 2003a) administration. When examining the locomotor response to repeated administration of the drug, EC rodents exhibit reduced locomotor sensitization to amphetamine (Bardo et al., 1995), cocaine (Smith et al., 1997), nicotine (Green et al., 2003a), methylphenidate (Wooters et al., 2011, Gill et al., 2013), morphine (Bardo et al.,

1997), and ethanol (Rueda et al., 2012). Interestingly, there were no differences in either heroin- or methamphetamine-mediated locomotor sensitization (El Rawas et al., 2009, Thiriet et al., 2011), however this may be due to rodent differences as these studies were focused on mice, whereas the former studies focused on rats. In a CPP paradigm, enrichment in rats increases the rewarding-like properties of amphetamine (Bowling and Bardo, 1994), cocaine (Green et al., 2010), and morphine (Bardo et al., 1997). When evaluating the effect of enrichment on the reinforcing properties of drugs of abuse, EC rats, in contrast to IC and SC rats, have decreased responding on FR schedules of reinforcement for amphetamine (Bardo et al., 2001), cocaine (Green et al., 2010), ethanol (Deehan et al., 2011) and methylphenidate (Alvers et al., 2012), in addition to decreased responding on PR schedules of reinforcement for cocaine (Green et al., 2010) and amphetamine (Green et al., 2002). Moreover, enrichment also decreases cocaine-taking behavior in an escalation paradigm in comparison to IC and SC rats (Gipson et al., 2011). However, it should be mentioned that the majority of the findings regarding EC-induced reductions in drug-taking behavior appear at low unit doses for the drug, where as the responding rates in EC and IC rats were found to be not significantly different at high unit doses. These behavioral adaptations are intriguing as enrichment appears to actually increase the euphoric effect of drugs of abuse, yet somehow blunts the drug-taking behavior. Overall, enrichment appears to have a preventative effect in the behavioral responses to drugs of abuse. Although the focus for the before mentioned work focuses on enrichment exposure before and during the drug exposure itself, it should be noted that enrichment has a potential curative effect as well. After drug-mediated behaviors have been established, exposing rodents to enrichment eliminated post-testing of cocaine-

mediated locomotor sensitization (Solinas et al., 2008), cocaine-induced CPP (Solinas et al., 2008); and cue- and stress-induced reinstatement of cocaine CPP (Chauvet et al., 2009) and self-administration (Thiel et al., 2010, Thiel et al., 2011).

Collectively, exposure to environmental enrichment increases acute locomotor sensitivity to drugs of abuse, decreases repeated drug-induced locomotor sensitization, increases the rewarding properties of drugs of abuse, and decreases the reinforcing properties of drugs of abuse. Accordingly, environmental enrichment produces powerful behavioral adaptations in response to drugs of abuse, which are likely controlled by enrichment-induced neuroadaptations. The environmental enrichment paradigm allows researchers to critically examine how extreme conditions, as represented by the IC condition, contribute to drug abuse vulnerability preclinically, while also allowing researchers to control every aspect of the environmental conditions. Although, environmental conditions are very diverse across humans, studies have shown clinical relevance with regard to environmental influence in individual vulnerability to drug abuse. In particular, previous research has determined that vulnerability to drug abuse is highly dependent on environmental factors (Leshner, 2000, Rhee et al., 2003, Compton et al., 2005). Additionally, shared genetic factors involved in addiction vulnerability can be influenced by environmental conditions (Hopfer et al., 2003, McGue et al., 1996). Thus, the environmental enrichment paradigm is able to extend the clinical findings of the effect of environmental conditions on the behavioral aspects of individual susceptibility to drug addiction in a preclinical rodent model.

1.12 PREFRONTAL CORTEX: KEY AREA FOR ENRICHMENT-INDUCED BASAL ADAPTATIONS

The neurochemical correlates of an enriched environment stretch across numerous brain regions, but one critical “hotspot” that is targeted by enrichment is the PFC. The PFC is of particular importance for this review in that the PFC is a heterogenous structure involved in regulating reward processing, habit learning, impulsivity, and drug seeking behaviors (Perry et al., 2011). The PFC receives dense ascending dopaminergic innervations from the NAc and VTA, while sending reciprocal descending glutamatergic projections back to these areas (Grace et al., 2007). Thus, by altering the basal state of the PFC, environmental enrichment may allow animals to better adapt to the drug-induced response.

Structurally, enrichment increases dendritic spine density, dendritic length, and dendritic branching within the PFC of primates. Importantly, there were no significant differences in the number of neurons in PFC regions, suggesting that increases in dendritic arborization play a relatively stronger role (Kozorovitskiy et al., 2005). These prefrontal structural modifications have also been supported in rats as enrichment-induced alterations in spine density, dendritic branching, and dendritic length were found in the PFC of male and female rats (Mychasiuk et al., 2014). Additionally, indirectly measuring the number of synapses through synaptophysin levels, enrichment increases the number of synapses in the PFC (Kozorovitskiy et al., 2005, Nithianantharajah et al., 2008). Together, these studies provide convincing evidence that enrichment modifies prefrontal structure. What remains relatively underexplored is the way in which enrichment alters prefrontal function.

The dopaminergic system within the PFC is highly vulnerable to enrichment-mediated alterations. IC gerbils have reductions in DA innervations into the medial and orbital portions of the PFC in comparison to EC gerbils (Winterfeld et al., 1998, Neddens et al., 2001). Enrichment has been shown to have no effect on DA levels in the rodent PFC (Naka et al., 2002, Zhu et al., 2004). While examination of the DA metabolite, 3,4-Dihydroxyphenylacetic acid (DOPAC) has been shown to be decreased in rats in response to enrichment (Zhu et al., 2004); there appears to be no differences in DOPAC or homovanilic acid (HVA) content found in mice (Naka et al., 2002). The seemingly disparate results of decreased DOPAC content in the PFC of EC rats with no changes to the DA levels, may be a result of modifications to the dopamine transporter (DAT). In fact, enrichment decreased the maximal velocity of [³H] DA uptake with no differences in B_{max} or K_d values of DA binding sites (Zhu et al., 2004), suggesting a potential DAT trafficking-dependent mechanism induced by enrichment. In support of this view, while no DAT immunoreactivity was observed in the PFC of EC or IC rats, cell surface biotinylation assays revealed a reduction in cell surface DAT expression in EC rats with no differences seen in total DAT levels (Zhu et al., 2005b). Additionally, EC rats have been shown to be more sensitive to prefrontal DAT internalization induced by protein kinase C-dependent mechanisms (Wooters et al., 2011). While there were no differences found in baseline DA clearance from the synapse between EC and IC rats, there does appear to be some evidence that the DA clearance rate is slower in EC rats in comparison to IC rats (Zhu et al., 2007a). Thus, these data heavily supports the idea that enrichment is capable of modifying DAT function as a result of a DAT trafficking-dependent mechanism. There is also additional evidence for enrichment-mediated alterations to the

dopaminergic system at the cell surface. EC rats have decreased D1 receptor density in the PFC (Del Arco et al., 2007a). This was accompanied by experiments showing that perfusion of the D1 agonist, SKF38393, into the PFC increases local acetylcholine (ACh) release in IC rats only, suggesting that EC rats decrease the expression and function of prefrontal D1 receptors. Interestingly, we have found that EC rats have increased D2 receptor mRNA compared to IC rats within the PFC (unpublished data), while others have shown that compared to SC rats, IC rats have decreased D2 receptor expression (Fitzgerald et al., 2013). These data support the idea that an enriched environment likely increases the dopaminergic inhibitory tone in the PFC, while potentially decreasing the dopaminergic stimulatory tone within the PFC.

In opposition, enrichment appears to increase the glutamatergic tone within the PFC. Microdialysis experiments have determined that there are no differences in the basal levels of PFC glutamate levels between EC and IC rats (Melendez et al., 2004; Rahman and Bardo, 2006); however, the function and expression of cell surface glutamate receptors appear susceptible to enrichment-mediated adaptations. Enriched mice have been shown to have increased AMPA receptor subunit GluR1, and NMDA receptor subunits NR2B and NR2A expression, with no change to NR1 expression in the mouse forebrain compared to standard mice (Tang et al., 2001). Yet, others have found no differences in AMPA GluR1 levels (Nithianantharajah et al., 2008) and NR2A or NR2B expression (Shum et al., 2007) between enriched and non-enriched mice. However, the latter study did show an enrichment-induced enhancement of the NR2B:NR2A current ratio, which further corresponded to increased AMPA-mediated long-term potentiation as well as decreased AMPA-mediated long-term depression.

Moreover, enrichment alters metabotropic-glutamate receptor (mGluR) subunit composition in a region-specific manner within the PFC (Melendez et al., 2004). Due to the fact that a group 1 mGluR agonist and a mGluR2/3 antagonist failed to elevate extracellular glutamate in IC rats, it is likely that EC rats have enhanced prefrontal mGluR transmission compared to IC rats (Melendez et al., 2004). In conclusion, enrichment increases glutamatergic tone within the PFC in addition to decreasing the dopaminergic tone within the PFC.

Although the emphasis of enrichment-induced alterations in the literature appears to be concentrated on the dopaminergic and glutamatergic systems, enrichment is not limited to these systems. Enrichment has been found to have no effect on norepinephrine levels or its metabolites in the rodent PFC (Naka et al., 2002, Brenes et al., 2008). Additionally, enrichment's effects on the serotonergic system is conflicting with one group reporting no changes in prefrontal serotonin levels or its metabolites (Naka et al., 2002), and another reporting that enrichment increased serotonin levels in the PFC of rodents (Brenes et al., 2008). Also, no changes in basal levels of acetylcholine in the PFC have been found (Del Arco et al., 2007b, Segovia et al., 2008). Aside from the classical neurotransmitter systems, corticosterone levels and glucocorticoid receptors remain unchanged in the PFC between EC and IC rats (Garrido et al., 2013). Moreover, studies of the PFC have revealed that enrichment decreases basal BDNF levels in mice (Rueda et al., 2012); however, in rats BDNF levels have been found to be unaltered between EC and IC rats (Chen et al., 2005).

Enrichment also mediates downstream targets beyond the cell surface. EC rats have been shown to have decreased basal levels of pDARPP-32 Thr34, but no changes to

pDARPP-32 Thr75 or total DARPP-32 levels in the PFC (Gomez et al., 2012). Additionally, EC rats have lower pCREB levels, with no change in total CREB, within the PFC compared to IC and SC rats (Gomez et al., 2012). The enrichment-mediated alterations in basal DARPP-32 and CREB activity may reflect the dysregulated dopaminergic and glutamatergic systems within the PFC of EC rats. There also appears to be strong evidence that enrichment increases prefrontal basal levels of immediate early genes including c-fos, Δ FosB, zif-268, and Arc (Pinaud et al., 2001, Shum et al., 2007, Lehmann and Herkenham, 2011, Venebra-Munoz et al., 2014). In summary, while there has been a concerted effort to understand the enrichment-induced neuroadaptations that occur within the PFC, the fact that enrichment-dependent alterations appear at the cellular and sub-cellular levels leave researchers with a great deal still left to understand.

1.13 PREFRONTAL CORTEX: POTENTIAL KEY AREA FOR ENRICHMENT-INDUCED PROTECTION TO DRUG-INDUCED NEUROADAPTATIONS

The PFC receives dense ascending dopaminergic innervations from the NAc and VTA, while sending reciprocal descending glutamatergic projections back to these areas (Grace et al., 2007). Through this circuitry the PFC has the ability to modulate the behavioral output induced by drugs of abuse. As mentioned previously, enriched rats have a neuroprotective-like phenotype to the behavioral effects of drugs of abuse, and also have dramatic basal neuroadaptations in the PFC. As a result, the PFC represents a potential culprit in mediating the behavioral-induced adaptations observed in rats raised in an enriched environment. However, little is known in terms of the molecular

underpinnings of environment-mediated neuroadaptations within the PFC in response to addictive drugs.

As seen with enrichment's effect basally, the DAT appears to be altered in EC rats in response to psychostimulants. After rats were prenatally treated with cocaine, and then placed postnatally in EC or IC conditions, post-enrichment was shown to attenuate the nicotine-mediated response of the DAT within the PFC (Neugebauer et al., 2004). Furthermore, in response to acute nicotine EC rats had dramatic increases in the dopamine clearance within the PFC in comparison to IC rats, suggesting that EC rats have altered DAT function in response to nicotine (Zhu et al., 2007a). Moreover, acute methylphenidate administration was shown to decrease DA uptake in the PFC of EC, but not IC rats (Wooters et al., 2011). In this fashion, environmental enrichment appears to modulate the dynamics of the DAT in response to psychostimulants as well as basally. Moreover, additional research has determined that EC mice further decrease the low basal BDNF levels in the PFC in response to repeated ethanol exposure compared to SC mice (Rueda et al., 2012). With respect to examining intracellular activity, there have been very few studies. In response to chronic nicotine exposure, enrichment increases the sensitivity of pDARPP-32 Thr34 and pCREB activity in the PFC of rats (Gomez et al., 2012); as well as diminishes the enrichment-induced baseline increase in Δ FosB within the PFC in comparison to standard mice (Venebra-Munoz et al., 2014). This is the current extent of the research regarding drug-induced neuroadaptations in the PFC of rats raised in an environmental enrichment. Accordingly, more extensive studies are needed to better characterize the neurobiological consequences of enrichment on drug-mediated neuroplasticity within the PFC.

1.14 SIGNIFICANCE REVISITED

These findings provide a framework whereby environmental enrichment may serve as a neuroprotective factor in nicotine-mediated behaviors by mediating environmental-induced neuroadaptations, which are likely to be mediated by miR-dependent intracellular signaling processes in response to nicotine exposure within the PFC. The overarching goal of these studies was to identify whether environmental enrichment alters nicotine-mediated behaviors via distinct alterations in nicotine-mediated intracellular signaling (please see figure 1.2 for overview). As a follow-up, these studies sought to determine the potential regulatory mechanisms controlling enrichment-dependent changes in nicotine-mediated intracellular signaling. Thus, by dissecting the prefrontal molecular mechanisms of an enriched environment in nicotine-mediated behaviors, we may better understand individual vulnerability to nicotine addiction.

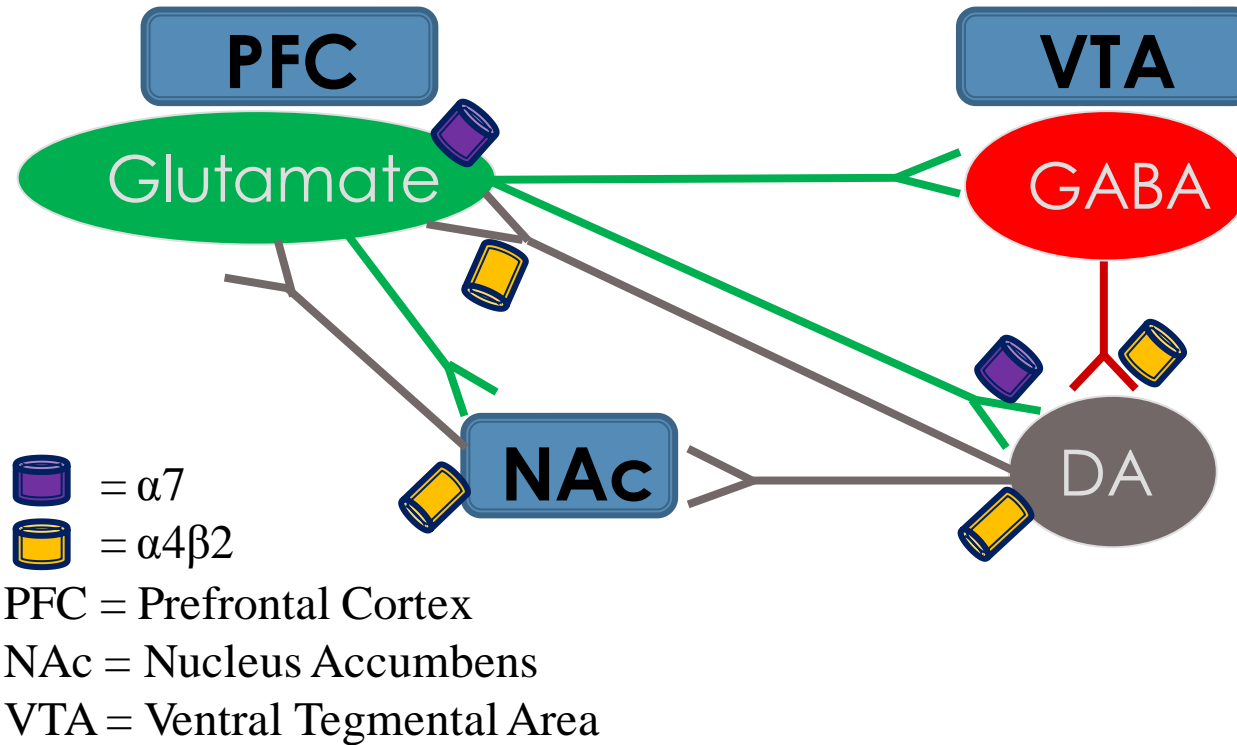


Figure 1.1. An integrated model depicting the role of nicotinic acetylcholine receptors in the mesocorticolimbic brain circuitry. Nicotine binds to nicotinic acetylcholine receptors allowing for the release of numerous neurotransmitters which regulates the rewarding and reinforcing properties of nicotine. Repeated exposure to nicotine produces neuroadaptations within this brain circuitry leading to nicotine addiction.

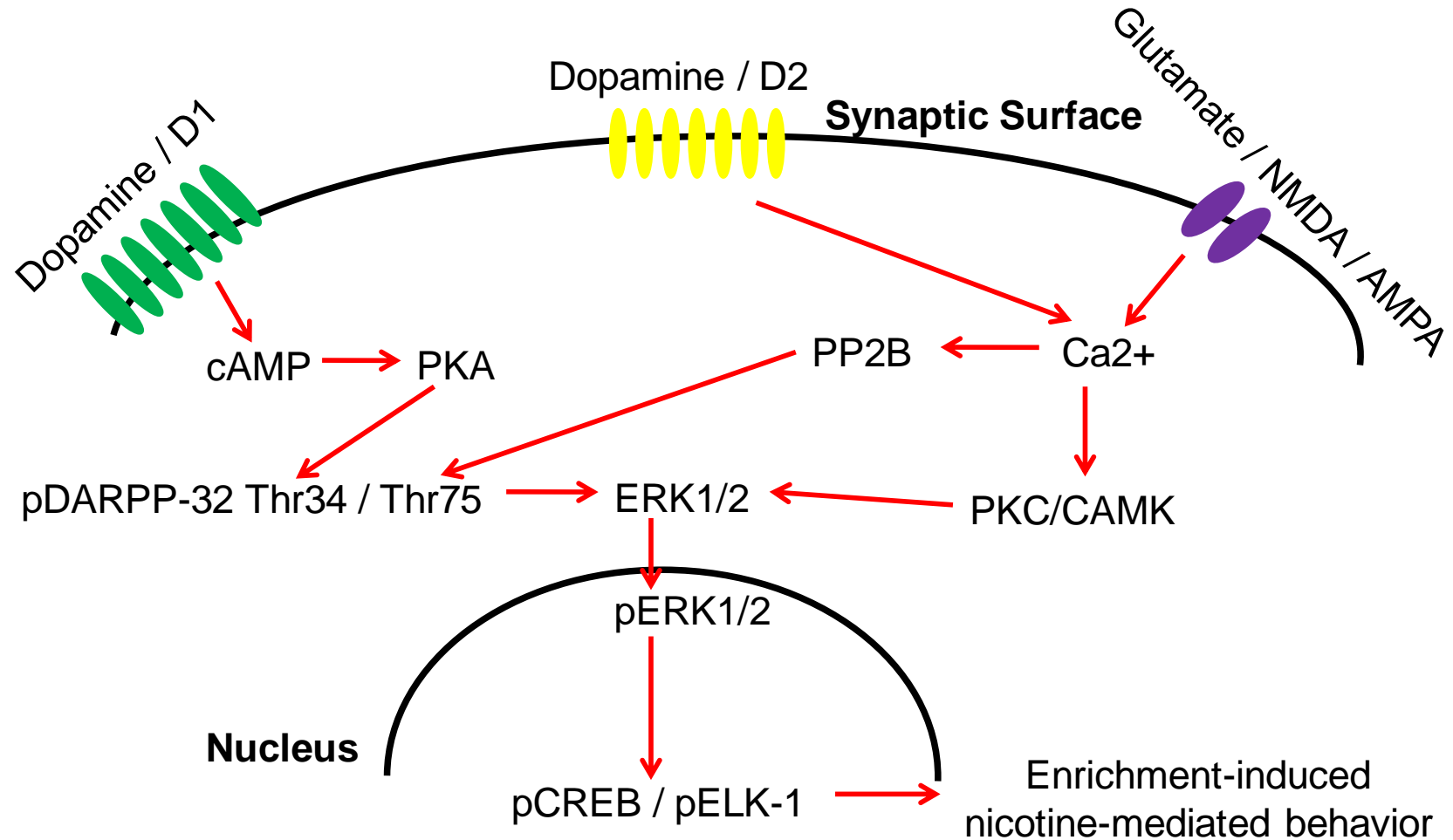


Figure 1.2. An integrated signaling cascade involved in nicotine-mediated intracellular signaling cascades responsible for the neuroadaptations that underlie nicotine-induced behavioral alterations. The central hypothesis is that environmental enrichment alters nicotine-mediated signaling which is responsible for the enrichment-induced behavioral adaptations in response to repeated nicotine administration.

CHAPTER 2

EFFECTS OF ENRICHMENT ON NICOTINE-MEDIATED SENSITIZATION

2.1 INTRODUCTION

Environmental factors contribute to individual vulnerability for drug abuse (Leshner, 2000, Rhee et al., 2003). The environmental enrichment paradigm has been widely used to address how environmental factors influence susceptibility to drug abuse. In this animal model, rats are raised in one of the three different conditions during adolescence: an enriched condition (EC), an impoverished condition (IC) or a standard condition (SC); which differs in novelty, handling, social cohorts, and physical activity. Exposure within the environmental enrichment paradigm results in robust neurobiological adaptations, particularly within the prefrontal cortex (PFC) of the mesocorticolimbic dopaminergic system (Zhu et al., 2004, Bowling et al., 1993, Hall et al., 1998, Del Arco et al., 2007a). This neural circuit, in part, organizes motivated behavior and environmental enrichment-dependent alterations to this system are suggested to be protective against drug-induced maladaptive behaviors (Gomez et al., 2012, Zhu et al., 2013, Stairs and Bardo, 2009, Bardo et al., 2001).

The behavioral effects of nicotine are primarily mediated by its stimulating actions on nicotinic acetylcholine receptor-mediated dopamine (DA) release within the mesocorticolimbic system (Laviolette and van der Kooy, 2004). Long term exposure to nicotine induces neuroadaptations within the mesocorticolimbic circuitry to induce a

behavioral sensitization (Clarke and Kumar, 1983b, Kalivas, 1995, Clarke and Kumar, 1983a). Although behavioral sensitization is not a direct measure of drug reward or reinforcement, this procedure is sensitive to the behavioral and molecular changes produced by the psychostimulant effects of abused drugs (Robinson and Berridge, 1993, Berridge and Robinson, 1998, Wise and Bozarth, 1987). EC rats exhibit reduced nicotine-mediated locomotor activity compared to IC and SC rats (Green et al., 2003b), which may be mediated by enriched environment-induced alterations of dopaminergic pathways. Indeed, drug-naïve EC rats exhibit diminished DA transporter function (Zhu et al., 2004), less synaptic DA levels in medial prefrontal cortex (Bowling et al., 1993, Hall et al., 1998), and show decreased D1 receptor function and expression in the PFC compared with IC and SC groups (Del Arco et al., 2007a). In contrast, repeated nicotine administration profoundly increases DA clearance and 3,4-Dihydroxyphenylacetic acid (DOPAC) levels in the PFC of EC rats but not in IC rats (Zhu et al., 2007b). Therefore, EC rats may have a lower dopaminergic tone compared to IC rats under basal conditions, which may contribute to differential behavioral responses to psychostimulants. Acute and repeated nicotine administration activates the DA/D1 receptors/cAMP/protein kinase pathway, including DA- and cAMP-regulated phosphoprotein-32 (DARPP-32), extracellular signal-regulated kinase1/2 (ERK1/2, two isoforms with overlapping structure and function), and cAMP-response element-binding protein (CREB) (Nakayama et al., 2001, Hamada et al., 2004, Valjent et al., 2004a). Moreover, alterations in the phosphorylation states of these proteins have been shown to alter the expression of nicotine-induced behavioral sensitization (Valjent et al., 2006b, Addy et al., 2007). As a result, nicotine addiction involves stable, long-term alterations in neuroadaptations that

are the result of nicotine's indirect action on the plasticity of these intracellular signaling proteins via nAChR-dependent increases in dopamine and glutamate release (Dajas-Bailador and Wonnacott, 2004).

Activation of the DA/D1 receptor/cAMP/protein kinase A (PKA) pathway increases phosphorylation of DARPP-32 at the site Threonine 34 (pDARPP-32 Thr34), but decreases phosphorylation of DARPP-32 at Threonine 75 (pDARPP-32 Thr75) (Nishi et al., 2000). In contrast, phosphorylation of DARPP-32 at Thr75 by cyclin-dependent kinase 5 has a major inhibitory effect on pDARPP-32 Thr34, thereby reducing D1 DA signaling through the DARPP-32/ protein phosphatase-1 (PP-1) cascade (Nishi et al., 2000, Bibb et al., 1999). In addition, activation of this PKA pathway enhances phosphorylation of the transcription factor CREB at serine 133, (Nairn et al., 2004, Svenningsson et al., 2005, Dash et al., 1991). In an opposing fashion, activation of Ca²⁺-dependent calcineurin by D2 receptors and glutamate-activated N-methyl-D-aspartate (NMDA) receptors results in dephosphorylation of pDARPP-32 Thr34 (Lindskog et al., 1999). ERK1/2, a member of the mitogen-activated protein kinase (MAPK) signaling pathway also signals to CREB at Ser133 via glutamate-dependent NMDA receptor activation of calcium-mediated calcium/calmodulin-dependent protein kinase II (CAMKII) activation, in addition to DARPP-32 being able to control ERK activation via PP-1 (Valjent et al., 2000, Valjent et al., 2005). Consequently, these intracellular signaling proteins are essential for neuronal plasticity in response to repeated nicotine exposure. By acting as multifunctional platforms that mediate the activity between dopaminergic and glutamatergic receptor activation at the cell surface with downstream transcription factors responsible for gene expression (Greengard et al., 1999, Nairn et al.,

2004, Beaulieu and Gainetdinov, 2011), these proteins play a critical role in drug-induced neuroplasticity.

Understanding the molecular mechanisms of enrichment-induced changes in these intracellular signaling cascades may provide important insights into how environmental enrichment reduces susceptibility to psychostimulant drugs. Collectively, these studies aimed to determine the molecular underpinning(s) of enrichment-induced alterations in the intracellular signaling events implicated in nicotine-mediated locomotor sensitization. Ultimately, I proposed that environmental enrichment-induced alterations in basal and nicotine-induced locomotor activity are associated with adaptations in the basal and nicotine-mediated phosphorylation states of DARPP-32, ERK1/2, and CREB within the mesocorticolimbic circuitry.

2.2 METHODS

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA). Rats arrived at the age of 21 days and were housed with food and water *ad libitum* in a colony room in the Division of Laboratory Animal Resources at the University of South Carolina. The colony room was maintained at 21 ± 2 °C, 50 ± 10 % relative humidity on a 12-h light/dark cycle with lights on at 07:00 AM. All of the experimental procedures using animals were performed according to the National Institute of Health guidelines for AAALAC accredited facilities. The experimental protocol for this study was approved by the Institutional Animal Care and Use

Committee (IACUC) at the University of South Carolina in compliance with animal welfare assurance.

Environmental conditions

Upon arrival at postnatal day 21, rats were randomly assigned to EC, IC, or SC groups. EC rats were group-housed (10-15 per cage) in a metal cage (120 cm length × 60 cm width × 45 cm height). Twelve hard non-chewable plastic objects were randomly placed in the cage. On a daily basis half of the objects were replaced with new objects, and the remaining objects were rearranged. IC rats were individually housed in wire mesh hanging cages (25 cm length × 18 cm width × 17 cm height) with solid metal sides and wire mesh floor. SC rats were pair-housed in a clear polycarbonate cage (43 cm × 20 cm width × 20 cm height) with a wire cage top. EC rats were handled each day as to change the novelty of the environment on a daily basis. IC and SC rats were neither handled nor exposed to any object except food and water; however, all rats were handled extensively throughout behavioral testing so that novelty and the number of cohorts were the only factors that differed among the groups throughout behavioral paradigms. The SC condition represents the standard housing conditions set in the NIH Guide for the 1996 version of the *NIH Guide for the Care and Use of Laboratory Animals*. Rats were raised in these conditions from 21 to 53 days of age and were maintained in these conditions throughout all experiments.

Nicotine administration and locomotor activity

Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline (0.9% sodium chloride). The nicotine solution (freebase) was prepared immediately prior to injection and neutralized to pH 7.4 with NaOH to reduce

irritation. Locomotor activity was assessed using Digipro System Software (v.140, AccuScan Instruments) to detect movement in 16 square (40 × 40 cm) chambers (Hamilton-Kinder Inc., Poway, CA). Movement was detected by infrared photocell interruptions; each chamber has 32 emitter/detector pairs capable of measuring horizontal and vertical (rearing) activity. Each beam was spaced 2.5 cm apart and 7.0 cm above the chamber floor. The chambers were converted into round (~ 40 cm diameter) compartments by adding clear Plexiglas inserts; photocell emitter/detector pairs were tuned by the manufacturer to handle the extra perspex width. Horizontal activity was measured as all beam breaks in the horizontal plane, and rearing activity was measured as all beam breaks in the vertical plane. All activity monitors were located in an isolated room that is separate from the animal colony.

Beginning at 54 days of age, all animals were habituated to the locomotor activity chambers for two 60 min sessions, once/day with no injection. Twenty-four hours after the second habituation session, all rats were habituated to the locomotor chambers for 30 min prior to injection, and then injected subcutaneously with saline and placed into the activity chambers for 60 min to measure baseline activity. The behavioral sensitization procedure began 24 h after the saline baseline measurement. All rats received a 30 min habituation period in the testing chamber prior to nicotine (0.35 mg/kg) or saline injection based on previous studies from our laboratory and others showing a dose of 0.35 mg/kg, s.c produces robust behavioral sensitization across sessions to repeated nicotine (Addy et al., 2007, Midde et al., 2011, Gomez et al., 2012). This was done so that the onset of nicotine's effects did not overlap with the period that rats showed the most exploratory behavior in the chamber, which was during the first 15 min (Harrod et al., 2008, Harrod

and Van Horn, 2009). After the 30 min habituation session, rats were administered nicotine (0.35 mg/kg; s.c.) or saline. Subsequently, horizontal and rearing activities were assessed during the subsequent 60 min session. Rats received saline or nicotine injections once/day for a total of 15 days; however, locomotor activities with regard to 30 min pre-injection sessions and 60-min post-injection sessions were recorded every other day, i.e., on days 1, 3, 5, 7, 9, 11, 13, and 15. During the “off” days of locomotor testing, rats were still transported to the same room where rats were injected for locomotor testing and then returned to home cages after nicotine or saline injection. On day 16 after completion of the behavioral sensitization phase, all rats were injected with saline or nicotine and brains were removed by rapid decapitation 20 min after the last injection. Brain regions were dissected in a chilled matrix for Western blot analyses.

Western blot analysis

Rats were sacrificed by rapid decapitation and brain regions were dissected in a chilled matrix and sonicated immediately on ice in a homogenization buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 0.4 M NaCl, 5 mM MgCl₂, 20% glycerol, 1 mM PMSF, phosphatase inhibitor cocktails I (P2850, Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors (P8340, Sigma-Aldrich, St. Louis, MO, USA). Homogenates were then centrifuged at 12,000 g for 15 min at 4°C. The supernatants were collected and stored at -80°C. Protein concentrations were determined in triplicate using Bio-Rad DC protein detection reagent. In brief, proteins (30 µg per PFC and NAc, 15 µg per STR tissue samples) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes that were transferred with proteins were preincubated with blocking buffer (5% dry milk powder in PBS containing 0.5% Tween-

20) and then incubated overnight at 4 °C in blocking buffer with primary antibodies: DARPP-32 (1:2000, 374-DARPP, PhosphoSolutions, Aurora, CO), pDARPP-32 Thr34 (1:1000, p1025-34, PhosphoSolutions, Aurora, CO), pDARPP-32 Thr75 (1:1000, p1025-75, PhosphoSolutions, Aurora, CO), CREB (1:1000, 9104, Cell Signaling, Danvers, MA), pCREB (1:1000, 9196L, Cell Signaling, Danvers, MA), total ERK1/2 (1:5000, V114A, Promega, Madison, WI, USA), and pERK1/2 (1:1000, SC-16982R, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were washed 10 min × 3 times with wash buffer (PBS containing 0.5% Tween 20) at room temperature, and then incubated in blocking buffer containing the following secondary affinity-purified, horseradish peroxidase-conjugated antibodies: anti-rabbit IgG (111-035-144, Jackson ImmunoResearch, West Grove, PA; 1:20000 for Total DARPP-32, 1:7500 for pDARPP-Thr34 and pDARPP-Thr75); anti-rabbit IgG; (1:20,000) from Jackson ImmunoResearch (West Grove, PA, USA) for total ERK1/2 and 1:5000 for pERK1/2; anti-mouse IgG (7076, Cell Signaling, Danvers, MA; 1:2000 for both Total CREB and pCREB). Blots were then washed another 10 min × 3 times with wash buffer (PBS containing 0.5% Tween 20) at room temperature. Immunoblots were detected using enhanced chemiluminescence (ECL-plus) and developed on Hyperfilm (Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). After detection and quantification of these proteins, each blot was stripped in a Re-blot plus mild antibody stripping solution (CHEMICON, Temecula, CA, USA) and reprobred for detection of β -tubulin (1:5000; H-235, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) to monitor protein loading among samples. Multiple autoradiographs were obtained using different exposure times,

and immunoreactive bands within the linear range of detection were quantified by densitometric scanning using Scion image software (Scion Corp., Frederick, MD, USA).

Effect of SL327-induced blockage of ERK on acute nicotine-mediated activity

To determine the role of the ERK pathway in nicotine-mediated activity in rats raised in different conditions, we determined the effects of ERK signaling blockage by SL327, an inhibitor of ERK activation, on acute nicotine-mediated activity. A separate group of rats were raised in different housing conditions as described above. At 54 days of age, all rats were habituated to the locomotor activity chambers for two consecutive days of 60 min sessions with no injections. Twenty-four hours after the second habituation session, all rats were placed in the locomotor chambers for 30 min and horizontal activity was recorded, and then injected subcutaneously with saline and placed into the activity chambers for 60 min to measure baseline activity. Twenty-four hours after the baseline activity measurement, all rats received a 30 min habituation period in the testing chamber, and then administered DMSO or SL327 (50 mg/kg, s.c.) intraperitoneally in a volume of 1.5 ml/kg. A dose of 50 mg/kg SL327 was chosen because SL327 at this dose has been reported to reduce the number of active ERK-positive neurons by 80% but had no effect on the spontaneous locomotor activity (Valjent et al., 2006b, Shi and McGinty, 2006). One hour later, rats were administered saline or nicotine (0.35 mg/kg, s.c.) and placed in the locomotor chambers for an additional 60 min recording of horizontal activity. In the current study, rats were randomly assigned to four treatment groups (DMSO/saline, DMSO/nicotine, SL327/saline and SL327/nicotine). Twenty minutes after the 60 min locomotor recording session, rat brains were removed

by rapid decapitation and brain regions were dissected in a chilled matrix for Western blot analyses.

Data analyses

Data are presented as mean \pm standard error of the mean (SEM), and *n* represents the number of subjects for each group. Locomotor activity data were analyzed by mixed-factor analyses of variance (ANOVA) with housing condition (EC, IC, or SC) and treatment (saline or nicotine) as between-group factors, and with day and/or time as within-subject factors. A housing \times day \times time ($3 \times 2 \times 12$) mixed factorial ANOVA was used to analyze data from the 2 habituation days, and a housing \times time (3×12) factorial ANOVA was conducted on the saline baseline day. The pre-injection habituation part of the experiment was analyzed using a housing \times treatment \times day \times time ($3 \times 2 \times 8 \times 12$) ANOVA. The effect of repeated nicotine injection on total horizontal activity was analyzed using a housing \times treatment \times day \times time ($3 \times 2 \times 8 \times 12$) factorial ANOVA, with housing and treatment as between-group factors, and day and time as within-subject factors. Additionally, nicotine-mediated sensitization in EC, IC and SC groups was analyzed by linear regression. To evaluate the effects of repeated nicotine administration on the activity of signaling proteins (total DARPP-32, pDARPP-32 Thr34, pDARPP-32 Thr75, total CREB, pCREB, pERK1/2, and ERK1/2); separate two-way ANOVAs (housing condition \times treatment) were performed on PFC, NAc, and striatum with environment and treatment as between-group factors. Simple effect comparisons were made for *post hoc* analyses. To determine whether a relationship existed between locomotor activity and immunoreactivity of DARPP-32, pDARPP-32 Thr34, pDARPP-32 Thr75, pERK1, and pERK2; separate Pearson correlations were conducted. When the

effect of SL327 on acute nicotine-induced locomotor levels and pERK1/2 immunoreactivity was assessed, a $3 \times 2 \times 2$ factorial ANOVA with housing condition (EC, IC, or SC), SL327 treatment (DMSO or SL327), and nicotine treatment (saline or nicotine) was performed. Simple effect comparisons were made for post hoc analyses. All statistical analyses were performed using SPSS (standard version 19.0, Chicago, IL). Differences were considered significant at $p < 0.05$.

2.3 RESULTS

Environmental enrichment decreased locomotor response to context novelty

Habituation refers to a progressive decrease in locomotor activity following repeated exposure to a particular context. Novelty-elicited locomotor behavior was measured during the two days of habituation to the locomotor apparatuses for EC, IC, and SC rats. The total activity that occurred for 60 min during the two habituation days is shown in Figure 2.2 (A-D). A housing condition \times day \times time ANOVA ($3 \times 2 \times 12$) revealed main effects of housing condition ($F_{(2, 70)} = 46.48, p < 0.001$), day ($F_{(1, 70)} = 317.41, p < 0.001$) and time ($F_{(11, 770)} = 148.57, p < 0.001$), and a significant housing condition \times day \times time interaction ($F_{(22, 770)} = 2.48, p < 0.001$). All animals showed the most activity at the first 10 min of the habituation session, the activity decreased over the 20 min period, and all groups achieved asymptote for the remaining 30 min of the session (Figure 2.2B and D). EC rats exhibited less locomotor activity than IC and SC rats during the habituation session ($p < 0.01$, Bonferroni t -test). On the third day, total activity was recorded for all groups after a saline injection to determine baseline activity prior to the induction of the sensitization phase of the experiment (Figure 2.2E and F). The housing

condition \times time ANOVA revealed a main effect of housing condition ($F_{(2, 70)} = 79.02, p < 0.001$) and time ($F_{(11, 770)} = 108.96, p < 0.001$), and a housing condition \times time interaction ($F_{(22, 770)} = 4.09, p < 0.001$). In general, all animals showed the most activity during the first 10 min of the saline baseline day, acquired asymptotic levels of activity more quickly, and showed a lower asymptote compared to that of the habituation sessions (Figure 2.2F). None of the comparisons indicated differences between IC and SC rats (all $p > 0.05$) during the habituation and saline baseline sessions.

Environmental enrichment increased sensitivity to nicotine-mediated locomotor sensitization

All animals were placed into locomotor chambers for 30 min prior to the activity measurement to produce within-session habituation of activity in response to the context prior to nicotine or saline injection. Total activity in saline control and nicotine-treated group during the 30 min habituation period across the 15-day treatment was recorded as shown in Figure 2.3A and B. A mixed-factor housing condition \times treatment \times day \times time ANOVA ($3 \times 2 \times 8 \times 6$) revealed main effects of housing condition ($F_{(2, 67)} = 333.90, p < 0.001$), treatment ($F_{(1, 67)} = 5.16, p < 0.05$), day ($F_{(7, 469)} = 298.97, p < 0.001$), time ($F_{(5, 335)} = 47.77, p < 0.001$) and a significant day \times housing condition interaction ($F_{(14, 469)} = 7.75, p < 0.001$). There were no main effects of treatment and interactions containing this factor. EC rats exhibited less locomotor activity than did IC and SC rats across the pre-injection habituation sessions ($p < 0.01$, Bonferroni t -test).

To determine whether differential environments alter nicotine-mediated locomotor sensitization, I analyzed locomotor activity during daily administration of nicotine (0.35 mg/kg) or saline during Days 1-15 in EC, IC, and SC rats (Figure 2C and

D). A housing condition \times treatment \times day ANOVA analysis revealed significant main effects of housing condition ($F_{(2, 67)} = 104.29, p < 0.001$), treatment ($F_{(1, 67)} = 333.57, p < 0.001$) and day ($F_{(7, 469)} = 139.97, p < 0.001$). A significant housing condition \times treatment \times day interaction ($F_{(14, 469)} = 2.76, p < 0.05$) was found. As shown in Figure 2.3D, linear regression analysis revealed that nicotine-induced locomotor activity gradually increased across treatment days in EC ($F_{(1, 6)} = 20.97, p < 0.01$), IC ($F_{(1, 6)} = 27.94, p < 0.01$) and SC ($F_{(1, 6)} = 33.11, p < 0.01$), which are identically observed in three housing groups ($F_{(2, 20)} = 22.48, p < 0.001$), indicating that repeated nicotine injection produces behavioral sensitization in EC, IC, and SC rats.

Based on the significant three-way interaction, separate two-factor ANOVAs were conducted. In saline-treated groups, a housing condition \times day ANOVA analysis revealed significant main effects of housing condition ($F_{(2, 34)} = 80.77, p < 0.001$) and day ($F_{(7, 238)} = 2.52, p < 0.05$). Also, a significant housing condition \times day interaction ($F_{(14, 238)} = 1.84, p < 0.001$) was found, indicating repeated overall difference in total activity among the different housing groups. Post hoc tests (Bonferroni *t*-test) showed that EC rats were significantly less active than were IC or SC rats across treatment days ($p < 0.001$) and no difference between IC and SC rats was found ($p > 0.05$). In nicotine-treated groups, two-way ANOVA analysis revealed that significant main effects of housing condition ($F_{(2, 33)} = 32.57, p < 0.001$) and day ($F_{(7, 231)} = 243.45, p < 0.001$), and post hoc tests showed no difference between the IC and SC groups. There was a significant housing condition \times day interaction ($F_{(14, 231)} = 5.51, p < 0.001$), suggesting EC rats have lower nicotine-mediated locomotor activity across treatment days. Overall, all rats

treated with nicotine displayed greater locomotor activity on Days 3-15 than on Day 1 ($p < 0.05$).

The time course effect of nicotine in EC, IC, and SC rats on Days 1 and 15 is illustrated in Figure 2.4. A housing condition \times treatment \times day \times time ANOVA revealed significant main effects of housing condition ($F_{(2, 67)} = 108.16, p < 0.001$), treatment ($F_{(1, 67)} = 243.44, p < 0.001$), day ($F_{(1, 67)} = 209.67, p < 0.001$) and time ($F_{(11, 737)} = 231.97, p < 0.001$). In addition, there was a significant housing condition \times treatment \times day interaction ($F_{(2, 67)} = 3.66; p < 0.05$). When the data were expressed as a percentage change relative to the respective saline controls, on Day 1 acute nicotine increased locomotor activity (243 %) in EC ($p < 0.001$, Bonferroni t -test) and (116%) in IC, but decreased activity (20%) in SC rats ($p < 0.05$, Figure 2.4A). No differences in activity between nicotine- and saline-treated IC rats were observed ($p > 0.05$). On day 15, nicotine produced hyperactivity in EC rats (661%), IC (215%) and SC (195%) relative to their respective saline control. In addition, compared to Day 1, repeated nicotine administration elevated activity to a greater extent in EC rats (271%), IC (185%), and SC (243%) on Day 15, suggesting that the EC rats exhibit increased sensitivity to behavioral sensitization. The time course data in these rats on Day 1 and Day 15 are illustrated in Figure 2.4C and D. *Post hoc* tests showed that all rats had greater activity during the first 10 min and that nicotine-treated rats exhibited higher activity counts throughout the remainder of the 60 min.

Acute nicotine administration regulated DARPP-32 in EC and IC rats in a dose- and region-dependent manner

To determine whether environmental enrichment regulates DARPP-32 signaling in response to nicotine in a dose-dependent manner, we examined acute effects of nicotine (0.1, 0.3, and 0.8 mg/kg) on pDARPP-32 Thr34, pDARPP-32 Thr75, and total DARPP-32 in the PFC, NAc, and striatum in EC and IC rats (Table 2.1). In the EC group, nicotine (0.1 mg/kg) increased pDARPP-32 Thr34 levels (42%) in NAc ($F_{(1,6)} = 5.2, p < 0.05$), but not in the PFC and striatum compared to saline control. However, EC rats treated with nicotine (0.3 mg/kg) showed an increase in pDARPP-32 Thr34 in the PFC (45%, $F_{(1,6)} = 7.5, p < 0.05$), NAc (116%, $F_{(1,6)} = 9.5, p < 0.05$) and striatum (101%, $F_{(1,6)} = 10.2, p < 0.05$). Following 0.8 mg/kg nicotine administration, EC rats had a significant increase of pDARPP-32 Thr34 in the PFC (447%, $F_{(1,6)} = 12.1, p < 0.01$) and in the NAc (59%, $F_{(1,6)} = 8.1, p < 0.01$), but not in striatum. In addition, nicotine (0.3 mg/kg) increased pDARPP-32 Thr75 in striatum (58%, $F_{(1,6)} = 7.9, p < 0.01$) in EC rats. Acute nicotine had no effects on total DARPP-32 in EC and IC rats. These findings demonstrated that nicotine dose-dependently increased pDARPP-32 Thr34 levels most prominently in the EC-reared condition and that 0.3 mg/kg acute dose of nicotine was the optimal dose to elicit the most robust changes in pDARPP-32 Thr34 among the measured brain regions of EC and IC rats.

In the saline control groups, the levels of pDARPP-32 Thr34 were lower in all regions of EC rats relative to IC rats, whereas no significant differences in the levels of pDARPP-32 Thr75 between EC and IC rats. These results suggest environmental enrichment diminishes the basal levels of phosphorylated DARPP-32 at the Thr34 site. *Repeated nicotine administration differentially regulated phosphorylation of DARPP-32 in EC, IC, and SC rats.*

The pathway of DARPP-32 signaling is crucial for the neuroadaptations in response to repeated nicotine administration (Addy et al., 2007, Abdolahi et al., 2010). Therefore, we determined whether DARPP-32 levels and phosphorylation states were altered in parallel with the differential behavioral response to nicotine in EC, IC, and SC rats. The levels of DARPP-32 and pDARPP-32 Thr34 in the PFC, NAc and striatum of the EC, IC, and SC rats, directly from the behavioral measurements, are illustrated in Figure 2.5. No differences in total DARPP-32 and pDARPP-32 Thr75 in PFC, NAc, and striatum among the three housing conditions were found (data not shown).

Two-way ANOVA on the levels of pDARPP-32 Thr34 in the PFC of the rats treated with saline or nicotine revealed a main effect of housing condition ($F_{(2, 53)} = 3.43$, $p < 0.05$) and a trend of housing \times treatment interaction ($F_{(2, 53)} = 2.87$, $p = 0.06$), and no significant effect of treatment (Figure 2.5A and D). In saline controls, one-way ANOVAs revealed the level of pDARPP-32 Thr34 in the PFC in the EC group was lower than that in both the IC and SC groups ($p < 0.01$), but no difference between the IC and SC groups was found. Following repeated nicotine administration, the level of pDARPP-32 Thr34 in PFC was increased in EC ($50 \pm 2.8\%$, $F_{(1, 16)} = 8.32$, $p < 0.05$) and IC ($18 \pm 0.2\%$, $F_{(1, 16)} = 3.12$, $p < 0.05$) rats compared to the respective saline controls. No difference between SC-Sal and SC-Nic groups were found. In the NAc, two-way ANOVA on the levels of pDARPP-32 Thr34 revealed a main effect of housing condition ($F_{(2, 53)} = 4.21$, $p < 0.05$), and no significant effect of treatment and their interaction (Figure 2.5B and E). EC rats exhibited decreased basal pDARPP-32 Thr34 levels compared to IC and SC rats ($47 \pm 3.5\%$ and $39 \pm 2.8\%$, respectively). Repeated nicotine significantly increased pDARPP-32 Thr34 level in the EC rats ($30 \pm 2.1\%$, $p < 0.05$), but not in IC or SC rats ($p > 0.05$). In

the striatum, no difference in pDARPP-32 Thr34 level was found among EC, IC and SC rats with nicotine or saline injection (Figure 2.5C and F).

Repeated nicotine administration differentially regulated phosphorylation of CREB in EC, IC, and SC rats

In a parallel study, we examined whether environmental enrichment changed CREB and pCREB in the PFC, NAc, and striatum in the EC, IC, and SC groups. As shown in Figure 2.6, no significant differences in total CREB were found in these regions among the groups. With respect to the ratio of pCREB /CREB in the PFC (Figure 5A and D), a main effect of housing condition ($F_{(2, 18)} = 21.22, p < 0.001$) and treatment ($F_{(1, 18)} = 98.64, p < 0.001$), and a significant housing condition \times treatment interaction ($F_{(1, 18)} = 4.69, p < 0.05$) were found. *Post hoc* analysis revealed that the ratio of pCREB /CREB was lower in EC-Sal than in IC-Sal ($F_{(1, 6)} = 11.44, p < 0.05$) and SC-Sal ($F_{(1, 6)} = 58.00, p < 0.001$), indicating that environmental enrichment decreases the basal levels of pCREB. Repeated nicotine administration significantly increased pCREB levels in PFC of EC ($185 \pm 16\%$, $F_{(1, 6)} = 88.57, p < 0.001$), IC ($75 \pm 12\%$, $F_{(1, 6)} = 29.57, p < 0.01$), and SC ($39 \pm 2.9\%$, $F_{(1, 6)} = 21.35, p < 0.01$) nicotine-treated groups compared to the respective saline control groups.

With respect to the ratio of pCREB /CREB in the NAc (Figure 2.6B and E), a main effect of housing condition ($F_{(2, 18)} = 9.34, p < 0.05$) was found. There was no significant effect of treatment and their interaction. The ratio of pCREB /CREB was lower in EC-Sal than in IC-Sal groups ($F_{(1, 6)} = 5.92, p < 0.05$). Repeated nicotine administration increased the level of pCREB in EC-Nic rats ($42 \pm 3.0\%$, $F_{(1, 6)} = 6.71, p < 0.05$) but not in IC-Nic and SC-Nic rats. In the striatum, no differences in pCREB and

total CREB levels were found among the EC, IC and SC rats treated with nicotine or saline (Figure 2.6C and F).

Repeated nicotine administration differentially regulates pERK1/2 levels in the PFC among EC, IC, and SC rats

The ERK1/2 pathway is crucial for nicotine-induced neuroadaptations and nicotine-associated behaviors (Zhai et al., 2008). Additionally, we chose to further pursue the PFC due to our previous findings showing that DARPP-32 and CREB changes were most robust in this brain region within EC rats. Therefore, we determined whether the levels of ERK1/2 activity in the PFC are differentially regulated in EC, IC, and SC rats following repeated nicotine or saline treatment. As shown in Figure 2.7A and B, no differences in total ERK1/2 were found among the three housing conditions (data not shown). A two-way ANOVA on the levels of phosphorylated ERK1 revealed a main effect for housing condition ($F_{(2,30)} = 4.5, p < 0.05$) and a significant interaction of housing condition \times treatment ($F_{(2,30)} = 4.2, p < 0.05$). No significant main effect of treatment was found. In saline controls, one-way ANOVAs revealed the levels of pERK1 in the EC group was higher than that in both the IC ($73.5 \pm 2.3\%, p < 0.05$) and SC ($95 \pm 12.5\%, p < 0.05$) groups ($F_{(2,17)} = 11.3, p < 0.05$). Following repeated nicotine administration, the level of pERK1 was significantly increased in IC ($52 \pm 6.5\%; t_{(10)} = 2.8, p < 0.05$) rats and a trend for significance was observed in SC ($49 \pm 6.9\%; t_{(10)} = 1.6, p > 0.05$) rats. No significant effects were found in EC rats between nicotine-treated and saline control groups.

With regard to the level of pERK2, a two-way ANOVA revealed a main effect of housing condition ($F_{(2,30)} = 3.4, p < 0.05$) and treatment ($F_{(1,30)} = 9.1, p < 0.05$); however, no

significant housing condition \times treatment interaction was found (Figure 2.7A and B). In saline controls, one-way ANOVA revealed a significant effect for basal pERK2 levels ($F_{(2,17)} = 4.0, p < 0.05$) with the EC group having significantly increased pERK2 levels compared to IC ($39.7 \pm 2.7\%, p < 0.05$) and SC rats ($54.2 \pm 6.7\%, p < 0.05$). Repeated nicotine significantly increased pERK2 level in IC rats ($46 \pm 3.1\%; t_{(10)} = 3.1, p < 0.05$) and a trend for significance in the SC groups ($42 \pm 3.1\%; t_{(10)} = 1.7, p > 0.05$). No difference in pERK2 level between EC-S and EC-N was found ($p > 0.05$). Thus, these results suggest that an enriched environment attenuates nicotine's effect on ERK1/2 activity.

Alterations of locomotor behavior are associated with pDARPP-32 Thr34 and pERK1 levels in PFC

To determine whether the basal level of DARPP-32 activity was associated with the results of behavior tests, correlations of locomotor activity and DARPP-32 activity were examined. Figure 2.8A illustrates the correlations of immunoblot densities of pDARPP-32 Thr34 in PFC of all saline control rats and their respective locomotor counts collected from last day (Day 15, Session 8) of behavioral tests. There were no correlations regarding total horizontal activity and total DARPP-32 protein levels ($p = 0.69$, Pearson $r = -0.082$, data not shown) or pDARPP-32 Thr75 protein levels ($p = 0.79$, Pearson $r = 0.051$, data not shown). However, pDARPP-32 Thr34 protein levels were correlated positively with mean total horizontal activity (Fig. 2.8A, $p < 0.01$, Pearson $r = 0.5113$). Correlations were also examined in the nicotine-treated rats; however, no correlations were found (data not shown). Together, these results suggest that pDARPP-32 Thr34 levels in the PFC are responsible, at least in part, for EC

reductions in locomotor basal levels and possibly involved in repeated nicotine-induced locomotor sensitization. We have recently shown that the basal phosphorylation state of DARPP-32 at threonine-34 site in the PFC from EC, IC, and SC saline control rats was positively correlated with their respective baseline locomotor activity, and that EC rats displayed greater sensitization to nicotine compared to IC and SC rats (Gomez et al., 2012). To explore whether ERK1/2 activity, a downstream signaling protein of DARPP-32, was also associated with locomotor activity, we examined the correlations of immunoblot densities of the ratios of pERK1/2 to total ERK1/2 in the PFC of all rats treated repeatedly with nicotine or saline to their respective locomotor counts collected from the last day (Day 15, Session 8) of behavioral tests (Fig. 2.8B). There were no correlations regarding the ratios of pERK1/2 to total ERK1/2 in the PFC of rats treated repeatedly with nicotine and their respective horizontal activity (data not shown). However, the ratio of pERK1 to total ERK1 in the PFC of all saline control rats was negatively correlated with total horizontal activity (Figure 2C, $p < 0.05$, Pearson $r = -0.54$). These results indicate that ERK activity, in particular pERK1, may be critical for enriched environment-induced basal changes in locomotor activity. However, the fact that no correlations were found with nicotine-mediated pERK1/2 levels may be due to the extremely high basal levels of pERK1/2 in EC rats which could cloud the interpretation. Rather this may suggest that the basal pERK1/2 levels affect the subsequent nicotine-mediated pERK1/2 response.

Effect of SL327-induced blockade of ERK on acute nicotine-mediated locomotor activity among EC, IC, and SC rats

To determine whether blockade of ERK signaling by SL327 contributes to environmental enrichment-induced increased activity in response to acute nicotine, we assessed the effects of the systemic administration of SL327 on activity following acute administration of nicotine (0.35 mg/kg) or saline. As shown in Figure 2.9, a housing condition \times SL327 treatment (DMSO or SL327) \times nicotine treatment (saline or nicotine) ANOVA on total horizontal activity during a 60 min period revealed main effects for housing ($F_{(2,56)} = 15.9, p < 0.001$) and nicotine treatment ($F_{(1,56)} = 25.3, p < 0.001$); as well as a significant housing \times SL327 treatment interaction ($F_{(2,56)} = 3.6, p < 0.05$). Neither a main effect for SL327 treatment nor interactions for housing \times SL327, treatment \times nicotine treatment, housing \times SL327 treatment \times nicotine treatment were significant. In saline controls, a housing condition \times SL327 treatment ANOVA revealed main effects of housing condition ($F_{(2,31)} = 20.8, p < 0.001$) and SL327 treatment ($F_{(1,31)} = 4.5, p < 0.05$); as well as a significant housing \times SL327 treatment interaction ($F_{(2,31)} = 4.8, p < 0.05$). One-way ANOVA revealed that the administration of SL327 significantly decreased activity in SC rats ($F_{(1,10)} = 36.4, p < 0.001$, Fig. 2.9C) but not in EC or IC rats. In nicotine-treated groups, a housing \times SL327 ANOVA revealed a significant main effect for housing condition ($F_{(1,24)} = 4.5, p < 0.05$), but not for SL327 treatment. There was no significant housing \times SL327 treatment interaction.

Effect of blockade of ERK by SL327 on acute nicotine-mediated pERK1/2 activity in PFC of EC, IC, and SC rats

We determined whether ERK1/2 levels and phosphorylation states of ERK1/2 were altered in the PFC of SL327-pretreated EC, IC, and SC rats that underwent acute nicotine administration (Figure 2.10). A housing condition \times SL327 treatment \times nicotine

treatment ANOVA on the levels of pERK1 revealed a main effect of housing condition ($F_{(2,132)} = 4.0, p < 0.05$) and a significant SL327 treatment \times nicotine treatment interaction ($F_{(1,132)} = 7.5, p < 0.05$). There was a trend of main effect of nicotine treatment ($F_{(1,132)} = 3.4, p = 0.068$) and a housing \times nicotine treatment interaction ($F_{(2,132)} = 2.8, p = 0.063$). With regard to the ratio of pERK2/ β -tubulin, a significant main effect for housing condition ($F_{(2,132)} = 4.3, p < 0.05$), and a significant interaction for SL327 treatment \times nicotine treatment ($F_{(1,132)} = 5.6, p < 0.05$) were found. There was no significance for a main effect of nicotine treatment ($F_{(1,132)} = 2.8, p = 0.095$) nor a housing \times nicotine treatment ($F_{(2,132)} = 2.4, p = 0.097$) interaction. In saline controls, separate housing condition \times SL327 treatment ANOVAs revealed a main effect of SL327 treatment ($F_{(2,66)} = 5.3, p < 0.05$) on the level of pERK1, but not pERK2. There were no significant interactions of housing condition \times SL327 treatment in the levels of either pERK1 or pERK2. *Post hoc* analysis revealed that pretreatment with SL327 increased the levels of pERK1/2 in the SC group (Fig. 2.10C) but not in EC or IC group.

In nicotine-treated groups, separate housing condition \times SL327 treatment ANOVAs revealed significant main effects of housing condition on the levels of pERK1 ($F_{(1,66)} = 8.0, p < 0.01$) and pERK2 ($F_{(1,66)} = 7.9, p < 0.01$). However, no significant interactions of housing condition \times SL327 treatment in either pERK1 or pERK2 were found. Acute nicotine administration increased the levels of pERK1 ($F_{(1,22)} = 10.3, p < 0.01$) and pERK2 ($F_{(1,22)} = 18.4, p < 0.001$) in the PFC of DMSO-pretreated SC rats but not EC or IC rats, whereas pretreatment with SL327 attenuated the nicotine-induced increased pERK1/2 levels in SC rats (Fig. 2.10C), suggesting that ERK may not play a significant role in the acute nicotine effects.

2.4 DISCUSSION

These findings demonstrate that exposure to an enriched environment decreases basal locomotor levels as well as increases locomotor sensitivity in response to both acute and repeated nicotine administration in comparison to IC or SC rats. Additionally, exposure to an enriched environment alters the levels of phosphorylated DARPP-32, ERK1/2, and CREB under control conditions (i.e. after a saline injection) and following repeated nicotine administration. Specifically, the effects of enrichment on activity of DARPP-32, ERK1/2, and CREB are profoundly found within the PFC relative to the NAc and striatum, suggesting adaptations in the activity of these signaling proteins within the PFC, may at least, in part, be responsible for enrichment-mediated locomotor changes. While repeated nicotine administration produced locomotor sensitization in EC, IC, and SC rats; when the nicotine-mediated activity was expressed as percent changes from the respective saline controls EC rats displayed greater sensitization to nicotine than IC and SC rats. EC rats had diminished basal levels of pDARPP-32 Thr34 and pCREB, yet enhanced basal levels of pERK1/2. Repeated nicotine administration increased pERK1/2 levels in the PFC of IC and SC rats but not EC rats. In addition, the magnitude of change from saline control in nicotine-induced enhancement of pDARPP-32 Thr34 and pCREB in the PFC were strikingly increased in EC rats relative to IC and SC rats. Together, the findings demonstrate a novel role for prefrontal signaling proteins in enriched environment-induced neuroplasticity and the associated behavioral changes in response to repeated nicotine administration.

Notably, because EC rats have lower baseline levels of ambulatory activity relative to IC and SC rats, it is important to point out how to express environment-

dependent differences in drug effects when the saline-treated control groups differ. In the current study, when the nicotine-mediated locomotor activities in EC, IC, and SC groups on Days 1 and 15 were expressed as percent changes from the respective saline controls; EC rats actually show greater locomotor sensitization to both acute and repeated nicotine than IC and SC rats. However, when the nicotine-induced activities in the three housing groups were expressed as absolute values, EC rats have less sensitivity to nicotine-induced sensitization than that in IC and SC rats corresponding to other studies (Green et al., 2003b, Coolon and Cain, 2009). This difference in data presentation could complicate the interpretation of locomotor effects in response to nicotine on enriched environment-induced changes in nicotine-mediated motivation. Since baseline differences in behavior represent intrinsic differences between EC and IC rats, these results highlight the importance of considering the multiple assessments of the data when attempting to depict potential environment-dependent differences in response to various drug effects.

One key point of interest is that relative to the IC condition, the complexity of an enriched environment paradigm comprises multiple components: a large space, physical exercise, novel objects, and social cohorts. We acknowledge that one limitation of the current study is that the results do not address which of these components, or combination of components, is specifically responsible for the environmental induced behavioral and neurochemical changes. To examine this issue completely, several variations of “control” conditions would be needed, including manipulations to study the effects of cages size, numbers of social partners, presence of novel objects, amount of exercise, and so on, which is beyond the scope of the current study. In this study, we chose a social control condition with only one other animal in a small cage because this

represents the NIH standard housing condition, which is the most typical housing condition used across various laboratories and allows for cross comparisons with results published in the literature. However, some studies have used other controls, such as single-housed animals with novel objects or social caged animals (n= 8–10 per cage) without novel objects. For example, in a recent study (Gipson et al., 2011) an SC condition (using the larger EC cages with no toys), and a novelty condition (NC, using the isolated IC cages with two plastic toys rotated daily) were used as control conditions for the EC group. When the escalation of cocaine (0.1 mg/kg) was examined in a self-administration paradigm, only NC and IC rats showed escalation, suggesting that social cohorts may be the primary factor in the behavioral effects of an enriched environment. In addition, the effect of exercise has been shown to have dramatic effects on reducing drug-taking behavior as well as influencing a variety of neurochemical changes (Kanarek et al., 1995, Meeusen and De Meirleir, 1995, Lynch et al., 2010). Moreover, when only novelty was used to distinguish between enriched and standard environments (same size cage and number of cohorts), enrichment in mice eliminated both behavioral sensitization and conditioned place preference to cocaine (Solinas et al., 2008), suggesting novelty acts as the main neuroprotective factor. Thus, all the various components seem to contribute to at least some aspect of the neuroprotective phenotype of enrichment. The findings from the current study, as well as the reported above, suggest that the effects of each enrichment component on behavioral and neurochemical changes is an interesting topic for future study. This topic will remain a limitation, even if not directly discussed, throughout the rest of this dissertation.

The present study demonstrates that EC rats exhibit a decrease in baseline locomotor activity compared to IC and SC rats under basal conditions, as well as increased locomotor sensitivity to both acute and repeated nicotine administration (Zhu et al., 2004, Wooters et al., 2011, Gomez et al., 2012). The decreased baseline activity in EC rats is accompanied by parallel decreases in dopamine transporter function (Zhu et al., 2004, Zhu et al., 2005b), the number of D1 receptors (Del Arco et al., 2007a), and the basal phosphorylation levels of DARPP-32 at threonine-34 and CREB at serine 133 (Gomez et al., 2012) within the PFC. In particular, the levels of phosphorylated DARPP-32 at threonine-34 in the PFC of EC, IC, and SC rats are positively correlated with their respective basal locomotor activities under saline control conditions. In opposition, the basal levels of pERK1/2 in the PFC were higher in EC rats than that in IC and SC rats in the saline control group, and the pERK1/2 levels were negatively correlated with their respective baseline locomotor activity. The opposite effects of an enriched environment on the phosphorylation levels of DARPP-32 and ERK1/2 may be due to a compensatory response of ERK1/2 activity to drastically low levels of phosphorylated DARPP-32 at threonine-34. When mice had a point mutation of the threonine-34 residue via replacement with an alanine residue, pERK1/2 activity was actually increased in the PFC (Valjent et al., 2005), suggesting that the increased basal pERK1/2 observed in EC rats is a compensatory response to the drastically low basal levels of pDARPP-32 Thr34. Moreover, the stimulation of prefrontal D2 receptors could be secondary to upregulation of ERK1/2 activity (Stanwood, 2008). Recent results from our laboratory using microarrays for gene expression show that mRNA expression levels of the D2 receptor in the PFC is upregulated in EC rats compared to IC rats (unpublished data). With regard to

the basal differences in EC rats between pERK1/2 and pCREB, this may be due to the fact that pERK1/2 can target transcriptions factors not specific to CREB. ERK1/2 has been shown to also control gene expression by signaling to ets-like-1 protein (ELK-1) via related MAPKs (Davis et al., 2000). Both DARPP-32 and ERK1/2 are regulated by dopaminergic and glutamatergic signaling pathways (Valjent et al., 2005, Greengard et al., 1999). However, our previous study demonstrates that there were no changes in the number of NMDA receptors or NMDA subunit expression in the PFC among EC, IC and SC rats within saline control groups (Gomez et al., 2012). However, others have observed that enrichment increases metabotropic receptor-mediated glutamate release in the PFC of rats; GluR1, NR2B, and NR2A subunits in the mouse forebrain; and enhanced AMPA mediated excitatory neurotransmission in the rat cortex (Tang et al., 2001, Melendez et al., 2004, Nichols et al., 2007). Therefore, it is possible that environmental enrichment produces an imbalance of D1 and D2 receptors, as well as dramatic alterations in the glutamatergic system to alter associated downstream signaling within the PFC. Ultimately; this may lead to a down-regulation of dopaminergic tone and an up-regulation of glutamatergic tone under basal conditions. Thus, it is likely that enrichment-dependent prefrontal plasticity may contribute to the inherent difference in baseline locomotor activity, and furthermore basal alterations are likely to contribute to the behavioral and neurochemical manifestations in response to nicotine exposure.

Acute nicotine regulated the phosphorylation levels of DARPP-32 at Thr34 and Thr75 sites in a dose-dependent manner. While acute nicotine (0.1 mg/kg) only increased Thr34 levels in the NAc of EC rats and had no effects on Thr75, the high dose of nicotine (0.8 mg/kg) increased Thr34 levels in the PFC and NAc levels in EC rats.

However, nicotine (0.3 mg/kg) notably increased Thr34 levels in all regions and Thr75 levels in the striatum in EC rats, suggesting an enhanced maximal action of nicotine on the phosphorylation of DARPP-32. *In vitro* studies suggest nicotine at a low concentration decreases Thr34 levels; whereas a high concentration increases Thr34 and decreases Thr75 levels in mouse striatal slices (Hamada et al., 2005, Hamada et al., 2004). Although systemic acute nicotine (0.8 mg/kg, s.c.) increased phosphorylation of both Thr34 and Thr75 in the mouse striatum 15 min after injection (Zhu et al., 2005a), another report showed no effects on Thr34 and Thr75 in the striatum and NAc of rats 20 min after a single injection of nicotine (0.35 mg/kg, s.c.) (Addy et al., 2007). Thus, nicotine-mediated regulation of DARPP-32 activity is largely dependent on the species, dosage, route of administration, and the time needed to harvest brains. Importantly, in the current study, nicotine produces robust increases of pDARPP-32 Thr34 in EC rats, which may be caused by intrinsic difference in basal levels of Thr34 between EC and IC rats. The action of nicotine on Thr34 is regulated by activation of D1 receptor-mediated PKA pathway (Hamada et al., 2004), and this cascade contributes to nicotine-induced motivation (Svenningsson et al., 2004). The current results show that the behaviorally-relevant dose of nicotine (0.35 mg/kg) produces hyperactivity in EC and IC rats, but hypoactivity in SC rats on Day 1. Thus, the differential regulatory effects on pDARPP-32 Thr34 levels in response to acute nicotine in EC and IC rats may play a role in different locomotor response to nicotine between EC and IC rats. We next decided to further follow this up in a model of repeated nicotine.

Repeated nicotine administration eliminated the basal difference in pDARPP-32 Thr34 observed between the EC and IC rats and increased pDARPP-32 Thr34 in the PFC

of EC rats relative to IC rats. Nicotine also elicited Thr34 increases in the NAc of EC rats, despite not being as robust as in the PFC. This implicates that the processes that mediate the lower basal levels of pDARPP-32 Thr34 in the PFC of EC rats do not prevent repeated nicotine from regulating DARPP-32 signaling. Rather, compared to their respective saline controls, the magnitude of change in nicotine-induced Thr34 levels in the PFC is greater in EC than in IC and SC rats. DA D1 receptor activation has been demonstrated to increase Thr34 levels (Svenningsson et al., 1998) and it is possible that increased nicotine-induced Thr34 levels in the PFC of EC rats may represent a compensatory D1 receptor-mediated down-regulation in response to nicotine-stimulated enhancement of DA transmission. While the current results show no effects of repeated nicotine on pDARPP-32 Thr34 levels in the striatum were found in EC, IC, and SC rats, a previous study has shown that repeated nicotine (0.35 mg/kg) produces a clear increase in pDARPP-32 Thr34 in the dorsal striatum of rats (Addy et al., 2007). One possibility for the discrepancy could be due to the different ways of sample preparations, for example, the entire rat head 15 min after last nicotine injection was immediately frozen in chilled 2-methyl butane and brains were later dissected (Addy et al., 2007), whereas in the current study, brain regions were immediately dissected and sonicated in sample buffer as described previously (Midde et al., 2011). Similarly, our previous study using *in vivo* voltammetry assay has demonstrated that basal DA clearance in the medial PFC is lower in EC rats than IC rats under saline control condition, whereas systemic acute nicotine injection only increases the DA clearance in EC rats, but not in IC rats (Zhu et al., 2007b). The decrease in DA clearance in medial PFC of EC rats is the result of reduced DA transporter surface expression (Zhu et al., 2005b). Given that DA induces

internalization of D1 receptors in HEK293 cells (Vickery and von Zastrow, 1999), it is possible that a greater proportion of D1 receptors in IC rats are internalized by nicotine-induced DA release resulting in a reduced molecular response to nicotine in IC rats. Although little evidence shows that enrichment-induced manipulations of DA signaling within the mesolimbic circuit attenuate nicotine-mediated sensitization, the current results demonstrate a complex regulatory mechanism underlying the differential molecular effects in response to nicotine between EC and IC rats. Thus, enrichment-induced decreases in basal Thr34 levels may allow EC rats to have an increased molecular response to nicotine.

In addition to DARPP-32, this study also determined the effects of enrichment on CREB activity following repeated saline or nicotine administration. Enrichment-mediated changes in phosphorylated CREB at Ser133 are in parallel with the changes of pDARPP-32 Thr34 levels, which are associated with PKA activation (Nairn et al., 2004, Dash et al., 1991). Basal pCREB levels were lower in the PFC and NAc of EC rats than IC and SC rats in the saline control group, which is in agreement with a previous report showing decreased levels of pCREB in the NAc of EC rats (Green et al., 2010). However, the robust nicotine-induced increases in pCREB in the PFC and NAc were reflected more so in EC rats than in IC and SC rats. At the cellular levels, phosphorylation of DARPP-32 at Thr34 by PKA converts to a potent inhibitor of the PP-1, which controls the phosphorylation state of CREB (Greengard et al., 1999, Hemmings et al., 1984). Activation of D1 receptors has been shown to increase the levels of phosphorylation of Thr34 and pCREB in the rat PFC (Hotte et al., 2006). Thus, enriched

environment-induced changes in the levels of both pDARPP-32 Thr34 and pCREB in the PFC is consistent with a regulatory role of the D1/cAMP/PKA signaling pathway.

Nicotine has also been shown to elevate the phosphorylation levels of ERK1/2 both *in vitro* and within the mesocorticolimbic circuitry (Nakayama et al., 2001, Valjent et al., 2004b). The present results demonstrate that a significant increase in nicotine-mediated pERK1/2 in the PFC was observed in IC rats, but not in EC rats in our nicotine sensitization model, suggesting that an environmental enrichment attenuates nicotine-mediated pERK1/2 levels. These results are in disagreement with our previous findings that the magnitude of changes in nicotine-induced phosphorylated DARPP-32 at threonine-34 and the DA clearance rate were greater in EC rats than IC and SC rats, based on their basal levels (Zhu et al., 2007a, Gomez et al., 2012). The attenuation of nicotine-mediated activation of dopaminergic signaling within the PFC by environmental enrichment may therefore represent compensatory enrichment-dependent plastic changes within the PFC in response to nicotine stimulation. This interpretation is supported by repeated nicotine-induced elimination of basal differences in DARPP-32 and ERK1/2 activity between EC and IC rats in our current and previous studies (Gomez et al., 2012). Consistent with the enrichment-induced molecular changes, the behavioral studies show that EC rats display greater sensitization to nicotine and amphetamine than IC and SC rats (Gomez et al., 2012, Bardo et al., 1995). However, the current and previous studies (Gomez et al., 2012) show that nicotine-mediated phosphorylated DARPP-32 and ERK1/2 levels in the PFC were not correlated with their respective nicotine-mediated locomotor activity in these animals. It is possible that EC rats have relatively high basal levels of pERK1/2 in the PFC, which may mask the nicotine-induced elevation of the

phosphorylation of this signaling protein, thereby producing a ceiling effect on nicotine-mediated pERK1/2 in EC rats. Additionally, the nicotine-mediated activity was not correlated with the levels of pDARPP-32 Thr34 in the PFC, NAc, or STR. It is possible that nicotine elevated pDARPP-32 Thr34 levels in EC and IC rats to its maximum potential thereby causing a ceiling effect on nicotine-mediated pDARPP-32 Thr34. Evidence suggests that DARPP-32 and its phosphorylation at Thr34 have an inhibitory role in spontaneous locomotor activity, morphine- or cocaine-induced locomotor sensitization and nicotine-induced motor depression in mice (Hiroi et al., 1999, Zhu et al., 2005a, Zachariou et al., 2006, Valjent et al., 2010). There is also evidence showing that systemic nicotine produces profound enhancements of ERK1/2 in the PFC compared to other brain regions (Valjent et al., 2004b), indicating that the PFC plays a crucial role in nicotine-mediated ERK activity and the consequence of its behavioral effects. In accordance, the current findings show that an enriched environment produces neuroadaptations in the prefrontal ERK pathway, which may alter nicotine-enhanced activation of ERK1/2 and the relevant nicotine-mediated behaviors.

To further examine this, we administered SL327 to rats before undergoing an acute nicotine-induced locomotor session. Administration of SL327 has been previously shown to decrease the locomotor response to acute amphetamine in rats, along with attenuating locomotor sensitization to repeated amphetamine and cocaine in mice (Shi and McGinty, 2006, Valjent et al., 2006b). Acute nicotine (0.4 mg/kg) has been shown to induce PFC pERK activation (Valjent et al., 2004a); however, the contribution of SL327 to nicotine-induced activation of pERK as well as nicotine-induced locomotor levels is unknown. We observed SL327-induced decreases in both basal and acute nicotine-

induced locomotor levels within SC rats, although interestingly, we found no effect within EC or IC rats. This may suggest that EC and IC rats compensate or adapt to the locomotor effects of SL327 compared to SC rats. SC rats have the highest basal locomotor levels compared to IC or EC rats. This may represent a floor effect, particularly in EC rats, where the basal locomotor levels are unaffected by SL327 due to the considerably low levels of locomotor activity. Additionally, the SL327-induced decreased locomotor levels in SC rats in response to acute nicotine may be a result of SL327-induced decrease in basal locomotor levels as the percent change from the DMSO control group is roughly the same. There are conflicting reports, with one study showing that a 50 mg/kg i.p. dose of SL327 has no effect on basal locomotor levels, and another study showing a SL327-induced basal locomotor effect as we saw in our experiment (Shi and McGinty, 2006, Carr et al., 2009). This notion of SC selectivity was supported by immunoblotting as SL327 only decreased the nicotine-induced enhancements of pERK1/2 strictly in SC rats. One explanation for the unseen differences in EC and IC rats could be due to the acute nature of the study. Acute SL327 was able to drastically decrease ERK1/2 activation induced by cocaine and amphetamine; although there are mixed reports regarding the acute effect of SL327 on psychostimulant-mediated locomotor levels. Previous reports have shown that SL327 decreased locomotor levels induced by amphetamine in rats, but failed to decrease acute cocaine- and amphetamine-induced locomotor levels in mice (Valjent et al., 2006b, Shi and McGinty, 2006). Adding to these mixed reports, SL327 abolished cocaine- and morphine-induced expression of conditioned place preference (CPP), a measure of drug reward, but failed to have any effect on ethanol-induced CPP expression in mice (Valjent et al., 2006a, Groblewski et

al., 2011). Therefore, the behavioral effects of SL327 could depend on various factors such as rodent species or the type of drug. The failure of acute nicotine to induce prefrontal ERK activation in EC or IC rats, may also further explain why we did not see behavioral differences in EC or IC rats, as SL327 did not have to block any specific ERK activity in response to acute nicotine. Additionally, the failure of SL327 to decrease both locomotor levels and pERK1/2 levels in response to nicotine may be due to SL327 being injected systemically, and thus is affecting multiple brain regions including the NAc, STR, and VTA, which likely impacts the overall behavioral and molecular outcome. Future studies involving site-specific blockade of ERK activity by intra-PFC of U0126, a non-brain penetrating MEK inhibitor, in EC and IC rats undergoing nicotine-mediated locomotor sensitization may be better suited to clarify this potential mechanism.

Collectively, the current study demonstrates that enriched environment-induced alterations in prefrontal signaling proteins may be responsible for enrichment-mediated alterations in nicotine-associated behaviors. Given the important role of the phosphorylation at Thr34 of DARPP-32, pCREB, and pERK1/2 in drug-mediated behaviors (Zhang et al., 2006), the current results may also have relevance to environmental enrichment-induced potential resistance to drug-self-administration. In fact, EC rats display altered self-administration behavior to both amphetamine and cocaine (Gipson et al., 2011, Green et al., 2002). We speculate that intake of nicotine in EC rats might differ in a more reliable model of drug addiction, such as self-administration. These findings may also have important implications for preclinical studies involving the role of enrichment in individual differences in vulnerability to nicotine abuse. Manipulations of prefrontal intracellular signaling activity, particularly

with regard to ERK1/2, or the upstream mechanisms responsible for activation of these intracellular signaling cascades may therefore, represent an approach in the treatment of drug addiction. A full understanding of the neurobehavioral mechanisms underlying the enriched environment-induced resistance to psychostimulants is important for targeting those individuals most vulnerable to psychostimulant abuse and may aid in the discovery of novel pharmacological treatments for drug abuse.

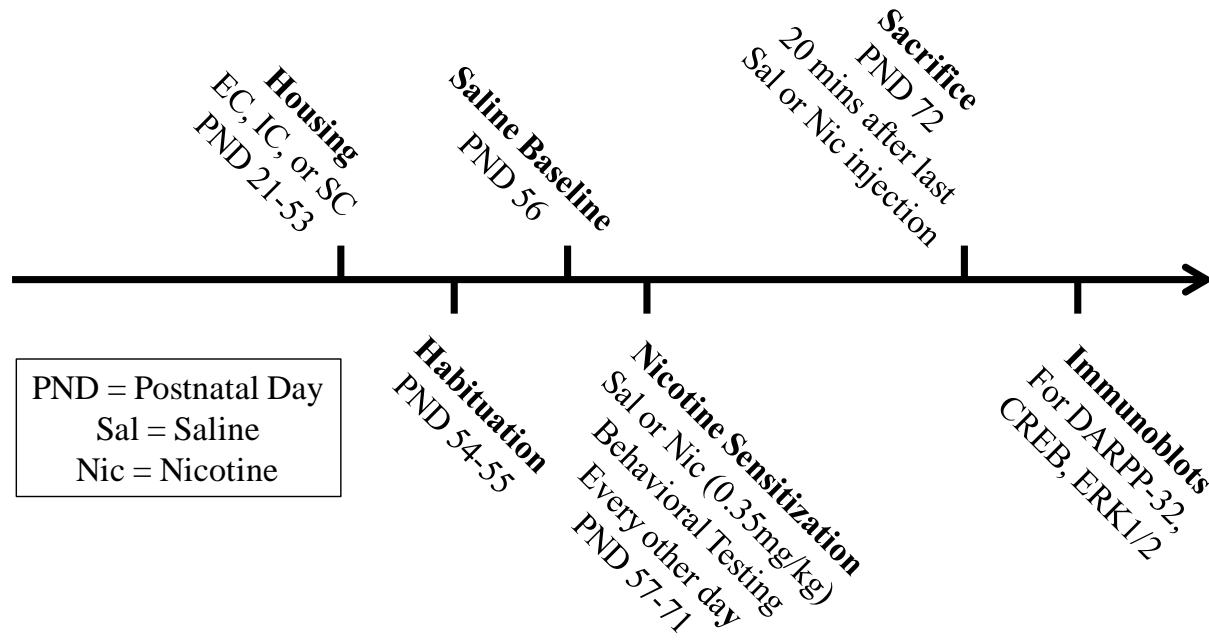


Figure 2.1. Timeline of the experimental paradigm starting on postnatal day 21 of EC, IC, and SC rats used for the nicotine sensitization experiments involving locomotor activity and assessment of intracellular signaling proteins.

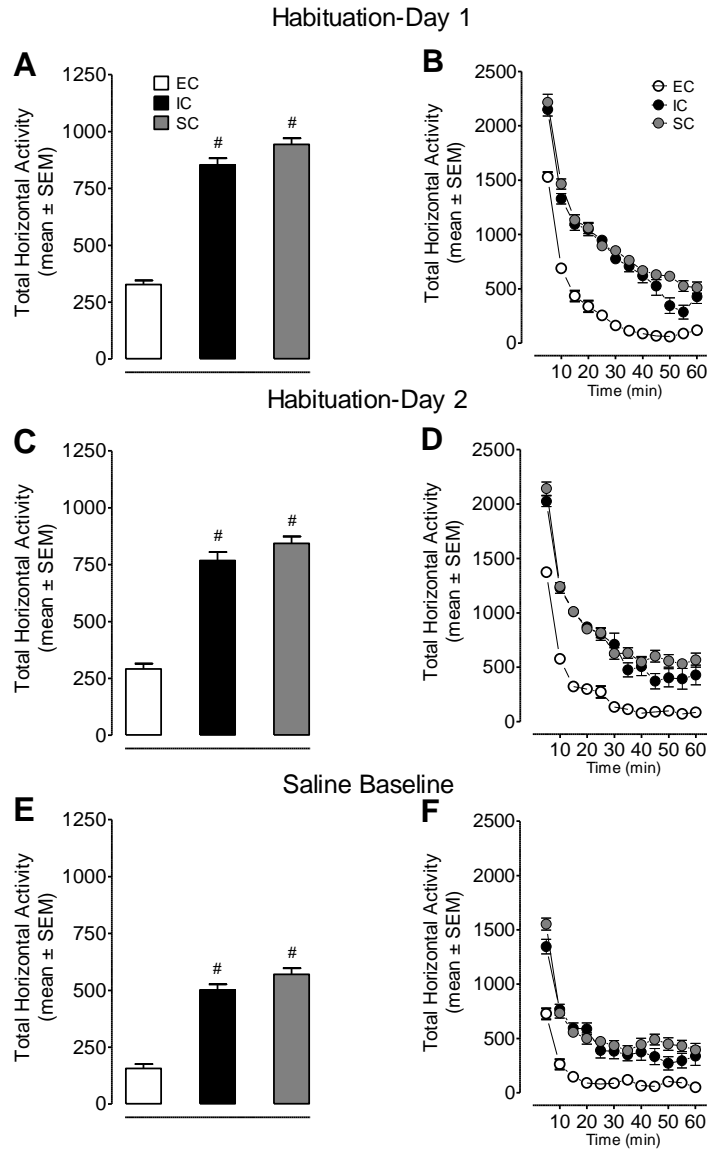


Figure 2.2. Environmental enrichment diminishes baseline activity. Panels A, C, and E show the total horizontal activity (mean ± SEM) across the two 60-min habituation periods and 60-min session post-saline injection. Panels B, D, and F show the time course of the total horizontal activity (mean ± SEM) during each 5-min interval across the two 60-min habituation periods and 60-min session post saline injection. Total horizontal activity revealed a significant effect of housing condition ($F_{(2, 70)} = 46.48, p < 0.001$), day ($F_{(1, 70)} = 317.41, p < 0.001$) and time ($F_{(11, 770)} = 148.57, p < 0.001$), and a significant housing condition × day × time interaction ($F_{(22, 770)} = 2.48, p < 0.001$). Overall total horizontal activity was lower in EC than in IC or SC ($p < 0.001$, Bonferroni *t*-test). # $p < 0.001$ denotes difference between EC and IC or SC groups ($n = 22$ - 27 rats/group). Published as (Gomez et al., 2012).

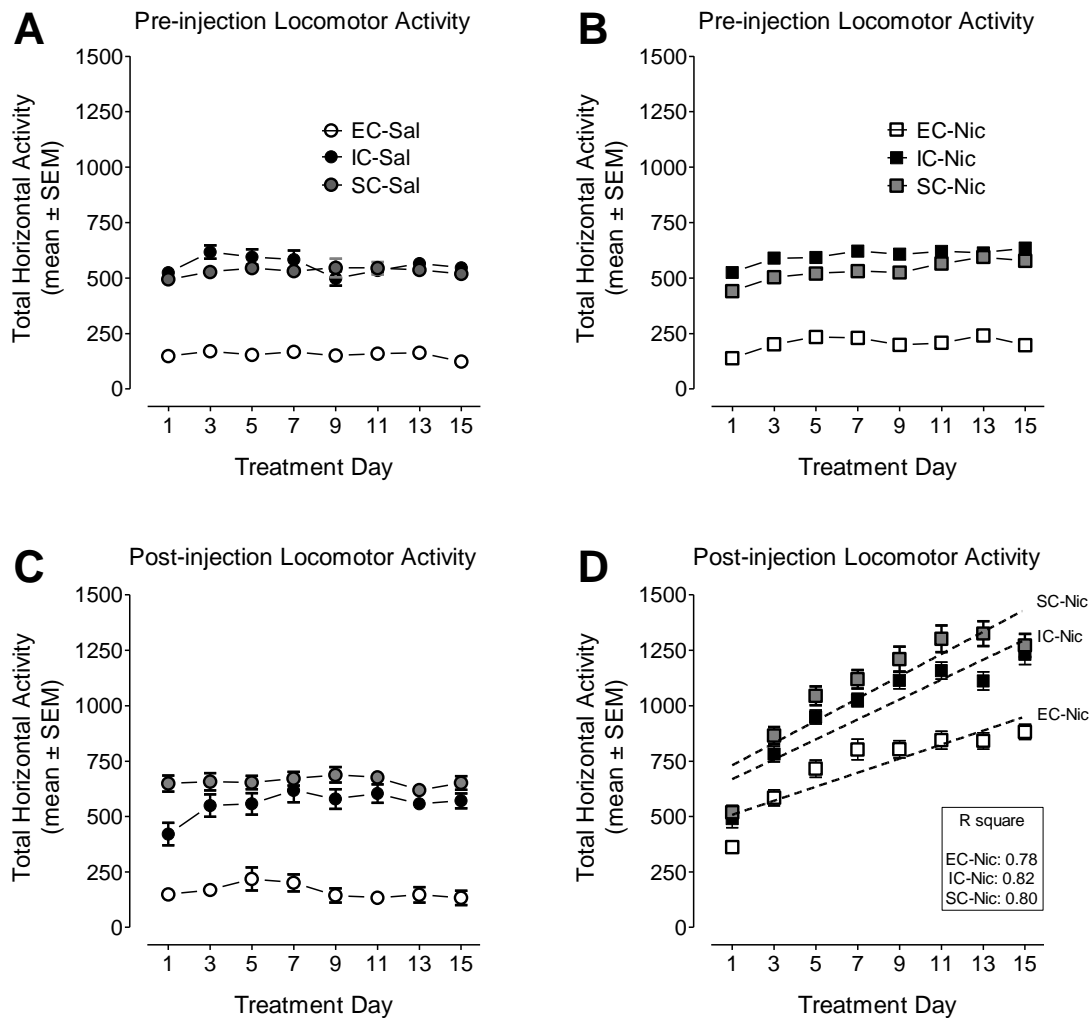


Figure 2.3. The time-course data during the behavioral sensitization phase. EC, IC or SC rats were administered nicotine (Nic, 0.35 mg/kg, s.c.) or saline (Sal) on Days 1-15. Panel A and B show the total horizontal activity (mean ± SEM) in saline control and nicotine-treated groups during the 30 min pre-injection habituation period. Panel C and D shows the total horizontal activity (mean ± SEM) during the 60 min following saline or nicotine injection with robust behavioral sensitization observed under all housing conditions (panel D). Total horizontal activity after a subcutaneous injection of saline or nicotine revealed a significant effect of housing condition ($F_{(2, 67)} = 104.29, p < 0.001$), treatment ($F_{(1, 67)} = 333.57, p < 0.001$) and day ($F_{(7, 469)} = 139.97, p < 0.001$). A significant interaction of housing condition × treatment × day ($F_{(14, 469)} = 2.76, p < 0.05$) and treatment × day ($F_{(7, 469)} = 115.42, p < 0.05$) were found. n = 12 rats/group. Published as (Gomez et al., 2012).

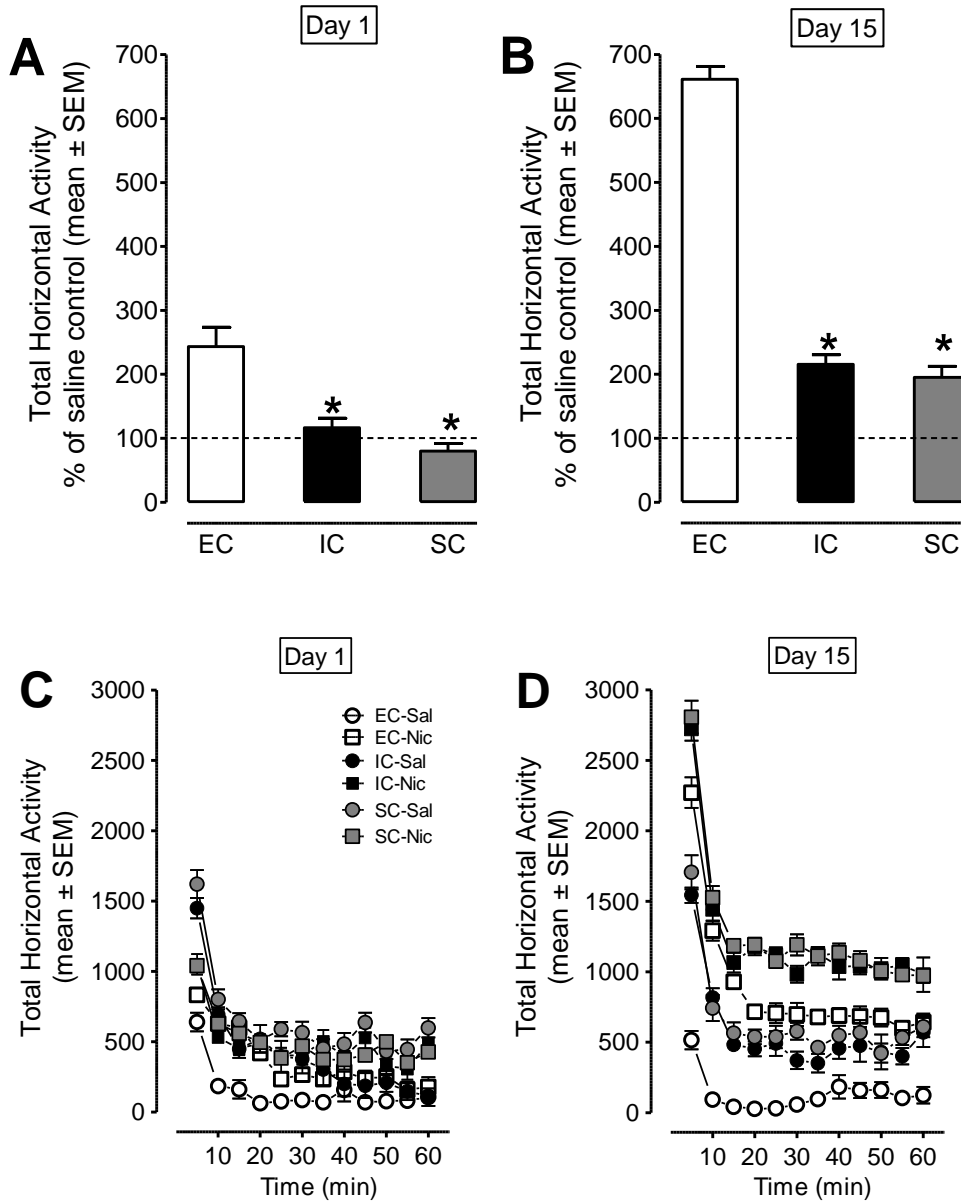


Figure 2.4. The time-course data for total horizontal activity during day 1 and day 15 of the behavioral sensitization phase. Panels A and B show the total horizontal activity (mean \pm SEM) across the 60-min session. Data are presented as percent of their saline controls for respective housing condition. Panels C and D show the time course of the total horizontal activity (mean \pm SEM) during each 5-min interval. * $p < 0.05$ difference between nicotine- and saline-treatment groups. # $p < 0.001$ difference between EC, IC or SC groups. $n = 12$ rats/group. Published as (Gomez et al., 2012).

Table 2.1. Activity of DARPP-32 Thr34 and Thr75 are differentially altered among EC and IC rats within the mesocorticolimbic circuitry basally and in an acute nicotine-dose dependent manner. Published as (Gomez et al., 2012).

* $p < 0.05$ denotes difference between the nicotine- and saline-treated groups.

$p < 0.05$ denotes difference between housing groups. n=10 rats/group.

	pDARPP-32Thr34/DARPP-32			pDARPP-32Thr75/DARPP-32		
	0.1 mg/kg	0.3mg/kg	0.8mg/kg	0.1 mg/kg	0.3mg/kg	0.8mg/kg
PFC						
EC-Sal	0.60 ± 0.04	0.69 ± 0.07	0.21 ± 0.07	0.92 ± 0.08	1.19 ± 0.12	1.05 ± 0.08
EC-Nic	0.64 ± 0.08	1.00 ± 0.09*	1.04 ± 0.09*	1.16 ± 0.15	1.15 ± 0.13	1.13 ± 0.11
IC-Sal	0.88 ± 0.09#	0.95 ± 0.08#	0.70 ± 0.09#	1.37 ± 0.16	1.26 ± 0.09	1.05 ± 0.12
IC-Nic	0.95 ± 0.10	1.03 ± 0.11	1.10 ± 0.11	1.08 ± 0.09	0.91 ± 0.08	0.74 ± 0.08
NAC						
EC-Sal	0.76 ± 0.07	0.55 ± 0.09	0.51 ± 0.06	1.02 ± 0.08	1.29 ± 0.15	0.76 ± 0.06
EC-Nic	1.08 ± 0.08*	1.19 ± 0.11*	0.86 ± 0.09*	1.16 ± 0.13	1.23 ± 0.13	0.85 ± 0.08
IC-Sal	1.10 ± 0.11#	0.89 ± 0.09#	1.01 ± 0.12#	1.09 ± 0.15	1.24 ± 0.15	0.97 ± 0.11
IC-Nic	1.11 ± 0.13	1.13 ± 0.12	0.89 ± 0.08	1.43 ± 0.17	1.39 ± 0.14	1.00 ± 0.13
Striatum						
EC-Sal	0.92 ± 0.08	0.68 ± 0.08	0.60 ± 0.09	1.33 ± 0.12	0.82 ± 0.07	0.78 ± 0.09
EC-Nic	0.94 ± 0.07	1.37 ± 0.12*	0.76 ± 0.08	1.47 ± 0.15	1.30 ± 0.13*	0.80 ± 0.09
IC-Sal	1.28 ± 0.06#	1.08 ± 0.09#	1.02 ± 0.11#	1.37 ± 0.17	1.10 ± 0.11	1.09 ± 0.13
IC-Nic	1.18 ± 0.12	1.33 ± 0.12	0.92 ± 0.11	1.40 ± 0.11	0.81 ± 0.09	0.98 ± 0.11

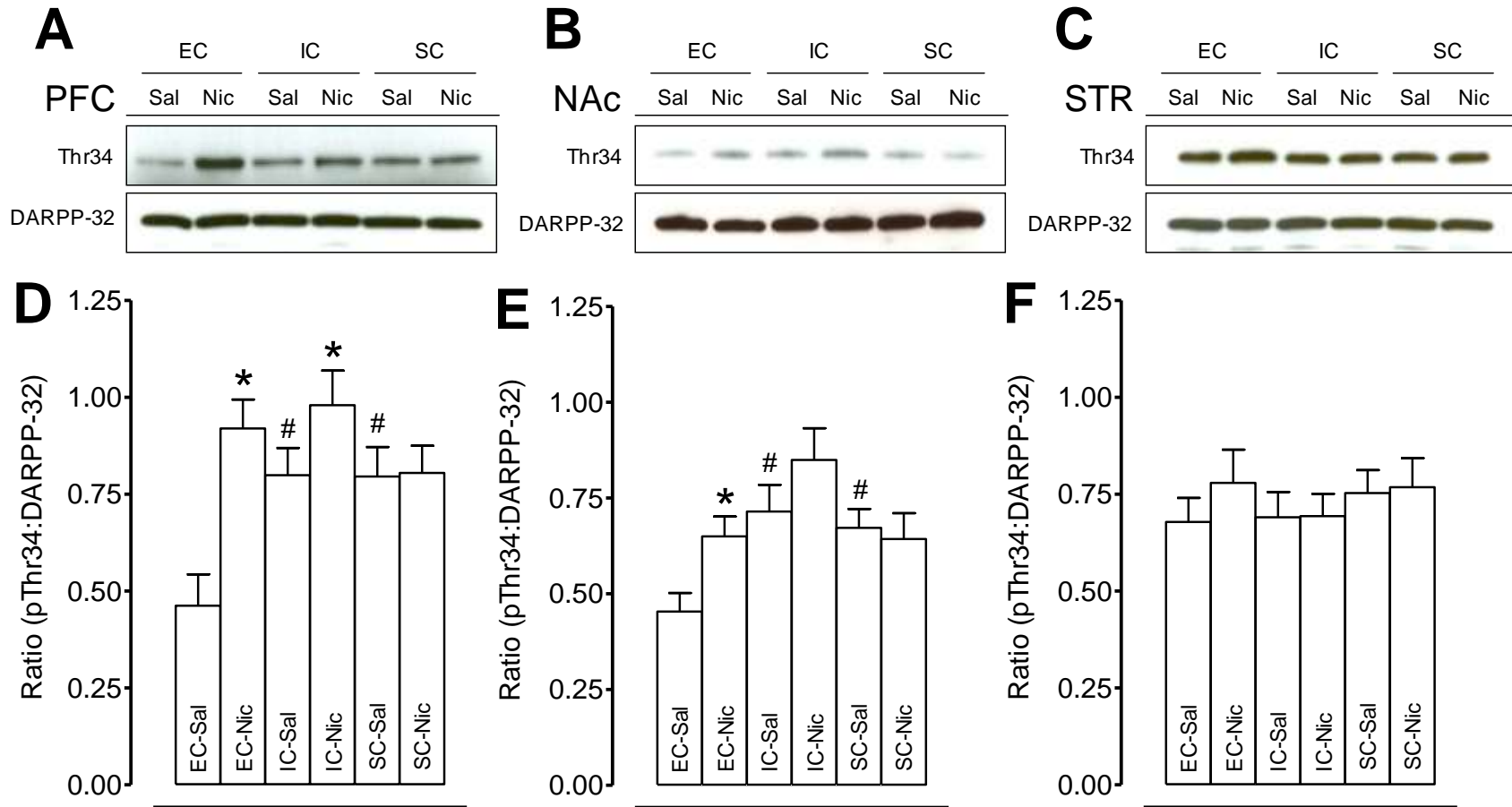


Figure 2.5. Levels of pDARPP-32 Thr34 and total DARPP-32 in the prefrontal cortex (PFC), nucleus accumbens (NAc) and striatum (STR) in EC, IC, and SC rats. Top panels show representative immunoblots of pDARPP-32 Thr34 and total DARPP-32 (Band = 32 kDa) in the PFC (A), NAc (B) and STR (C) in nicotine (Nic) or saline (Sal)-treated rats. Bottom panels show the ratio of levels of pDARPP-32 Thr34 to total DARPP-32 in the PFC (D), NAc (E) and STR (F) of Nic (0.35 mg/kg) or Sal-treated rats. Data are presented as the percentage of pDARPP-32 Thr34 to total DARPP-32 densitometry values. No differences were found with total DARPP-32 levels in any of the groups. * $p < 0.05$ denotes difference between the nicotine- and saline-treated groups. # $p < 0.05$ denotes difference between housing groups. $n=10$ rats/group. Published as (Gomez et al., 2012).

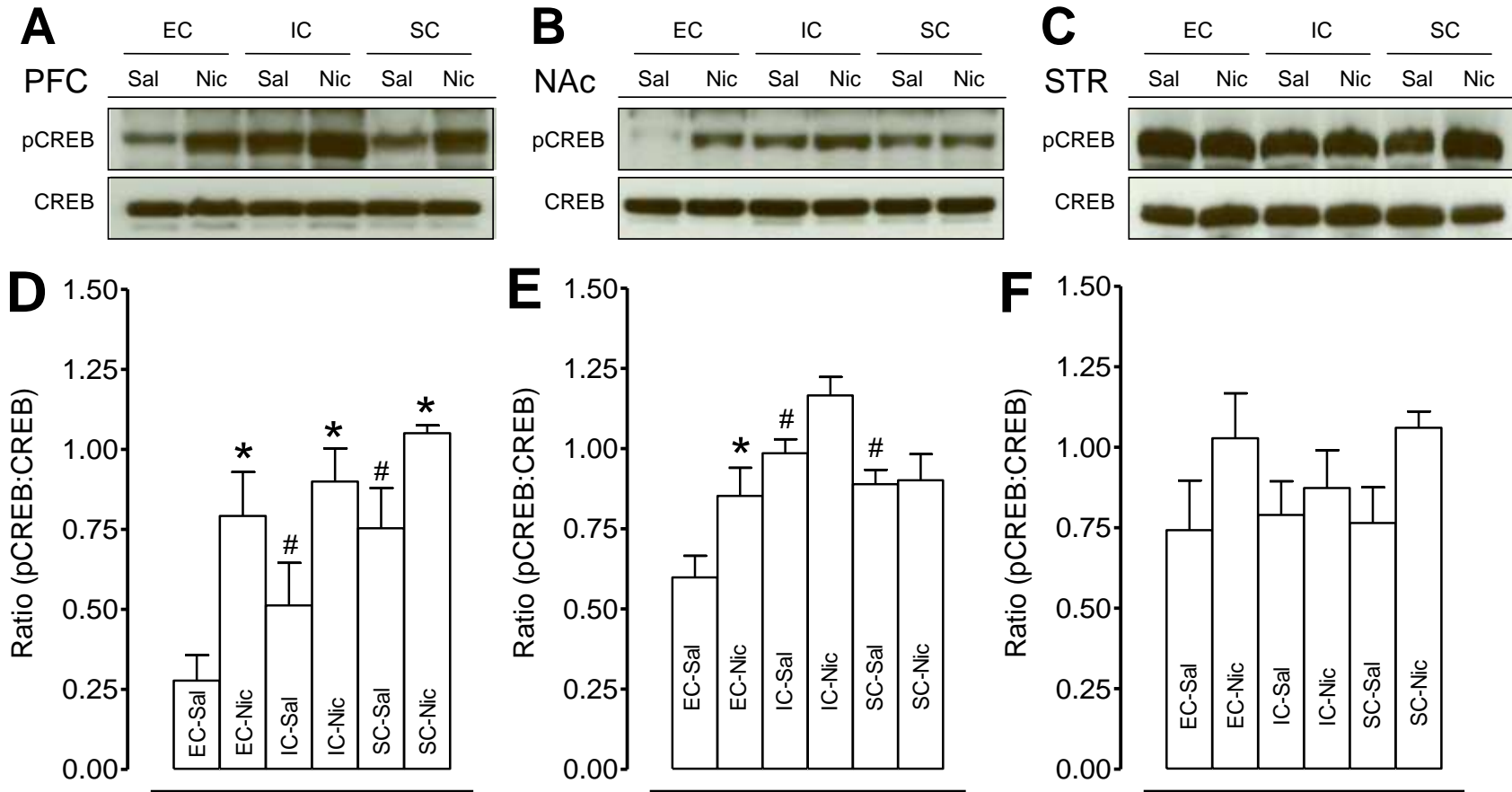


Figure 2.6. Levels of pCREB and total CREB in the prefrontal cortex, nucleus accumbens, and striatum in EC, IC, and SC rats. Top panels show representative Western blots of pCREB and CREB (Band = 43 kDa) in the prefrontal cortex (PFC, A), nucleus accumbens (NAc, B) and striatum (STR, C). Bottom panels show the ratio of phosphorylated CREB levels to total CREB in the PFC (D), NAc (E) and STR (F) of nicotine (0.35 mg/kg) or saline-treated rats. Data are presented as the percentage of pCREB to total CREB densitometry values of immunoreactivity. Histograms represent means and bars represent SEM. Total CREB and pCREB were run at the same time with the same loading volume. No differences were found with total CREB levels in any of the groups. * $p < 0.05$ denotes difference between the nicotine- and saline-treated groups. # $p < 0.05$ denotes difference between housing groups. $n = 10$ rats/group. Published as (Gomez et al., 2012).

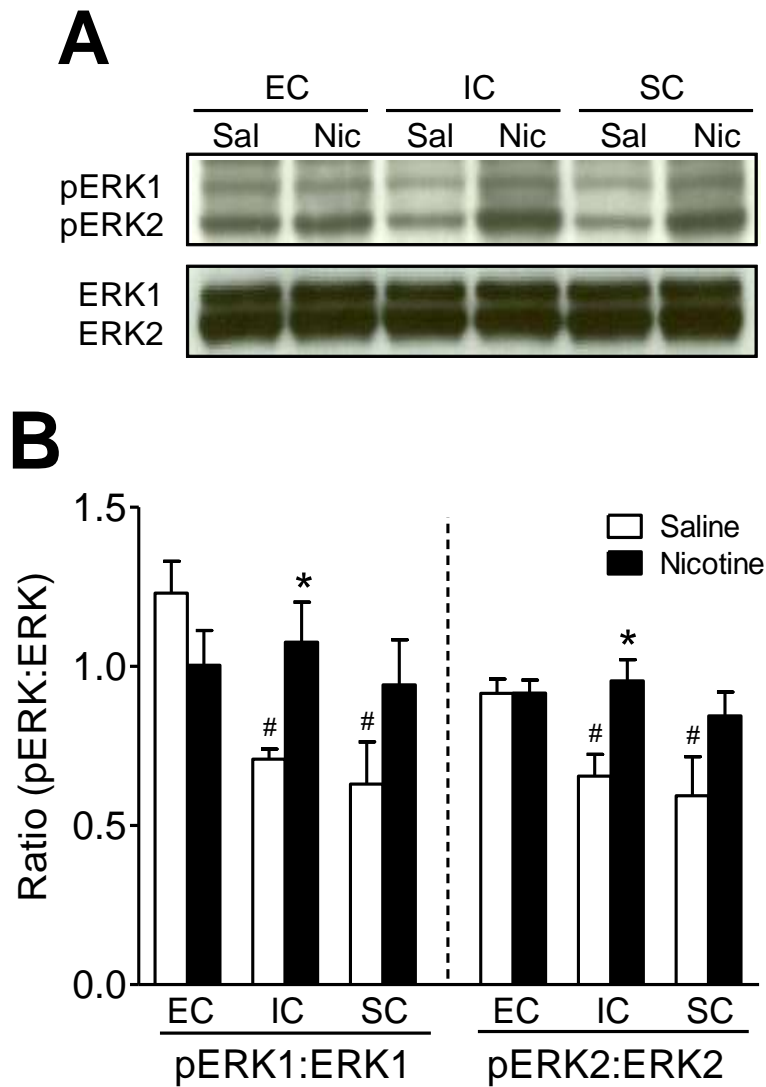


Figure 2.7. Levels of pERK1/2 and ERK1/2 in the prefrontal cortex (PFC) of EC, IC, and SC rats treated repeatedly with nicotine or saline. Panel A shows representative immunoblots of pERK1/2 and ERK1/2 immunoreactivity (Band 1 = 44 kDa, Band 2 = 42 kDa) in the PFC of EC, IC, and SC rats following repeated administration of nicotine (Nic; 0.35 mg/kg, s.c.) or saline (Sal). Panel B shows the ratio of pERK1/2 levels to total ERK1/2 levels in the PFC in corresponding rats. Data are presented as the percentage of pERK1/2 to total ERK1/2 densitometry values of immunoreactivity. Histograms represent means and error bars represent SEM. The levels of pERK1/2 and total ERK1/2 were measured at the same time with the same loading volume of protein. * $p < 0.05$ compared to the respective saline controls. # $p < 0.05$ compared to EC group. $n = 6$ rats/group.

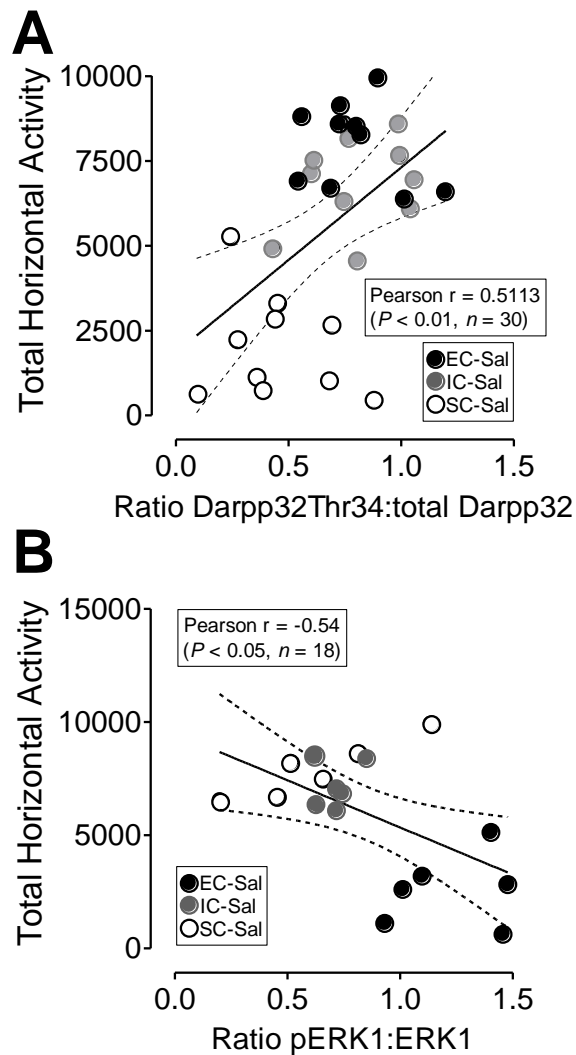


Figure 2.8. Panel A shows the correlation of the phosphorylation state of DARPP-32 at Thr34 in the PFC with locomotor activity for EC, IC, and SC saline-treated rats. Locomotor activity counts were collected from behavioral testing on day 15. Total horizontal activity is presented as mean values of each rat within the 60-min session. Phosphorylation levels of DARPP-32 at Thr34 are presented as the percentage of pDARPP-32 Thr34 to total DARPP-32 densitometry values of immunoreactivity. Dashed lines represent the 95% confidence interval of the linear regression fit (solid line). $n=10$ rats/group. Panel B shows correlation of the phosphorylation level of ERK1 in the PFC with total horizontal activity for saline treated EC, IC, and SC rats. Total horizontal activity counts of each rat within the 60-min session were collected from the last behavioral testing on day 15. Data for the ratio of pERK1 to total ERK1 densitometry values of immunoreactivity in the PFC of saline control rats were collected from panel B. Dashed lines represent the 95% confidence interval of the linear regression fit (solid line). $n=6$ rats/group. Published as (Gomez et al., 2012).

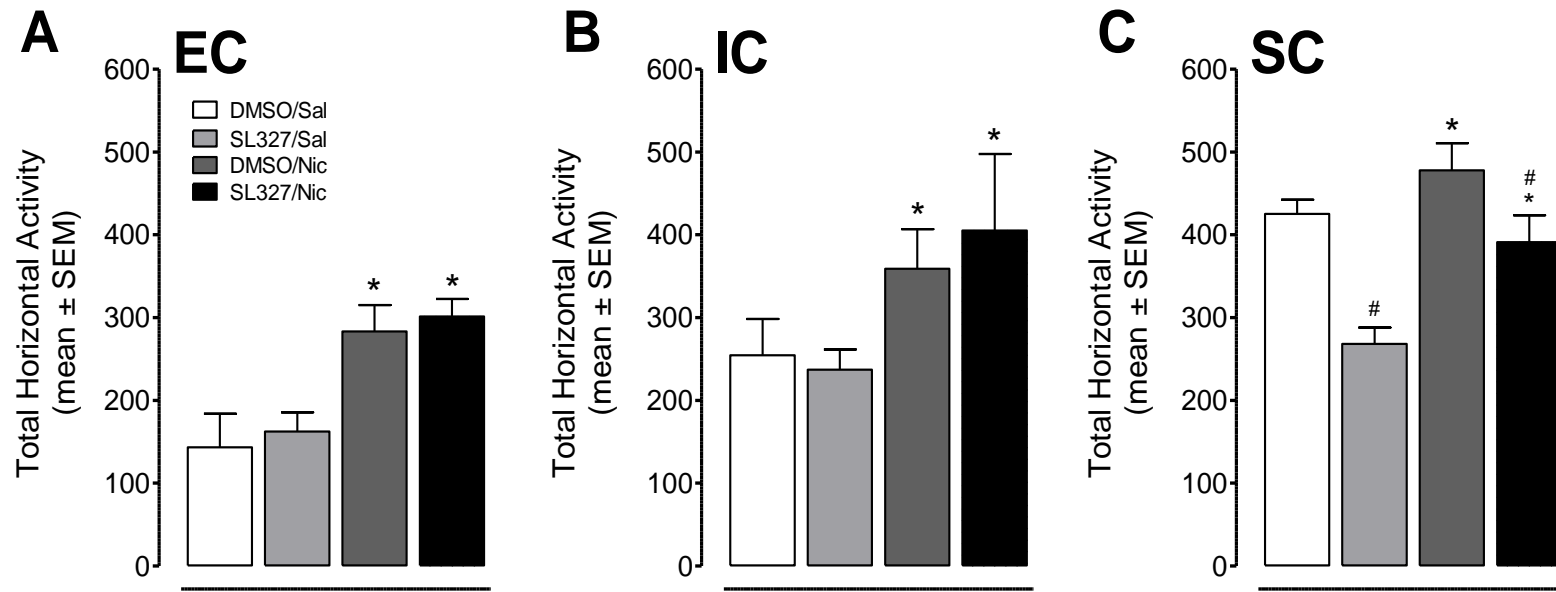


Figure 2.9. Effect of SL327 on acute nicotine-induced locomotion in EC, IC, and SC rats. Total horizontal activity (mean \pm SEM) across the 60-min session was measured in (A) EC, (B) IC, and (C) SC rats pretreated with DMSO or SL327 (50 mg/kg, i.p.), followed by an acute injection of saline (Sal) or nicotine (Nic; 0.35 mg/kg, s.c.). * $p < 0.05$ compared to the respective saline controls. # $p < 0.05$ compared to the respective DMSO controls. $n = 6$ rats/group.

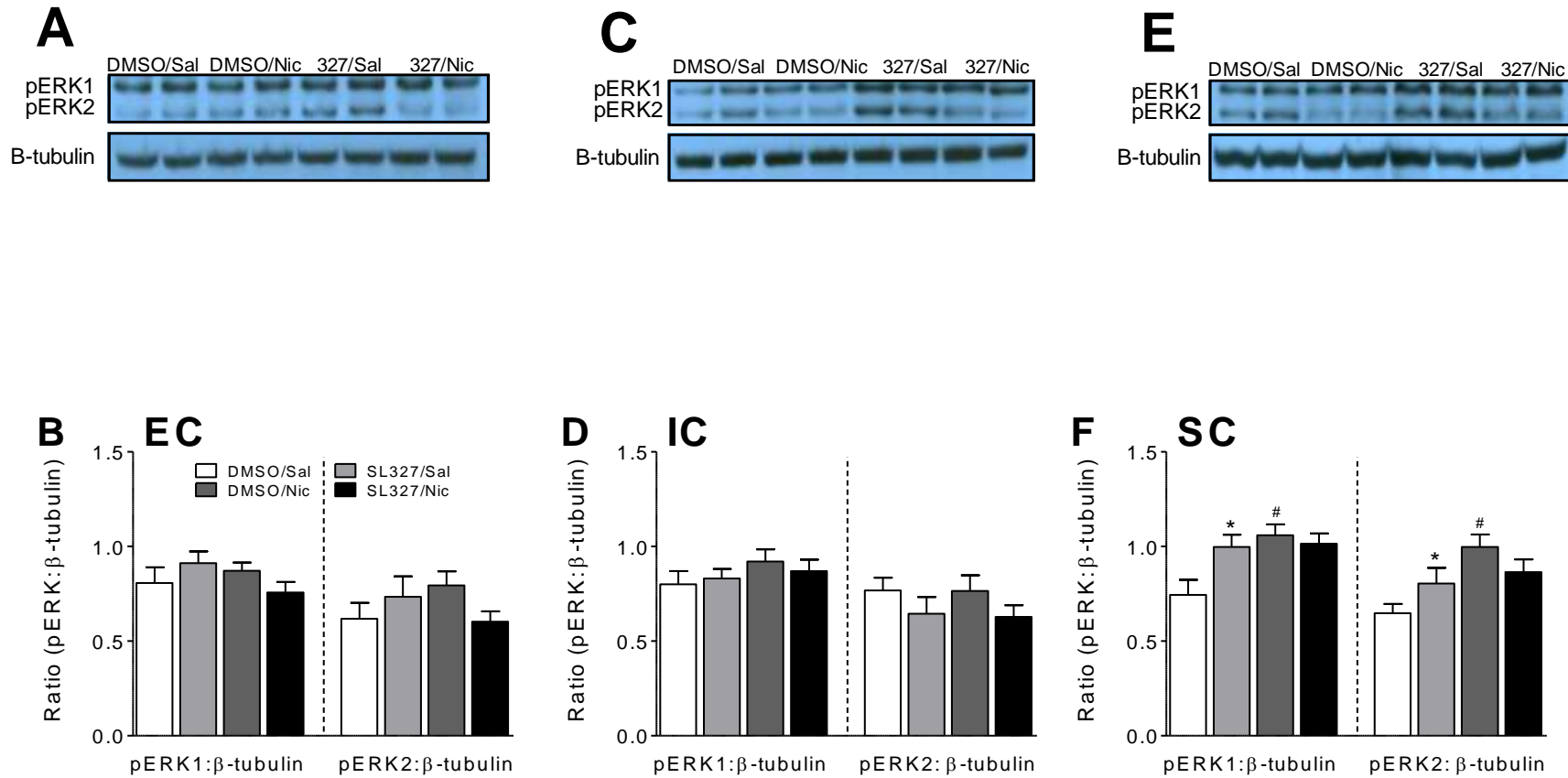


Figure 2.10. Levels of pERK1/2 and B-tubulin in the prefrontal cortex (PFC) of EC, IC, and SC rats pretreated with SL327 (50 mg/kg, i.p.) followed by an acute injection of nicotine (0.35 mg/kg, s.c.) or saline. Representative immunoblots of pERK1/2 and B-tubulin (Band 1 = 44 kDa, Band 2 = 42 kDa) immunoreactivity in the PFC of EC (A), IC (C) and SC (E) rats. The ratios of pERK1/2 levels to B-tubulin levels in the PFC of corresponding EC (B), IC (D) and SC (F) rats. Data are presented as the percentage of pERK1/2 to B-tubulin densitometry values of immunoreactivity. Histograms represent means and error bars represent SEM. The levels of pERK1/2 and B-tubulin were measured with the same loading volume of protein. * $p < 0.05$ compared to the respective saline controls. # $p < 0.05$ compared to the respective DMSO controls. $n = 6$ rats/group.

CHAPTER 3

ROLE OF MICRORNAs IN ENRICHMENT-INDUCED ALTERATIONS IN NICOTINE-MEDIATED SENSITIZATION

3.1 INTRODUCTION

The majority of people who experiment with drugs of abuse fail to persist as drug addicts (Benowitz, 2010). The coordinated involvement of genotype-environment interactions is essential for vulnerability to drug addiction. In particular, environmental factors are necessary components in nicotine abuse liability (Leshner, 2000, Rhee et al., 2003). Therefore, determining the underlying neurobiological mechanism(s) for the potential resistance to nicotine addiction represents a critical area that needs further investigation. Rats raised in an enriched condition (EC) exhibit a neuroprotective-like phenotype to psychostimulant-mediated behaviors compared to rats raised in an impoverished condition (IC) or a standard condition (SC) (Stairs and Bardo, 2009). Specifically, EC rats display altered sensitivity to the locomotor effects of both acute and repeated nicotine (Gomez et al., 2012). Hence, the environmental enrichment paradigm represents a preclinical model to dissect the neurobiological basis for individual vulnerability to nicotine addiction.

Nicotine acts on nicotinic acetylcholine receptor subtypes throughout the brain, in particular the prefrontal cortex (PFC), to activate neurotransmitter release and induce synaptic activity-dependent neuroadaptations (Laviolette and van der Kooy, 2004). The

PFC represents a critical modulator of psychostimulant-induced behaviors as it receives dense ascending dopaminergic (DAergic) projections from the nucleus accumbens (NAc) and ventral tegmental area (VTA), while also sending reciprocal descending glutamatergic projections (Grace et al., 2007). Moreover, disruptions to the PFC alter nicotine-mediated behaviors (Rezvani et al., 2008), suggesting that the PFC represents a fundamental link in the mesocorticolimbic circuitry in regulating nicotine-mediated behaviors. Coincidentally, enrichment-induced neuroadaptations occur most prominently within both prefrontal DAergic and glutamatergic systems (Tang et al., 2001, Melendez et al., 2004, Zhu et al., 2004, Zhu et al., 2005b, Del Arco et al., 2007a). Along these lines, we have previously shown that within the PFC EC rats exhibit altered sensitivity in glutamate- and DA-receptor mediated activity in the downstream intracellular signaling targets DA- and cAMP-regulated phosphoprotein-32 (DARPP-32), extracellular signal-regulated kinase1/2 (ERK1/2), and cAMP-response element-binding protein (CREB) in response to repeated nicotine administration (Gomez et al., 2012). Numerous studies have exemplified the importance of these signaling proteins in psychostimulant-regulated neuroplasticity which may underlie the behavioral alterations induced by drugs of abuse (Valjent et al., 2005, Valjent et al., 2006b, Shi and McGinty, 2006, Addy et al., 2007). Therefore, it's plausible that enrichment differentially affects nicotine's regulation of the DA/ D1 receptor/ cAMP/ protein kinase A (PKA) and the glutamate/ N-methyl-D-aspartate (NMDA) receptor/ mitogen-activated kinase (MEK)/ ERK signaling networks within the PFC. However, the regulatory processes underlying these environmental-induced intracellular signaling differences within the PFC in response to repeated nicotine remain unknown.

MicroRNAs (miRs) are small (~22 nucleotides), non-coding RNAs that have the ability to regulate synaptic-dependent neuronal plasticity (Kosik, 2006). As a result of binding with the 3'UTR of target mRNA, miRs can either post- transcriptionally silence gene expression via perfect complementarity or destroy target mRNAs via imperfect complementarity (Bartel, 2004). For this reason miRs are emerging as candidates for coordinating networks of gene expression (Li and van der Vaart, 2011) In particular, drugs of abuse target miRs to modify drug-induced maladaptive behaviors (Chandrasekar and Dreyer, 2009, Hollander et al., 2010, Im et al., 2010, Chandrasekar and Dreyer, 2011, Bahi and Dreyer, 2013, Tapocik et al., 2014). Escalation of cocaine intake increases striatal miR-212 in rats; however, striatal overexpression of miR-212 reverses the cocaine-taking behavior through BDNF-CREB homeostatic mechanisms (Hollander et al., 2010, Im et al., 2010). Additionally, when miR-206 was overexpressed in the medial PFC (mPFC), after miR-206 was found to be upregulated in alcohol-dependent rats, miR-206 mimicked increased alcohol consumption in non-drug exposed rats via inhibition of BDNF expression (Tapocik et al., 2014). Thus, by identifying and targeting selective brain region-specific miRs that are activated by drugs of abuse, researchers can functionally alter the miR-mediated intracellular signaling events that underlie drug-mediated behaviors. Previous studies have shown that nicotine can activate miR expression patterns in both *in vitro* and *in vivo* models (Huang and Li, 2009, Lippi et al., 2011, Taki et al., 2014), although the behavioral implication of these identified nicotine-responsive miRs remain unknown.

Here we propose that prefrontal miRs may be responsible for the enrichment-mediated alterations in intracellular signaling in response to repeated nicotine

administration. Additionally, identified miRs may subsequently regulate the differences in the sensitivity to the locomotor effects of repeated nicotine in differentially reared rats. Upon defining nicotine-mediated miRs between EC, IC, and SC rats, we may better understand the upstream neurochemical correlates involved in how environmental factors regulate nicotine susceptibility. Therefore, the goal was to identify novel nicotine-mediated miRs that are differentially expressed in the PFC of EC, IC, and SC rats and to assess if the identified miRs are responsible for the enrichment-mediated neuroadaptations in intracellular signaling and the enrichment-mediated increase in locomotor sensitivity in response to nicotine exposure.

3.2 METHODS

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA). Rats arrived at the age of 21 days and were housed with food and water *ad libitum* in a colony room in the Division of Laboratory Animal Resources at the University of South Carolina. The colony room was maintained at 21 ± 2 °C, $50 \pm 10\%$ relative humidity on a 12-h light/dark cycle with lights on at 07:00 AM. All of the experimental procedures using animals were performed according to the National Institute of Health guidelines for AAALAC accredited facilities. The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina in compliance with animal welfare assurance.

Environmental conditions

Upon arrival at postnatal day 21, rats were randomly assigned to EC, IC, or SC groups. EC rats were group-housed (10-15 per cage) in a metal cage (120 cm length \times 60 cm width \times 45 cm height). Twelve hard non-chewable plastic objects were randomly placed in the cage. On a daily basis half of the objects were replaced with new objects, and the remaining objects were rearranged. IC rats were individually housed in wire mesh hanging cages (25 cm length \times 18 cm width \times 17 cm height) with solid metal sides and wire mesh floor. SC rats were pair-housed in a clear polycarbonate cage (43 cm \times 20 cm width \times 20 cm height) with a wire cage top. EC rats were handled each day as to change the novelty of the environment on a daily basis. IC and SC rats were neither handled nor exposed to any object except food and water; however, all rats were handled extensively throughout behavioral testing so that novelty and the number of cohorts were the only factors that differed among the groups throughout behavioral paradigms. The SC condition represents the standard housing conditions set in the NIH Guide for the 1996 version of the NIH *Guide for the Care and Use of Laboratory Animals*. Rats were raised in these conditions from 21 to 53 days of age and were maintained in these conditions throughout all experiments.

Nicotine administration and locomotor activity

Nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline (0.9% sodium chloride). The nicotine solution (freebase) was prepared immediately prior to injection and neutralized to pH 7.4 with NaOH to reduce irritation. Locomotor activity was assessed using Digipro System Software (v.140, AccuScan Instruments) to detect movement in 16 square (40 \times 40 cm) chambers

(Hamilton-Kinder Inc., Poway, CA). Movement was detected by infrared photocell interruptions; each chamber has 32 emitter/detector pairs capable of measuring horizontal and vertical (rearing) activity. Each beam was spaced 2.5 cm apart and 7.0 cm above the chamber floor. The chambers were converted into round (~ 40 cm diameter) compartments by adding clear Plexiglas inserts; photocell emitter/detector pairs were tuned by the manufacturer to handle the extra perspex width. Horizontal activity was measured as all beam breaks in the horizontal plane, and rearing activity was measured as all beam breaks in the vertical plane. All activity monitors were located in an isolated room that is separate from the animal colony.

Beginning at 54 days of age, all animals were habituated to the locomotor activity chambers for two 60 min sessions, once/day with no injection. Twenty-four hours after the second habituation session, all rats were habituated to the locomotor chambers for 30 min prior to injection, and then injected subcutaneously with saline and placed into the activity chambers for 60 min to measure baseline activity. The behavioral sensitization procedure began 24 h after the saline baseline measurement. All rats received a 30 min habituation period in the testing chamber prior to nicotine (0.35 mg/kg) or saline injection as reported previously. This was done so that the onset of nicotine's effects did not overlap with the period that rats showed the most exploratory behavior in the chamber, which was during the first 15 min (Harrod et al., 2008, Harrod and Van Horn, 2009). After the 30 min habituation session, rats were administered nicotine (0.35 mg/kg) or saline. Subsequently, horizontal and rearing activities were assessed during the subsequent 60 min session. Rats received saline or nicotine injections once/day for a total of 15 days; however, locomotor activities with regard to 30 min pre-injection

session and 60 min post-injection session were recorded every other day, i.e., on days 1, 3, 5, 7, 9, 11, 13, and 15. During the “off” days of locomotor testing, rats were still transported to the same room where rats were injected for locomotor testing and then returned to home cages after nicotine or saline injection. On day 16 after completion of the behavioral sensitization phase, all rats were injected with saline or nicotine and brains were removed by rapid decapitation 20 min after the last injection. Brain regions were dissected in a chilled matrix for further analyses.

RNA Isolation and miR expression profiling

Brain tissues (~30 mg) were placed in 1ml of RNeasy Lysis Buffer (Ambion, Grand Island, NY, USA) whereby total RNA isolation was performed using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and RNA Integrity Numbers (RIN) ranged from 8.1 to 9.4. RIN is a measure of RNA quality which ranges from 1 (degraded RNA) to 10 (intact RNA).

For the miRNA microarray, total RNA samples from the PFC were 3’-end labeled and miRNAs were purified using the Micro Bio-spin 6 columns from Bio-Rad (Hercules, CA, USA). Labeled miRNA samples were vacuum dried, resuspended in nuclease-free water, and hybridized to rat miRNA microarrays Release 16.0 (8x15K, #G4473B, Agilent, Santa Clara, CA, USA) at 55 °C for 20 h. Both labeling and hybridizations were performed using miRNA Complete Labeling and Hyb Kit (5190- 0456, Agilent, Santa Clara, CA, USA). Arrays were scanned using an Agilent Microarray Scanner System (G2565CA) and the data were extracted from images with the Feature Extractor Software version 10.7.3.1 (Agilent, Santa Clara, CA, USA). Data were uploaded into GeneSpring

GX 11.5.1, log base 2 transformed, and normalized using the shift to the 75th percentile algorithm. Additionally, the data were median baseline transformed. Quality control of the data using principal components and correlation analysis, as well as other metrics available in the software, resulted in the elimination of 1 sample from the EC-nicotine group and 1 sample from the IC-saline group. Subsequently, data was filtered based on expression. Only spots with signals over the 20th percentile in at least 66% of the samples in any given experimental condition were considered for further analysis. A cutoff value of 2 was used to determine differences in miRNA levels.

qPCR

To further confirm the microarray findings, qPCR analyses of miR expression were subsequently performed. Complementary DNA (cDNA) was first synthesized from the 24 isolated RNA samples of the EC, IC, and SC rats previously used for microarrays using miRCURY LNA *Universal* RT microRNA PCR Universal cDNA synthesis Kit (EXIQON, Woburn, MA, USA). Following cDNA synthesis, quantitative PCR analyses were performed using EXIQON mercury LNATM *Universal* RT microRNA PCR and SYB Green Master Mix systems, along with stock primers as well as specific primers: rno-miR-221 LNA PCR primer set UniRT designed to detect the target sequence AGCUACAUUGUCUGCUGGGUUC; rno-miR-483 LNATM PCR primer set UniRT designed to detect the target sequence CACUCCUCCCCUCCCGUCUUGU; and rno-miR-423-3p LNATM PCR primer set UniRT designed to detect the target sequence AGCUCGGUCUGAGGCCCCUCAGU. All reactions were normalized to miR-423-3p, a stably expressed candidate miR used as an endogenous reference gene (control). Comparisons among groups were performed using the method of $2^{-\Delta\Delta C_t}$ where the

threshold cycle is at a significant detectable increase in fluorescence. ΔCt values were calculated by subtracting the Ct value for the endogenous control (miR-423-3p) from the Ct value of the miR of interest (miR-221 or miR-483). The $\Delta\Delta\text{Ct}$ value was calculated by subtracting the ΔCt value of the control sample from the ΔCt value of the experimental sample.

Cell culture

PC12 cells (#CRL-1721.1, ATCC, Manassas, VA, USA) were maintained *in vitro* using high glucose DMEM (Life Technologies, Grand Island, NY, USA) supplemented with penicillin/streptomycin (100 U/ml), 2.5% bovine calf serum (Hyclone Thermo Scientific, Logan, UT, USA), 15% horse serum (Life Technologies, Grand Island, NY, USA), and 2 mM glutamine (Life Technologies, Grand Island, NY, USA). HEK-293FT cells (Life Technologies, Grand Island, NY, USA) were maintained using high glucose DMEM, penicillin/streptomycin (100 U/ml), 1% sodium pyruvate, 1% non-essential amino acids, and 2 mM glutamine. Cells were cultured at 37°C in a humidified 95% air / 5% CO₂ incubator. Medium was replaced every 3 days with passages taking place every 4 days.

Generation of lentiviral (LV) miR-221 overexpression in PC12 cells

Viral constructs were purchased from Systems Biosciences (SBI, Mountain View, CA, USA), and consisted of pCDH-CMV-rno-miR-1-EF1-copGFP and pCDH-CMV-rno-miR-221-EF1-copGFP. Expression plasmids containing full length miR-1 and miR-221 with green fluorescent protein (copGFP) reporter genes were under the control of two separate promoters: cytomegalovirus (CMV) and elongation factor-1 (EF1) promoters, respectively. MiR-1 was selected as a control for miR-221 as this specific

miR is found primarily within the mammalian heart (Lee and Ambros, 2001, Lagos-Quintana et al., 2002, Mishima et al., 2007), and to our knowledge has no functional significance in the brain. There is also additional evidence of miR-1 as a control for drug-induced behavior (Hollander et al., 2010). Plasmid DNA from the viral constructs was propagated using *One Shot Top 10* chemically competent *E.coli cells* (C4040, Life Technologies, Grand Island, NY, USA). A plasmid isolation kit (Qiagen, Valencia, CA, USA) was used to purify plasmid DNA and sequencing was confirmed by restriction enzyme mapping and DNA sequencing at the University of South Carolina EnGenCore facility. LVs were then produced in HEK 293T cells using a third generation packaging system (pMDLg/pRRE+pRSV-Rev+pVSV-G) as described previously (Shtutman et al., 2010). In brief, HEK-293FT cells were transfected with 5 µg of specific miR plasmid DNA, 1 ml 150 mM NaCl, 20 µl Δ8.91 + VSV-G mix, and 30 µl PEI-transfection reagent. Media containing virus particles (10 mL) were harvested 48-72 h post-transfection. Viral containing medium was then filtered through 0.45 µm PVDF filters and combined with PC12 cell medium. PC12 cells were subsequently incubated for 24 h and fresh media was replaced. GFP visualization was verified using an Olympus IX81 fluorescent microscope and confirmation of miR-221 overexpression was confirmed by qPCR.

Nicotine treatment and ERK activity in PC12 cells

To optimize the assay of miR-221 on nicotine-induced pERK1/2 activity in PC12 cells, concentration- and time-dependent assays on nicotine activation of pERK1/2 were performed, and the optimized concentration and time point for nicotine exposure that caused a maximal increase in pERK1/2 levels in PC12 cells (data not shown) was

determined. In a separate experiment, LV-miR-1 and LV-miR-221 transfected PC12 cells were plated in 6-well poly-d lysine coated plates at a density of $\sim 2-5 \times 10^6$ cells / well. Media was replaced with serum-free media for approximately 3 h before nicotine exposure. After nicotine exposure, cells were washed twice with ice-cold 1X PBS, scraped, and then collected. Cells were then sonicated and centrifuged for 15 min at 12,000 rpm at 4 °C. The supernatant was then collected for western blot analysis.

Intra-mPFC microinjection of LV-miR-221

After rats were reared in initial housing conditions, on postnatal day 54, rats were first anesthetized by intraperitoneal injections of ketamine (66 mg/kg), xylazine (1.33 mg/kg), and equithesin (0.5 ml/kg). Anesthetized rats were then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and received 4 infusions (1.5 μ l / infusion) into the PFC at the intended injection sites according to stereotaxic sites relative to bregma (AP +3.0 mm, ML \pm 0.6 mm, DV -2.6 mm from dura according to (Paxinos and Watson, 2007). Viral supernatant concentration ranged from 4 - 5 $\times 10^7$ infection units/ml. Two small bilateral holes were then drilled through the skull where a microinjector was then lowered to the most ventral injection site, and 1.5 μ l of viral supernatant was delivered over 5 minutes (0.3 μ L / min). After the injection, the injector was left in place for an additional 5 minutes. The microinjector was then raised 1.0 mm to the most dorsal injection site and repeated. The two microinjections were repeated on the contralateral hemisphere. Upon completion of all injections, the drill holes were filled with bone wax and the scalp was sutured. Rats were given 7 days of recovery time, whereby antibiotic ointment was applied over the incision site for the first 3 days. After recovery, rats started the locomotor behavioral experiments as described in section 3.4.

LV placement and GFP confirmation

Upon completion of nicotine-mediated locomotor sensitization, brains were flash frozen in isopentane for ~ 20 sec and stored at -80°C for further analyses. The PFC was then sectioned coronally on a cryostat in 35 µm slices and placed onto slides. Slices were then analyzed for GFP verification using an Olympus IX81 fluorescent microscope.

Western blot analysis

Rats were sacrificed by rapid decapitation and brain regions were dissected in a chilled matrix and sonicated immediately on ice in a homogenization buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 0.4 M NaCl, 5 mM MgCl₂, 20% glycerol, 1 mM PMSF, phosphatase inhibitor cocktails I (P2850, Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors (P8340, Sigma-Aldrich, St. Louis, MO, USA). Homogenates were then centrifuged at 12,000 g for 15 min at 4°C. The supernatants were collected and stored at -80°C. Protein concentrations were determined in triplicate using Bio-Rad DC protein detection reagent. In brief, proteins (30 µg per PFC tissue samples) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes that were transferred with proteins were preincubated with blocking buffer (5% dry milk powder in PBS containing 0.5% Tween-20) and then incubated overnight at 4 °C in blocking buffer with primary antibodies: total ERK1/2 (1:5000, V114A) from Promega (Madison, WI, USA), and pERK1/2 (1:1000, SC-16982R) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membranes were washed and then incubated in blocking buffer containing secondary affinity-purified, horseradish peroxidase-conjugated anti-rabbit IgG; (1:20,000) from Jackson ImmunoResearch (West Grove, PA, USA) for total ERK1/2 and 1:5000 for pERK1/2.

Immunoblots were detected using enhanced chemiluminescence (ECL-plus) and developed on Hyperfilm (Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). After detection and quantification of these proteins, each blot was stripped in a Re-blot plus mild antibody stripping solution (CHEMICON, Temecula, CA, USA) and reprobbed for detection of β -tubulin (1:5000; H-235, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) to monitor protein loading among samples. Multiple autoradiographs were obtained using different exposure times, and immunoreactive bands within the linear range of detection were quantified by densitometric scanning using Scion image software (Scion Corp., Frederick, MD, USA).

Data analyses

Data are presented as mean \pm standard error of the mean (SEM), and n represents the number of independent experiments or subjects for each group. Data from microarray and quantitative PCR were analyzed by a 3×2 factorial analysis of variance (ANOVA) with housing condition (EC, IC or SC) and treatment (saline or nicotine) as between-subjects factors. One-way ANOVAs were used to evaluate data from RT-PCR confirmation analysis of miR-221 in PC12 cells along with concentration- and time-dependent effects of nicotine on pERK1/2 and ERK1/2 levels in PC12 cells. Mixed-factors ANOVAs were conducted on EC, IC, and SC groups in the nicotine-mediated locomotor sensitization experiments where groups (LV-miR-1 Sal, LV-miR-1 Nic, and LV-miR-221 Nic) were the between-subject factors and day (1, 3, 5, 9,11,13,and 15) were the within-subjects factors. To determine the effects of miR-221 on nicotine-mediated pERK1/2 levels, one-way ANOVAs on pERK1 and pERK2 levels were performed within each housing condition (EC, IC, and SC). Simple effect comparisons

(Bonferroni and Tukey) were conducted between groups where appropriate. All statistical analyses were performed using IBM SPSS Statistics version 20, and α level was set at $p < 0.05$ for all analyses.

3.3 RESULTS

Repeated nicotine upregulated miR-221 and miR-483 in the PFC of EC rats

To investigate the molecular basis for environmental enrichment-induced behavioral changes in response to repeated injections of nicotine, we identified the expression levels of miRs in RNA samples of the PFC from EC, IC, and SC rats treated repeatedly with nicotine or saline using a miR microarray. As shown in Fig. 3.2A, two-way ANOVA by GeneSpring revealed that a robust increase in the expression levels of six miRs (miRs-150, 202, 330, 380, 221 and 483) in the PFC were robustly increased in EC rats relative to IC and SC rats following repeated nicotine injection, indicating that these miRs may be involved in enrichment-induced neurobiological adaptations in response to nicotine.

Using qPCR, we further confirmed the expression levels of miR-221 and miR-483 in the PFC, NAc and striatum of EC, IC and SC rats. For miR-221 (Fig. 3.2B, C; Table 3.1), separate two-way ANOVA revealed a main effect of housing condition ($F_{(2, 18)} = 6.4, p < 0.05$) and treatment ($F_{(1, 18)} = 5.8, p < 0.05$), as well as a significant interaction between housing \times treatment ($F_{(2, 18)} = 4.2, p < 0.05$). Simple effect analyses revealed that repeated nicotine increased miR-221 level in EC rats ($170 \pm 20\%$, $p < 0.001$) and IC rats ($66 \pm 7\%$, $p < 0.05$), but not in SC rats relative to their respective saline controls, whereas no difference in miR-221 level was found among EC, IC and SC saline control rats.

Similarly, with regard to the expression level of miR-483 in the PFC (Table 3.1), a significant housing condition \times treatment interaction ($F_{(2, 18)} = 3.7, p < 0.05$) was found; however, neither the main effect of housing condition nor treatment was significant. Simple effect analysis revealed that repeated nicotine increased miR-483 level ($278 \pm 89\%, p < 0.001$) in EC rats but not in IC and SC rats compared with the respective saline controls. In contrast, there were no differences in expression levels of miR-221 or miR-483 among EC, IC and SC rats in response to nicotine or saline injection in the NAc and striatum, suggesting that nicotine-mediated upregulation of these miRs are expressed in a region-specific manner.

LV-miR-221 diminished nicotine-induced pERK1/2 increases in vitro

MiR-221 has been predicted to target multiple targets within the ERK1/2 signaling cascade, and its expression is highly responsive to activation of ERK1/2 activity (Terasawa et al., 2009, Hamada et al., 2012, Garofalo et al., 2009, Zhang et al., 2013). Nicotine has been shown to stimulate phosphorylation of ERK1/2 *in vitro* (Nakayama et al., 2001) and *in vivo* (Midde et al., 2011). To determine whether miR-221 is involved in the enriched environment-induced increase in the locomotor response to nicotine, we employed a LV-based transfection technique to determine whether overexpression of miR-221 regulates nicotine-mediated ERK1/2 activity in PC12 cells. First we confirmed the LV-miR overexpression in PC12 cells by fluorescent microscopy and miR qPCR. Figure 3.3 (A) illustrates both phase contrast and GFP expression in PC12 cells transfected with either LV-pmiR-1 (LV-miR-1) or LV-miR-221. Results from miR quantitative PCR showed that the expression level of miR-221 was significantly

increased in LV-miR-221 transfected cells ($F_{(2,6)} = 10.8, p < 0.05$) relative to cells without transfection and LV-pmiR-1 transfected cells (Fig. 3.3C).

Based on the concentration- and time-dependent studies for effects of nicotine on levels of pERK1/2 and total ERK1/2 in PC12 cells (data not shown), the optimized nicotine concentrations (1 and 100 μM) and exposure time (10 min) were chosen, which were also reported in previous studies (Steiner et al., 2007, Nakayama et al., 2001). Next, we determined whether overexpression of miR-221 suppresses the nicotine-mediated phosphorylated ERK1/2 levels in PC12 cells (Fig. 3.3B). As shown in Fig. 3.3D, a two-way ANOVA on pERK1 and pERK2 levels revealed a main effect of both LV condition (pERK1: $F_{(1,18)} = 27.0, p < 0.05$ and pERK2: $F_{(1,18)} = 6.47, p < 0.05$), treatment (pERK1: $F_{(2,18)} = 50.0, p < 0.05$ and pERK2: $F_{(2,18)} = 51.6, p < 0.05$), and a significant interaction of LV condition \times treatment (pERK1: $F_{(2,18)} = 3.7, p < 0.05$). No difference in pERK1/2 levels between LV-miR-1 and LV-miR-221 was found. Exposure to nicotine (1 or 100 μM) increased the levels of pERK1/2 in both LV-miR-1 and LV-miR-221, whereas the levels of pERK1 and pERK2 were lower in LV-miR-221 than LV-miR-1 ($p < 0.001$, Tukeys *post hoc*), suggesting that overexpression of miR-221 attenuates nicotine-induced increase in ERK1/2 activity.

LV-miR-221 in the mPFC further enhances acquisition of nicotine-mediated locomotor sensitivity in IC rats

We have previously found that EC rats have a lower baseline of locomotor activity, in addition to decreased nicotine-induced hyperactivity when compared to IC and SC rats. However, when nicotine-induced hyperactivity in differentially reared rats are expressed from baseline, EC rats actually show increased locomotor sensitivity in

response to acute and repeated nicotine in comparison to IC and SC rats (Gomez et al., 2012). To determine whether miR-221 is involved in the differential enriched environment-induced locomotor response to nicotine, we determined whether miR-221 overexpression in the mPFC alters the nicotine-mediated locomotor response in EC, IC, and SC rats. The mPFC was selected due to its profound role in goal-directed actions, impulsivity, and reward processing (Perry et al., 2011). First, after behavioral testing, we confirmed the viability of *in vivo* LV miR overexpression within the medial portions of the PFC by fluorescent microscopy and miR qPCR. Figure 3.5A and B illustrates coronal sections for the injection sites and GFP expression in the PFC of rats that received LV injections. Results from miR qPCR showed that the expression level of miR-221 was significantly increased in rats that received LV-miR-221 injections ($t_{(40)} = 4.44$, $p < 0.001$) in comparison to rats that received LV-miR-1 injections into the mPFC (Fig. 3.5C).

We then extended *in vivo* miR-221 overexpression within the mPFC of EC, IC, and SC rats to our nicotine-mediated locomotor sensitization paradigm. One week after receiving LV injections, all animals were placed into locomotor chambers for two 60 min habituation sessions to produce within-session habituation of activity in response to the context prior to nicotine or saline injection (data not shown). We should mention that LV-miR-221 Sal group was absent from the current study for two reasons: 1.) if miR-221 has an effect on basal locomotor levels, we would observe this in the LV-miR-221 Nic groups during the habituation, saline baseline, and 30 min pre-injection phases of the behavioral experiment, and 2.) LV-miR-221 in PC12 cells had no effect on basal pERK1/2 levels. Separate two-way ANOVAs with repeated measures (group \times day) revealed no differences across day 1 or day 2 of habituation between LV-miR-1 or LV-

miR-221 rats within EC, IC, or SC groups. However, all EC rats had decreased basal levels in comparison to IC or SC rats ($F_{(1, 2)} = 39.5, p < 0.001$) Furthermore, no differences within any of the EC, IC, or SC groups were found in the third day of locomotor testing during the 30 min pre-injection or the 60 min post-injection saline baseline sessions. However, EC rats were shown to have significantly lower basal levels of locomotor activity during the 30 min pre-injection saline baseline session ($F_{(2, 39)} = 26.0, p < 0.001$) and the 60 min post-injection saline baseline session ($F_{(2, 39)} = 32.97, p < 0.001$) in comparison to IC and SC rats. This data suggests that overexpression of miR-221 in the mPFC has no effect on basal locomotor levels; which is also in agreement with our previous findings demonstrating that EC rats exhibit decreased basal locomotor levels compared to IC and SC rats (Gomez et al., 2012).

All animals were then placed into locomotor chambers for 30 min prior to saline or nicotine injections across the 15-day treatment (Figs 3.6A-C). Corresponding to the habituation and saline baseline sessions, a two-way, repeated measures ANOVA (group \times time) revealed that there were no differences within EC, IC, or SC groups, although basal locomotor levels of EC rats were significantly lower than those in IC or SC rats ($F_{(1, 2)} = 89.3, p < 0.001$).

To determine whether overexpression of miR-221 in the mPFC alters nicotine-mediated locomotor sensitization within housing conditions, we analyzed locomotor activity during daily administration of nicotine (0.35 mg/kg) or saline during Days 1-15 in EC, IC, and SC rats (Figs. 3.6D-F). Separate two-way ANOVAs with repeated measures (group \times time) revealed that repeated nicotine produced locomotor sensitization in LV-miR-1 and LV-miR-221 treated EC ($F_{(1, 2)} = 71.6, p < 0.001$), IC ($F_{(1, 2)} = 34.4, p <$

0.001), and SC ($F_{(1,2)} = 4.3, p < 0.05$) rats. Furthermore, analyses for nicotine-treated groups within each housing condition revealed that LV-miR-221 IC rats showed a trend for greater sensitivity in response to nicotine than did LV-miR-1 IC rats ($F_{(1,1)} = 3.9, p = 0.76$). *Post hoc* analyses further revealed that LV-miR-221 IC rats had significantly higher locomotor levels in response to nicotine during days 3, 5, and 9 in comparison to LV-miR-1 nicotine-treated IC rats (Tukeys *post hoc*, $p < 0.05$). There was also a trend for significance on day 7 (Tukeys *t-test*, $p = .16$). After day 9 of nicotine injections, the increased locomotor sensitivity in LV-miR-221 IC rats started to diminish to a point where the locomotor activity of LV-miR-221 IC rats resembled the locomotor levels observed within LV-miR-1 IC rats. No differences were found between nicotine-treated LV-miR-1 EC and LV-miR-221 EC rats; as well as nicotine-treated LV-miR-1 SC and LV-miR-221 SC rats. This suggests that miR-221 plays a role in the expression of increased locomotor sensitivity to nicotine seen in IC rats, and potentially responsible for the EC-induced increases in locomotor sensitivity in response to repeated nicotine.

LV- miR-221 in the mPFC attenuates nicotine-induced pERK1 increases in IC rats

Our previous work has determined that IC rats exhibit increases in pERK1/2 levels in response to nicotine, whereas EC rats display an attenuated nicotine-mediated pERK1/2 response. We next determined if overexpression of miR-221 affects the phosphorylation state of ERK1/2 in EC, IC, and SC rats that underwent nicotine-mediated locomotor sensitization (Fig. 3.7D-F). A one-way ANOVA for groups on pERK1 and pERK2 levels within each housing condition revealed no differences in pERK1 or pERK2 levels in EC or SC housing groups. However, a significant effect for pERK1 ($F_{(1,2)} = 5.4, p < 0.05$) levels were found in IC groups, though a significant effect

of pERK2 levels were not found among IC rats. In agreement with our previous study, LV-miR-1 EC rats displayed an attenuated pERK1/2 in response to repeated nicotine. Additionally, LV-miR-221 in EC rats failed to have an effect on nicotine-mediated pERK1 or pERK2 levels. Further analyses for *Tukeys* multiple comparisons did reveal that nicotine increased pERK1 in LV-miR-1 IC rats ($t_{(10)} = 2.33$, $p < 0.05$), however LV-miR-221 IC rats significantly attenuated nicotine-mediated pERK1 increases in comparison to nicotine-treated LV-miR-1 IC rats ($t_{(10)} = 3.60$, $p < 0.05$). There were no differences found within IC groups for pERK2 levels. Moreover, no differences were found within any of the SC groups with regards to pERK1 or pERK2 levels. As well, there were no significant differences found with either total ERK1 or total ERK2 within any of the EC, IC, or SC groups. These data suggests that overexpression of miR-221 in IC rats diminishes nicotine-mediated pERK1 levels which may be responsible for the increased nicotine-mediated locomotor sensitivity observed in LV-miR-221 IC rats.

3.4 DISCUSSION

Our previous studies have demonstrated that the PFC represents a key brain region in enrichment dependent neuroadaptations, which may contribute to the altered behavioral and molecular responsiveness to nicotine stimulation (Zhu et al., 2007a, Gomez et al., 2012). In particular, we have previously shown that EC rats have an attenuated nicotine-induced pERK1/2 response, which may be mediating increased locomotor sensitivity in EC rats in response to repeated nicotine administration. In the current study, repeated nicotine administration upregulated six distinct miRs, specifically miR-221, within the PFC of EC rats but not in IC or SC rats; indicating that expression of

these miRs may be involved in the enrichment-induced neuroadaptations in response to nicotine. Overexpression of miR-221 *in vitro* diminished nicotine-induced increases in pERK1/2 activity, potentially supporting the notion that EC-induced attenuation of nicotine-induced pERK1/2 increases are mediated by miR-221. Injection of LV-miR-221 into the mPFC of EC, IC, and SC rats further enhanced nicotine-mediated locomotor sensitivity in IC rats alone. Additionally, in IC rats, LV-miR-221 attenuated prefrontal nicotine-mediated pERK1/2 increases. Ultimately, these findings suggest that miR-221 may mediate the increased locomotor sensitivity in response to repeated nicotine in EC rats by attenuating nicotine-induced pERK1/2 enhancements within the mPFC.

MiRs are becoming increasingly important in the regulation of synaptic-dependent plasticity underlying drug-mediated behaviors (Chandrasekar and Dreyer, 2011, Hollander et al., 2010, Im et al., 2010, Tapocik et al., 2014, Bahi and Dreyer, 2013). Due to our previous studies suggesting that the PFC represents a critical area involved in enrichment-dependent neuroadaptations involved in nicotine-mediated behavior (Zhu et al., 2007a, Gomez et al., 2012), we profiled prefrontal miR expression in EC, IC, and SC after receiving repeated nicotine injections. Our initial miR screening found 6 significant upregulated miRs, most notably miR-221 and miR-483, specifically in the PFC of EC rats in response to repeated nicotine exposure. These findings add to a new, growing body of literature illustrating that miRs are highly responsive to nicotine exposure; although the degree to which they are expressed are strikingly attributed to a variety of factors. The 6 significant miRs (miR-150, 202, 221, 330, 380, 483) found in the microarray screen appear to be distinctive to our study. Though, this may be due to the fact that the focus of the current study was not directly related to miR expression in

response to repeated nicotine administration, but rather to find distinctive nicotine-induced miR(s) between differentially reared rats.

Acute nicotine treatment (100 μ M) in PC12 cells either upregulates or downregulates 25 miRs, most notably miR-140* (Huang and Li, 2009). In a separate study, a distinct subset of 32 miRs was found to be differentially expressed in brains regions of mice treated repeatedly with nicotine (Lippi et al., 2011). Of note, these miRs were also differentially expressed in response to repeated cocaine or amphetamine, suggesting miR expression is both drug- and brain-specific. Moreover, miR expression may be related to dose-specific effects of nicotine. Acute nicotine treatment with a low (20 μ M) and high dose (200 μ M) in *C. elegans* significantly altered 40 miRs non-related to our current study. The low and high dose of nicotine also differentially altered not only the miRs themselves, but also the amount of fold change between the miRs (Taki et al., 2014). This may explain why we failed to find as robust of a miR-221 increase in rats repeatedly treated with a high dose of nicotine (0.6 mg/kg, s.c.) or in a nicotine self-administration paradigm (figure 5.3). We have previously shown that enrichment blunts nicotine intake in comparison to IC rats, and when we tested the PFC via qPCR, miR-221 was significantly increased in EC rats undergoing nicotine self-administration, although the increases were minimal, suggesting the amount of nicotine intake may impact miR expression. Thus, the expression of miRs appears to be dependent upon drug-, dose-, and brain-region specificity. Future studies profiling miR expression patterns in relevant brain regions associated with nicotine dependence in differentially reared rats, in addition to varying the nicotine dose, would be of particular interest to further determine the specificity of these miRs within this animal model.

MiR-221 was the focus for our follow-up studies as miR-221 has been strongly linked with the ERK1/2 signaling cascade. We have previously shown that EC rats have higher basal levels of pERK1/2 compared to IC and SC rats. Interestingly when rats underwent repeated nicotine administration, IC rats have increased pERK1/2 activation whereas EC rats display an attenuated nicotine-induced ERK1/2 response. Thus, these data insinuate that miR-221 may potentially be involved in the enrichment-dependent regulation of the ERK1/2 signaling cascade. In fact, bioinformatics and pathway analyses predicts miR-221 to target multiple proteins in the ERK1/2 signaling pathway; particularly MAPK isoforms MAPK6, MAPK10, and MAP3K14 which regulate the phosphorylation states of ERK1/2. Several growth factors including neurotrophin 3/4 (NT3/4), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) that signal to downstream ERK1/2 were also predicted to be targeted by miR-221. MiR-221 has also been predicted to target calcium/ calmodulin-dependent protein kinase II (CAMKII), a kinase that has been shown to be required for nicotine-induced activation of pERK1/2 (Steiner et al., 2007, Schmitt et al., 2005); however, preliminary studies (not shown) from our lab suggest miR-221 does not affect CAMKII protein levels expression specifically within EC or IC rats. Adapting the *in vitro* model implemented in the current work and assessing CAMKII activity may be better suited to fully assess the functional relevance of miR-221 and CAMKII. There have also been numerous *in vitro* reports linking miR-221 with upstream and downstream proteins involved in the ERK1/2 signaling pathway. Transient expression of miR-221 in endothelial progenitor cells (EPCs) reduced phosphorylation of RAF, MEK, and ERK1/2 by targeting and decreasing the expression of p21-activated kinase 1/2 (PAK1/2) (Zhang et al., 2013). In opposition,

miR-221 forced expression in H460 cells increased pERK1/2, pAkt, and PAK1 (Garofalo et al., 2009). Interestingly, nicotine has been shown to upregulate both ERK1/2 and PAK1/2 activity in primary human fibroblasts (Fang and Svoboda, 2005), suggesting PAK1/2 as a potential target of miR-221 in our model. MiRs have negative feedback loops which correspond to a report showing that in PC12 cells NGF-stimulated sustained activation of ERK1/2 increases miR-221 production (Terasawa et al., 2009). Additionally, the expression of miR-221 appears to be under positive control of the Ras-MAPK pathway (Cardinali et al., 2009). Although caution should be taken in extending these findings across cell lines and to *in vivo* models, there does appear to be strong evidence for homeostatic mechanisms between miR-221 expression and the phosphorylated states of ERK1/2, though the exact mechanism for these regulatory processes remains unclear.

Indeed, our results did establish a link between miR-221 and ERK1/2 activity, but this was found only in the presence of nicotine. We found that miR-221 diminished nicotine-induced pERK1/2 levels in PC12 cells, however, had no effect on the basal levels of pERK1/2 suggesting that this miR may be specific for nicotine-induced activation of pERK1/2. Acute and repeated nicotine has been shown to increase pERK1/2 levels both *in vitro* and *in vivo* (Nakayama et al., 2001, Valjent et al., 2004a). ERK1/2 integrates signals derived from extracellular stimuli to signal to immediate early genes and transcription factors to control gene expression. The fact that ERK1/2 is critical for the neuroadaptive response to nicotine stimulation supports the notion that miR-221 may alleviate potential neuroadaptations induced by nicotine exposure. Interestingly, miR-221 diminishes the nicotine enhancement more so in pERK1 than pERK2. ERK1 and ERK2

isoforms overlap considerably in both structure and function, however, ERK1-deficient mice have an enhanced response to sensitization and CPP to cocaine and morphine (Mazzucchelli et al., 2002, Ferguson et al., 2006) as well as further increasing ERK2-dependent downstream signaling (Ferguson et al., 2006). These findings emphasize ERK2 as the “more important” isoform; however, whether this extends to nicotine’s effects remains unknown. Nevertheless, the current results also provide a potential mechanism for the attenuation in nicotine-induced pERK1/2 increases we have previously observed in the PFC of EC rats.

Inhibition of ERK activation by systemic administration of SL327 and site-specific infusion of U0126, selective MEK inhibitors, attenuates cocaine- and D-amphetamine-mediated motivated behaviors (Valjent et al., 2006b, Shi and McGinty, 2006, Valjent et al., 2006a, Miller and Marshall, 2005), indicating a critical role for ERK1/2 in the behavioral adaptations produced by psychostimulants. Therefore, we aimed to determine if the diminished nicotine-induced pERK1/2 increases mediated by miR-221 translates to the differentially rearing-induced alterations in nicotine-mediated locomotor sensitization. EC rats display increased hyperactivity to acute and repeated nicotine administration in comparison to IC and SC rats when expressed from baseline measurements (Gomez 2012). We hypothesized that repeated nicotine would upregulate miR-221 in EC rats and subsequently alter nicotine-mediated hyperactivity by attenuating pERK1/2 levels in the mPFC. Interestingly, we found that LV-miR-221 overexpression into the mPFC further enhanced the acquisition of nicotine-mediated locomotor sensitization in IC rats only, while LV-miR-221 had no effect on basal locomotor levels. The behavioral adaptation in IC rats corresponded to the attenuating effect of LV-

miRNA-221 on nicotine-mediated pERK1 levels in the mPFC, which is consistent with the previous findings with regard to the *in vitro* effect of LV-miRNA-221 on nicotine-induced pERK1 levels. Expectedly, due to the high levels of miR-221 observed in EC rats in response to repeated nicotine during our initial microarray screen, we did not see an effect of medial prefrontal overexpression of LV-miR-221 on locomotor hyperactivity. MiR-221 also failed to further decrease the attenuated nicotine-induced pERK1/2 levels in EC rats, although this may be due to the fact that pERK1/2 are already upregulated basally. This suggests that endogenous miR-221 within EC rats may be functioning at its maximum level thereby eliminating the potential effect of LV-miR-221 overexpression. Future studies involving a knock down of miR-221 in EC rats to observe if there is a reversal of increased locomotor sensitivity in response to repeated nicotine would better help clarify this point. Following this study up by using siRNA technology to knock down miR-221 expression in the mPFC in EC rats, and observing if EC rats will then show decreased sensitivity to repeated nicotine administration would be immensely beneficial in implicating miR-221 as the main determinant underlying the behavioral sensitivity to repeated nicotine administration. Unexpectedly, we found that LV-miR-221 in the mPFC of SC rats was unsuccessful at altering the locomotor effects of repeated nicotine, and did not affect nicotine-mediated pERK1/2 levels. One possible explanation may be that nicotine failed to induce pERK1/2 activation within the PFC of LV-miR-1 treated SC rats, thereby miR-221 was void of having any effect. And, even though IC and SC rats exhibit similar behavioral responses to nicotine, we have previously seen that these two groups are distinct in how they respond to nicotine from an intracellular signaling viewpoint (Gomez et al., 2012), suggesting miR-221 may not have the same

effect in IC and SC rats as we had originally anticipated. Another interpretation may be due to the evidence that, although we did not see miR-221 differences in IC nicotine treated rats in the microarray, we did find a significant increase in miR-221 in the more sensitive approach of qPCR. Thus, there may be a threshold that miR-221 levels have to reach to induce a behavioral effect. In this fashion, LV overexpression in IC rats allows miR-221 levels to reach this threshold while LV overexpression in SC rats failed to meet this threshold. However, this seems unlikely as qPCR and GFP expression for LV-miR-221 confirmed an upregulation in SC rats.

The PFC has a modulatory role in nicotine-mediated motivated behaviors (Rezvani et al., 2008) by controlling NAc and VTA output generated by drugs of abuse (Grace et al., 2007). Lesions of the PFC by 6-hydroxydopamine increases nicotine-mediated hyperactivity and significantly alters nicotine self-administration in male and female rats (Rezvani et al., 2008), implicating that neuroadaptations within the PFC influences the behavioral response to nicotine. Therefore, it seems plausible that miR-221 attenuates certain nicotine-induced neuroadaptations within the PFC responsible for controlling the locomotor sensitivity to nicotine. In this manner, it appears that enrichment allows miR-221 to be upregulated in response to nicotine in the mPFC, which in turn, attenuates nicotine-mediated pERK1/2 levels in the mPFC to modulate the increased nicotine-mediated locomotor sensitivity.

Overall, the current studies implicate a highly unique role of miR-221 within the mPFC as a mediator of enrichment-induced increased sensitivity in nicotine-mediated hyperactivity. These findings also support the possibility that an enriched environment engages a novel regulatory circuit within the mPFC in which upregulation of miR-221

expression inhibits ERK signaling in response to repeated nicotine treatment. However, future studies will be essential in characterizing: 1) the mechanisms underlying brain region specific miR-221 upregulation in response to nicotine within EC rats, and 2) the specific target(s) of miR-221 which mediate an attenuation in nicotine-mediated pERK1/2 activity. Ultimately, the present study demonstrates that rearing environment has remarkable effects in altering the downstream signaling mechanisms that mediate nicotine-mediated behavior, and further provide a role for the implication of miRs in mediating aspects of nicotine addiction.

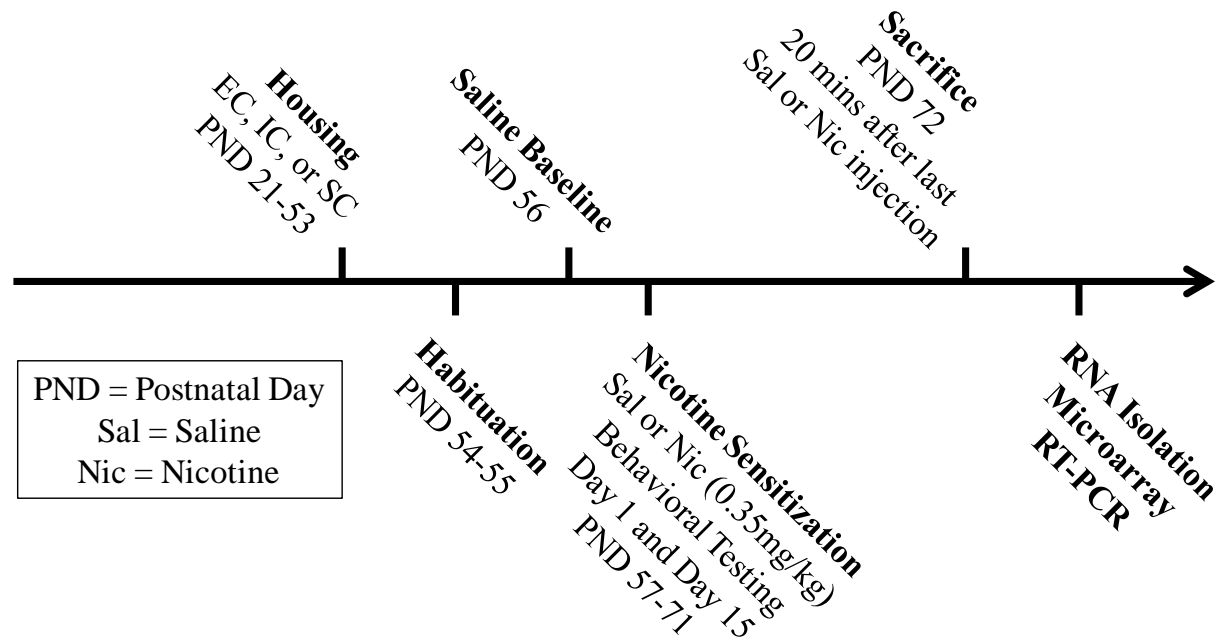


Figure 3.1. Timeline of the experimental paradigm starting on postnatal day 21 of EC, IC, and SC rats used for the identification of differentially expressed miRs via microarray in response to repeated.

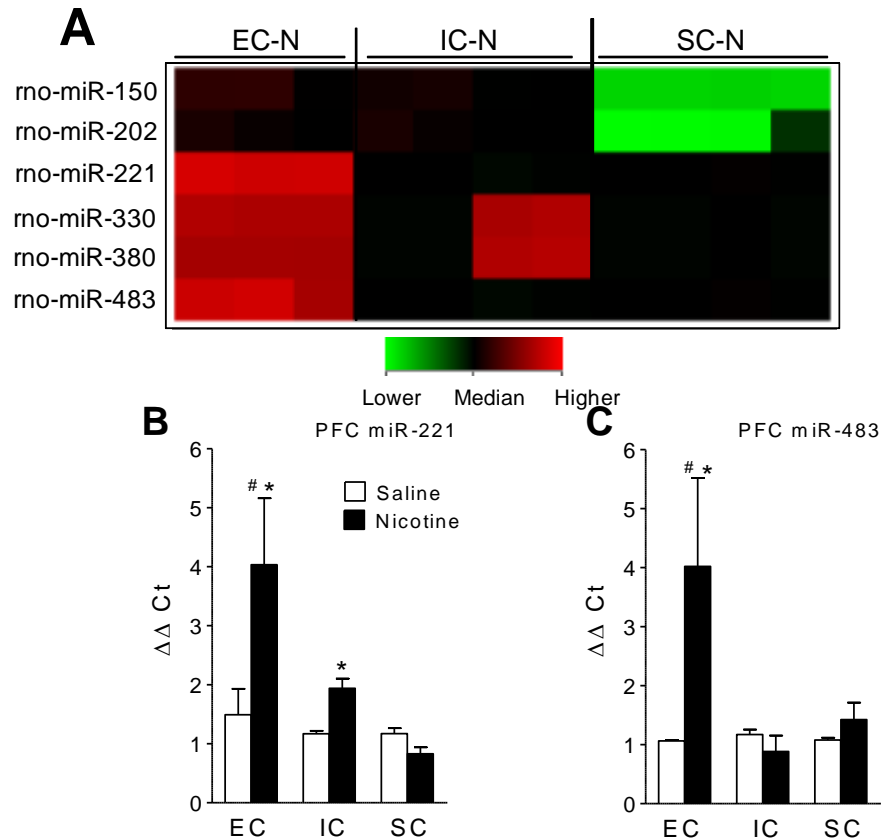


Figure 3.2. Environmental enrichment increases expression of specific miRNAs in the prefrontal cortex (PFC) of rats in response to repeated administration of nicotine. (A) Expression analysis using Agilent GeneSpring shows a significant increase in specific miRNAs in the PFC of EC, IC, and SC rats treated repeatedly with nicotine (N; 0.35 mg/kg, s.c.). Data are expressed as mean \pm SEM of log₂ normalized intensity values. Column #: 1-3, EC rats with nicotine treatment (EC-N); 4-7, IC rats with nicotine treatment (IC-N); 8-11, SC rats with nicotine treatment (SC-N). Rows display a list of 6 miRNAs that were found to be significant among groups with over 2-fold expression. (B) qPCR validation of miR-221 and miR-483 from the PFC of EC, IC, and SC rats undergoing repeated saline or nicotine injections. $\Delta\Delta$ Ct value is calculated by subtracting the Δ Ct value of the control sample (miR-423-3p) from the Δ Ct of miR-221 or miR-483. Data expressed as mean \pm SEM. * p < 0.05 denotes difference between the nicotine- and saline-treated groups. # p < 0.05 denotes difference in nicotine-treated groups among EC, IC and SC rats.

Table 3.1. $\Delta\Delta C_t$ values for miR-221 and miR-483 in different brain regions of EC, IC, and SC rats after repeated saline or nicotine administration (0.35 mg/kg for 16 days)

* $p < 0.05$ denotes difference within rearing groups between the nicotine- and saline-treated groups. # $p < 0.05$ denotes difference between housing groups. n=4 rats/group.

miR-221						
Brain region	EC-Sal	EC-Nic	IC-Sal	IC-Nic	SC-Sal	SC-Nic
PFC	1.49 ± 0.44	4.03 ± 1.13*#	1.17 ± 0.05	1.94 ± 0.16*	1.17 ± 0.09	0.83 ± 0.11
NAc	1.13 ± 0.08	1.64 ± 0.09	1.24 ± 0.09	1.04 ± 0.37	1.14 ± 0.07	0.98 ± 0.06
STR	1.04 ± 0.01	0.61 ± 0.14	1.20 ± 0.16	1.15 ± 0.40	1.09 ± 0.06	1.14 ± 0.17
miR-483						
Brain region	EC-Sal	EC-Nic	IC-Sal	IC-Nic	SC-Sal	SC-Nic
PFC	1.07 ± 0.02	4.02 ± 1.50*#	1.17 ± 0.09	0.89 ± 0.27	1.08 ± 0.04	1.43 ± 0.29
NAc	1.06 ± 0.05	1.01 ± 0.28	1.17 ± 0.09	1.12 ± 0.45	1.18 ± 0.08	1.02 ± 0.14
STR	1.03 ± 0.01	0.76 ± 0.21	1.17 ± 0.06	1.78 ± 0.45	1.19 ± 0.11	2.48 ± 1.00

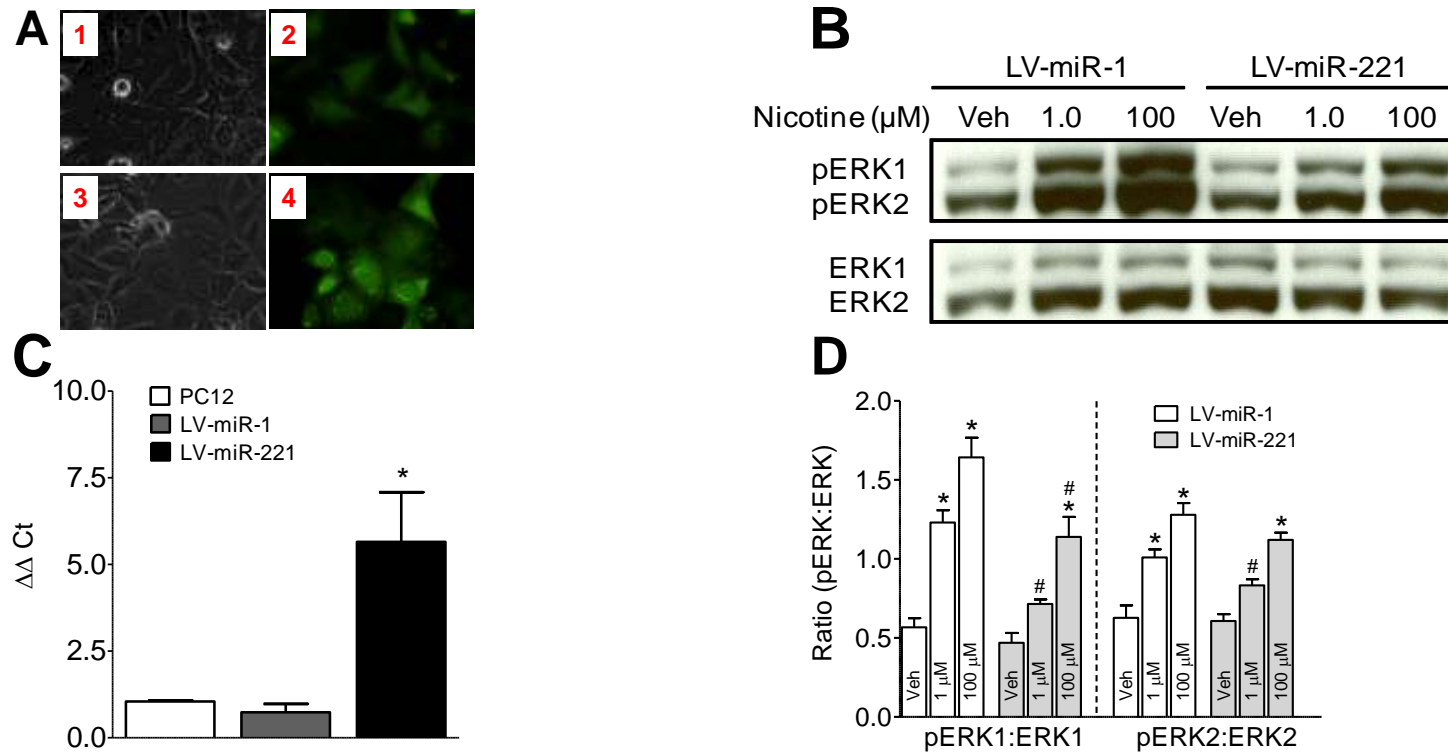


Figure 3.3. (A) Confirmation of LV-miR-221 expression in PC12 cells: Image (40X) of phase contrast (1 and 3) and GFP expression (2 and 4) in PC12 cells transfected with LV-miR-1 (1 and 2) or LV-miR-221 (3 and 4), respectively. (C) qPCR verification of increased miR-221 levels in PC12 cells untransfected, transfected with LV-miR-1, or transfected with LV-miR-221. PC12 cells without transfection were used as a negative control. $\Delta\Delta Ct$ values were calculated by subtracting the ΔCt values of miR-423-3p from the ΔCt of miR-221. * $p < 0.001$ different from PC12 cells alone. Transfected cells were first exposed to varying nicotine doses (1 μM or 100 μM) or vehicle (Veh) for 10 min. and then pERK1/2 levels were determined. (B) Representative immunoblots of pERK1/2 and ERK1/2 in LV-miR-1 or LV-miR-221 transfected cells following exposure to nicotine or Veh. (D) The ratio of pERK1/2 levels to total ERK1/2 in corresponding PC12 cell groups. Data are presented as the percentage of pERK1/2 to total ERK1/2 densitometry values. Histograms represent means and error bars represent SEM of four independent experiments. * $p < 0.05$ compared to the respective vehicle controls. # $p < 0.05$ compared to the respective LV-miR-1 group.

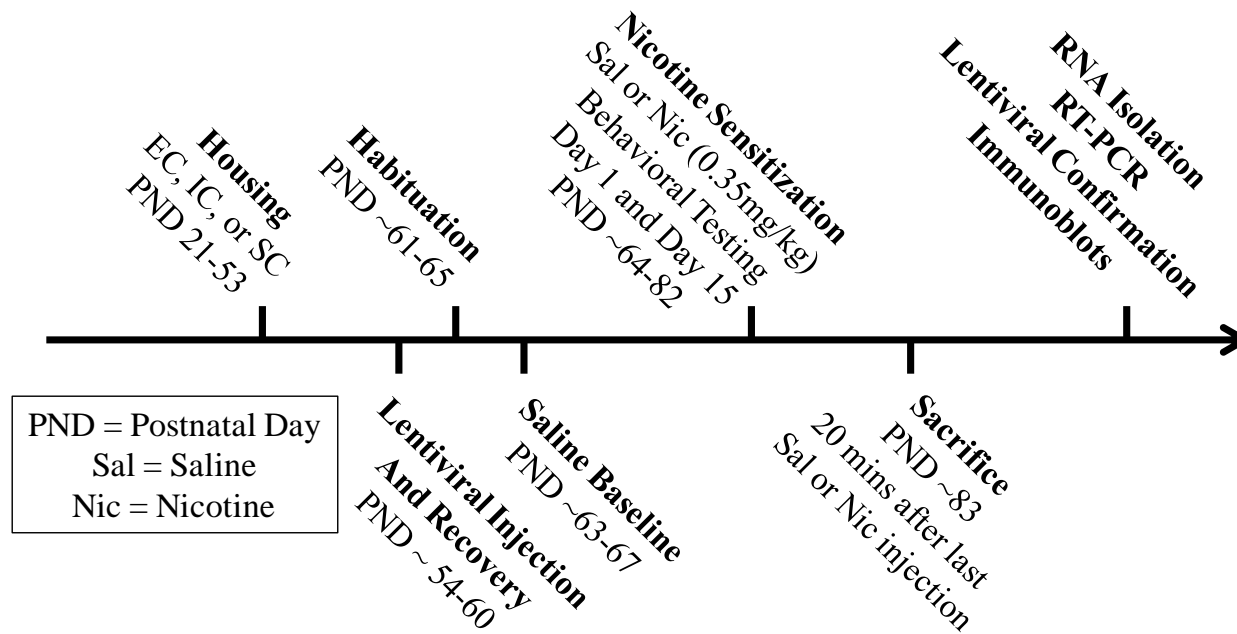


Figure 3.4. Timeline of the experimental paradigm starting on postnatal day 21 of EC, IC, and SC rats used for LV-miR-221 overexpression into the mPFC portion of the nicotine-induced sensitization experiments.

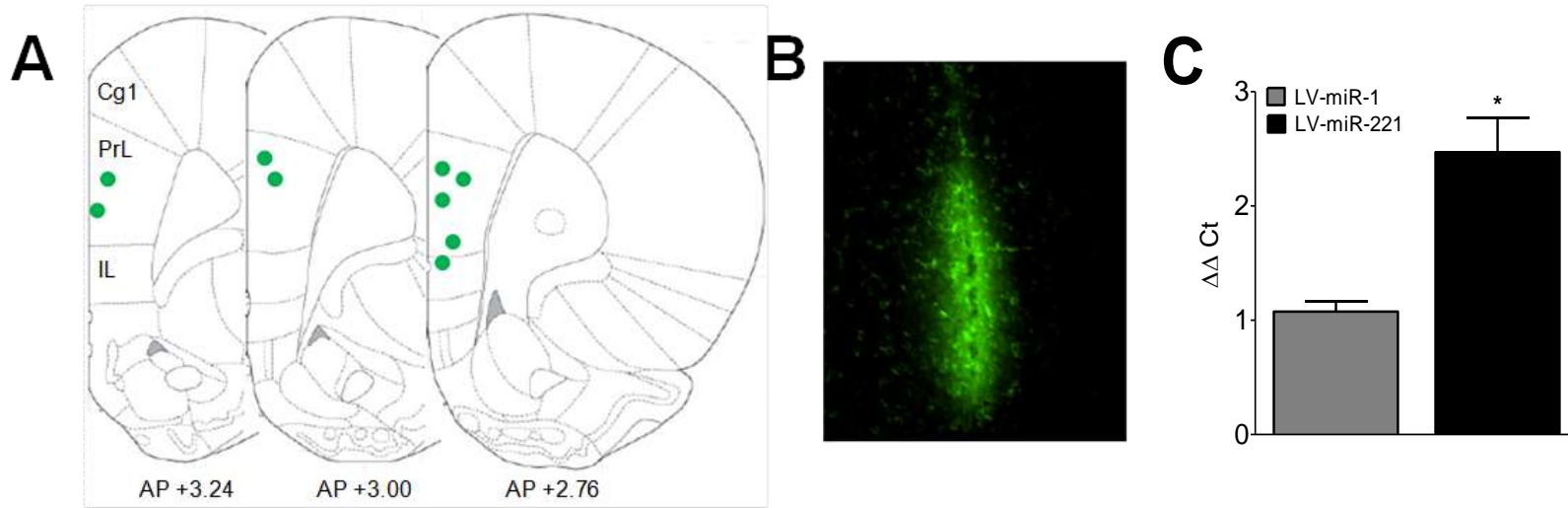


Figure 3.5. *In vivo* placement and confirmation of LV injection sites within the mPFC. (A) Representative placement mapping of coronal sections for LV injections within the mPFC, where green circles represent injection sites. (B) Coronal section (10X magnification) of LV injections tagged with GFP expression into the mPFC. (C) qPCR validation of miR-221 overexpression from the mPFC of rats injected bilaterally with either LV-miR-1 or LV-miR-221. $n = 7$ rats/groups. $*p < 0.05$ compared to LV-miR-1. Cg 1 = Cingulate cortex, PrL = Prelimbic, IL = Infralimbic.

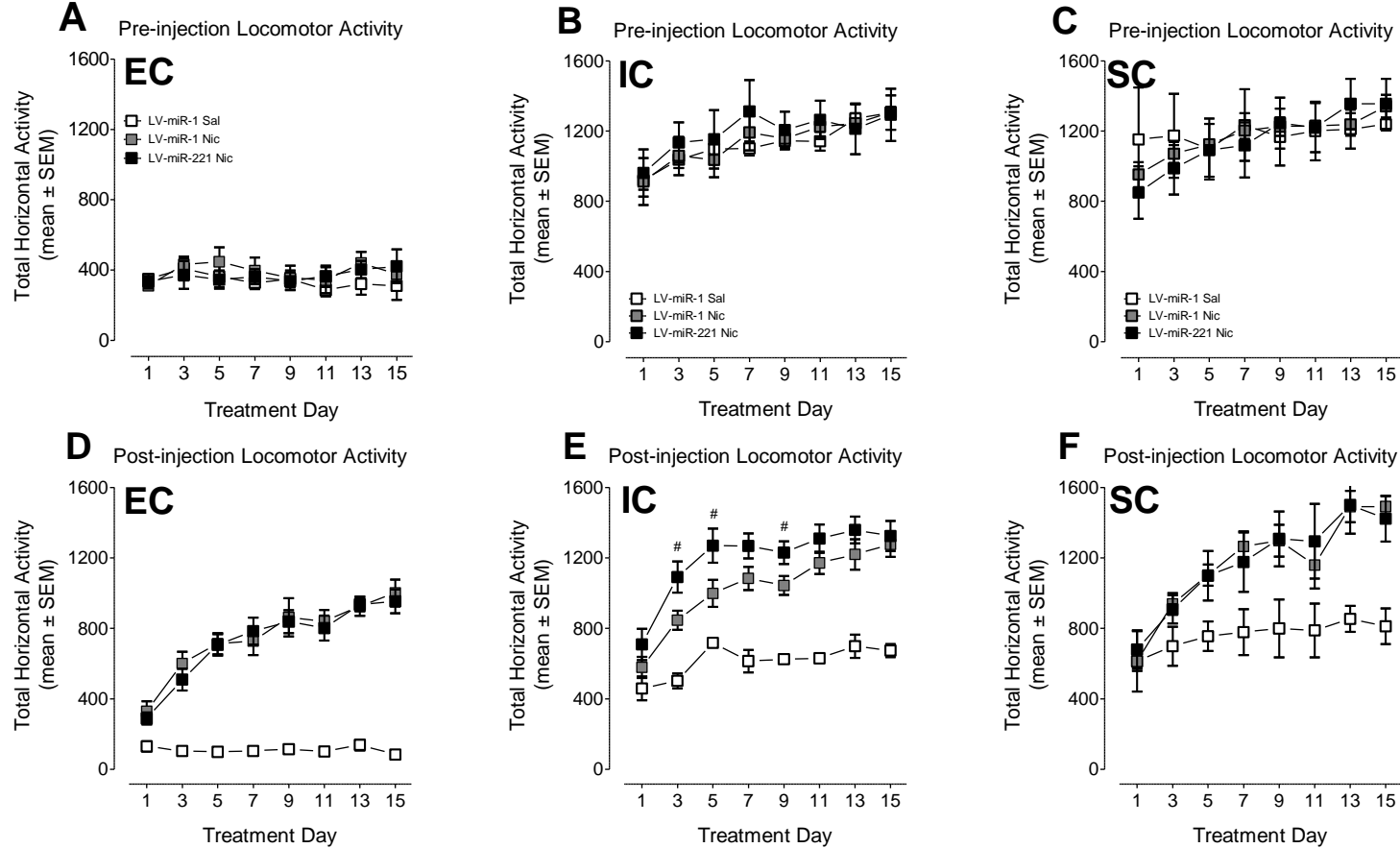


Figure 3.6. LV-miR-221 overexpression further enhances nicotine-mediated locomotor sensitivity in IC rats only. After 21-53 days of differential rearing conditions, EC, IC, and SC rats received bilateral injections of either LV-miR-1 or LV-miR-221 into the mPFC and were then administered nicotine (Nic, 0.35 mg/kg, s.c.) or saline (Sal) on Days 1-16 with locomotor measures assessed every other day. Top panels show the total horizontal activity in EC (A), IC (B), and SC (C) rats during the 30 min pre-injection habituation period across sessions. Bottom panels show the total horizontal activity in EC (D), IC (E), and SC (F) rats during the 60 min post-injection period across sessions immediately following Sal or Nic injection. Data are presented as mean \pm SEM. $n=4-6$ rats/group. # $p<0.05$ compared to LV-miR-1 Nic group within housing condition.

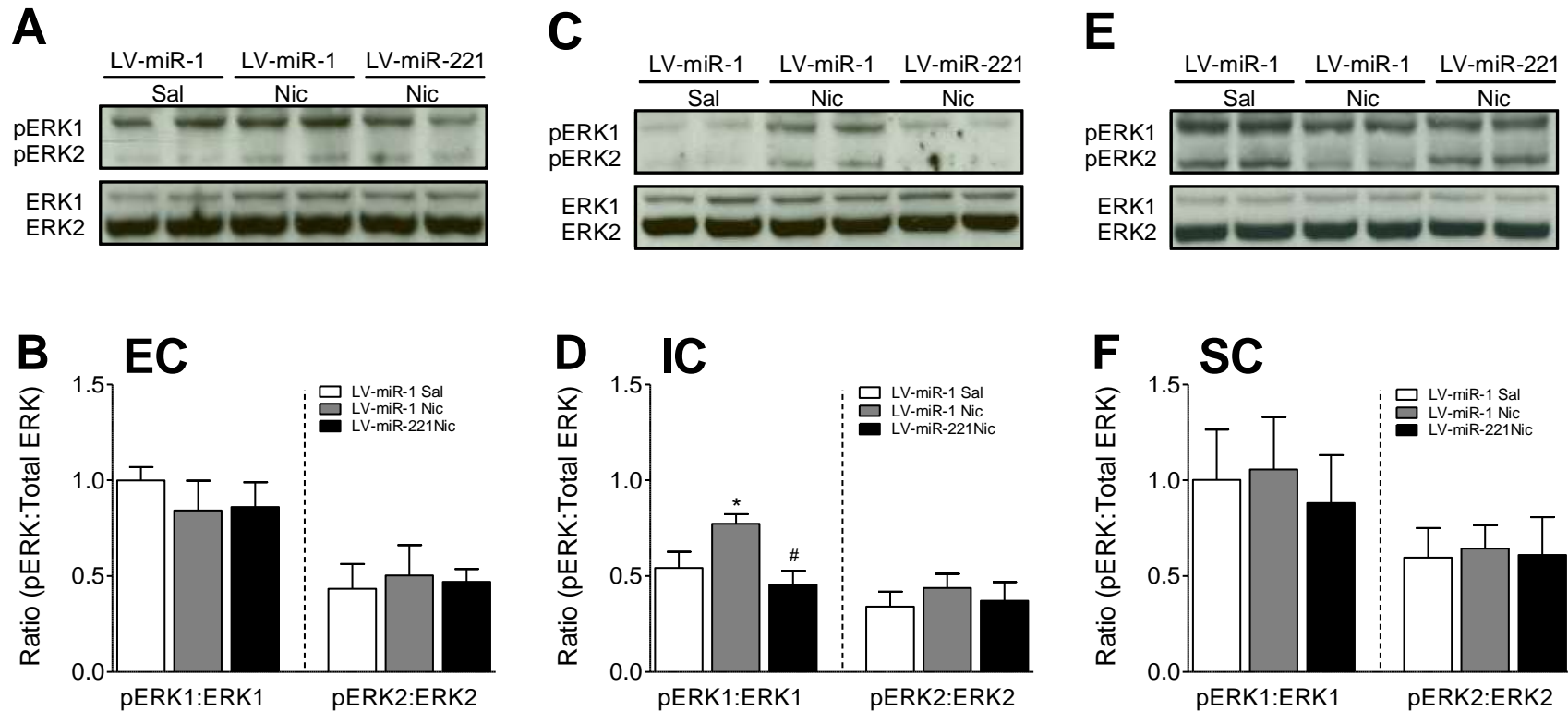


Figure 3.7. LV-miR-221 overexpression attenuates nicotine-mediated pERK1 levels in IC rats only. After undergoing locomotor sensitization paradigm, levels of pERK1/2 and ERK1/2 in the PFC of EC, IC, and SC rats that received either LV-miR-1 or LV-miR-221 were determined. Top panels show representative immunoblots of pERK1/2 and ERK1/2 immunoreactivity (Band 1 = 44 kDa, Band 2 = 42 kDa) in the PFC of EC (A), IC (C), and SC (E) rats 20 min after receiving the last (Day 16) injection of nicotine (Nic; 0.35 mg/kg, s.c.) or saline (Sal). Bottom panels show the ratio of pERK1/2 levels to total ERK1/2 levels in the PFC of corresponding EC (B), IC (D), and SC (F) rats. Data are presented as the percentage of pERK1/2 to total ERK1/2 densitometry values of immunoreactivity. Histograms represent means and error bars represent SEM. The levels of pERK1/2 and total ERK1/2 were measured at the same time with the same loading volume of protein. $n = 4-6$ rats/group. * $p < 0.05$ compared to LV-miR-1 Sal group. # $p < 0.05$ compared to LV-miR-1 Nic group.

CHAPTER 4

EFFECTS OF ENRICHMENT ON NICOTINE SELF-ADMINISTRATION

4.1 INTRODUCTION

Death from tobacco smoke remains the number one preventable cause of death, yet of the 60-70% of people who try cigarette smoking, only 20-25% progress to substance-dependent users (Ezzati and Lopez, 2003, Benowitz, 2010). Therefore, determining the underlying neurobiological mechanism(s) for the resistance to nicotine, the principal addictive component of tobacco smoke, abuse vulnerability is vital for establishing potential therapeutic strategies for the treatment of nicotine addiction. Environmental factors have garnered recognition in mediating vulnerability to nicotine addiction (Leshner, 2000, Rhee et al., 2003). The contribution of environmental factors can be studied under laboratory conditions utilizing the environmental enrichment paradigm where inbred rats are raised in either an enriched condition (EC) consisting of novel objects and social cohorts, or an impoverished condition (IC) consisting of no novelty and no social cohorts. Rats raised in the enriched condition exhibit a protective-like phenotype to addictive drugs allowing researchers to exploit this paradigm to dissect the neurobiological basis for individual vulnerability to nicotine addiction (Stairs and Bardo, 2009, Solinas et al., 2010).

In particular, in a self-administration paradigm, which is considered the most reliable and predictive experimental model for evaluating the reinforcing effects of drugs

in animals (Panlilio et al., 2008); EC rats have been shown to have decreased drug intake of amphetamine (Bardo et al., 2001), cocaine (Green et al., 2010), ethanol (Deehan et al., 2011) and methylphenidate (Alvers et al., 2012). However, examination of the enrichment-mediated protection in nicotine self-administration has yet to be determined. We have previously determined that EC rats have a decreased sensitivity to the locomotor effects of both acute and repeated nicotine administration in comparison to nicotine-treated IC rats (Gomez et al., 2012). Mirroring this finding, decreased locomotor sensitivity in EC rats has also been shown in response to cocaine (Smith et al., 1997) and amphetamine (Bardo et al., 1995). Thus, the decreased responding in amphetamine and cocaine self-administration observed within EC rats is likely to extend to nicotine-intake as well.

Moreover, the molecular mechanisms for the EC-induced decreases in drug self-administration have yet to be examined. In response to repeated nicotine, we have previously found that rearing-induced differential responding to the locomotor effects of nicotine are associated with neuroadaptations in extracellular-regulated kinase 1/2 (ERK1/2) activity within the prefrontal cortex (PFC). ERK1/2 is an intracellular signaling molecule central for linking extracellular activity with downstream transcriptional activity responsible for drug-mediated neuroplastic changes (Zhai et al., 2008). Acute and repeated systemic nicotine induce phosphorylation of ERK1/2 (Nakayama et al., 2001, Valjent et al., 2004b), suggesting that ERK1/2 may be mediating the neuroadaptations in response to experimenter-delivered nicotine. Therefore, ERK1/2 is likely mediating at least, in part, the neuroadaptations underlying the behavioral effects of

nicotine self-administration, although the role of ERK1/2 in nicotine self-administration has not been examined.

In this study, we investigated whether differential rearing conditions alters the behavioral response to the reinforcing properties of nicotine, and whether these behavioral differences may be mediated by changes in ERK1/2 activation states. Additionally, we sought to characterize the mechanism(s) controlling the change in ERK1/2 activation states via mRNA microarrays within the PFC of EC and IC rats that underwent nicotine self-administration. The central hypothesis of this study is that rats raised in an enriched environment have decreased responding to the reinforcing effects of nicotine in comparison to rats raised in an impoverished condition, and that the decreased responding in enriched rats is mediated by intracellular signaling mechanisms within the PFC.

4.2 METHODS

Animals

Male Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA). Rats arrived at the age of 21 days and were housed with food and water *ad libitum* in a colony room in the Division of Laboratory Animal Resources at the University of South Carolina. The colony room was maintained at 21 ± 2 °C, $50 \pm 10\%$ relative humidity on a 12-h light/dark cycle with lights on at 07:00 AM. All of the experimental procedures using animals were performed according to the National Institute of Health guidelines for AAALAC accredited facilities. The experimental protocol for this study was approved by the Institutional Animal Care and Use

Committee (IACUC) at the University of South Carolina in compliance with animal welfare assurance.

Environmental conditions

Upon arrival at postnatal day 21, rats were randomly assigned to EC, IC, or SC groups. EC rats were group-housed (10-15 per cage) in a metal cage (120 cm length \times 60 cm width \times 45 cm height). Twelve hard non-chewable plastic objects were randomly placed in the cage. On a daily basis half of the objects were replaced with new objects, and the remaining objects were rearranged. IC rats were individually housed in wire mesh hanging cages (25 cm length \times 18 cm width \times 17 cm height) with solid metal sides and wire mesh floor. SC rats were pair-housed in a clear polycarbonate cage (43 cm \times 20 cm width \times 20 cm height) with a wire cage top. EC rats were handled each day as to change the novelty of the environment on a daily basis. IC and SC rats were neither handled nor exposed to any object except food and water; however, all rats were handled extensively throughout behavioral testing so that novelty and the number of cohorts were the only factors that differed among the groups throughout behavioral paradigms. The SC condition represents the standard housing conditions set in the NIH Guide for the 1996 version of the *NIH Guide for the Care and Use of Laboratory Animals*. Rats were raised in these conditions from 21 to 53 days of age and were maintained in these conditions throughout all experiments.

Nicotine self-administration

Sucrose training: Operant chambers (ENV-008; Med-Associates, St. Albans, VT), housed within sound-attenuating enclosures, were controlled by Med-PC computer interface software. The front panel of the chamber allowed access to a recessed food

dipper (ENV-202M) through a 5 × 5 cm opening. Two retractable metal levers (ENV-112BM) on either side of the opening were located 7.3 cm above a metal grid floor. A dipper equipped with a 0.1 ml cup attached to the end of the arm was raised into the food receptacle, which allowed access to liquid sucrose following the completion of response requirements. A 28 V white cue light, 3 cm in diameter, located above each response lever, was used to signal timeouts. An infrared sensor (ENV-254-CB) was used to detect head entries into the food receptacle. During drug self-administration sessions a syringe pump (PHM-100) was used to deliver intravenous nicotine infusions through a water-tight swivel (PHM-115).

At 54 days of age, rats were food restricted in order to maintain 90% of free-feeding weight for three days prior to the beginning of dipper training. Dipper training and autoshaping (Reichel et al., 2008) were conducted according to previous research during which animals acquired the operant response for 26% (w/v) sucrose (Harrod et al., 2012, Lacy et al., 2012). During autoshaping, following the acquisition of the operant response, animals responded for 26% sucrose for two days according to a fixed-ratio 1 (FR-1) schedule. A response on the active lever resulted in a 4-sec access to sucrose whereas a response on the inactive lever resulted in no sucrose presentation.

Following 5 days of *ab libitum* access to food, rats were anesthetized with ketamine (100 mg/kg/ml, IP) and diazepam (5 mg/kg/ml, IP) and implanted with a catheter according to our published method (Harrod et al., 2012). In brief, one side of the catheter was inserted into the jugular vein, and the other was embedded in a dental acrylic head cap mounted to the top of the skull with four stainless steel jeweler's screws. The stainless steel catheter was capped by an aluminum standoff to prevent catheter damage

by rats housed in an enriched condition. Rats were allowed to recover from the surgery for 4 to 5 days. During recovery, the catheter of each rat was flushed twice daily with gentamicin (40 mg/ml) and sterile heparinized saline (0.2 %).

Beginning at 66-70 days of age, rats were allowed to self-administer nicotine (0.03 mg/kg per infusion) or saline (as a negative control for neurochemical measurement) on a FR-1 schedule of reinforcement during 3-h daily test sessions for a total of 21 days. Nicotine solution (freebase) was prepared immediately prior to testing and it was neutralized to pH 7.4 with NaOH. Nicotine doses were calculated based on the body weight of individual rats. Nicotine was infused (60 μ l, 3.3 s) following depression of the active level response, while responding on the inactive lever resulted in no nicotine infusion, but was recorded. Each drug infusion was followed by a 20-s time out. The time out occurred immediately after the active lever response and it was signaled by turning on the cue lights located above the response levers. No drug infusions were delivered if animals responded on either lever during the time-out. Twenty-four hours after last self-administration session (Day 21), brain regions were dissected in a chilled matrix for further analyses.

Western blot analysis

Rats were sacrificed by rapid decapitation 24 hrs after the last self-administration session and brain regions were dissected in a chilled matrix and sonicated immediately on ice in a homogenization buffer containing 20 mM HEPES, 0.5 mM EDTA, 0.1 mM EGTA, 0.4 M NaCl, 5 mM MgCl₂, 20% glycerol, 1 mM PMSF, phosphatase inhibitor cocktails I (P2850, Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors (P8340, Sigma-Aldrich, St. Louis, MO, USA). Homogenates were then centrifuged at 12,000 g

for 15 min at 4°C. The supernatants were collected and stored at -80°C. Protein concentrations were determined in triplicate using Bio-Rad DC protein detection reagent. In brief, proteins (30 µg per PFC tissue samples) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes that were transferred with proteins were preincubated with blocking buffer (5% dry milk powder in PBS containing 0.5% Tween-20) and then incubated overnight at 4 °C in blocking buffer with primary antibodies: total ERK1/2 (1:5000, V114A) from Promega (Madison, WI, USA), and pERK1/2 (1:1000, SC-16982R) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membranes were washed and then incubated in blocking buffer containing secondary affinity-purified, horseradish peroxidase-conjugated anti-rabbit IgG; (1:20,000) from Jackson ImmunoResearch (West Grove, PA, USA) for total ERK1/2 and 1:5000 for pERK1/2. Immunoblots were detected using enhanced chemiluminescence (ECL-plus) and developed on Hyperfilm (Amersham Biosciences UK Ltd., Little Chalfont Buckinghamshire, UK). After detection and quantification of these proteins, each blot was stripped in a Re-blot plus mild antibody stripping solution (CHEMICON, Temecula, CA, USA) and reprobed for detection of β -tubulin (1:5000; H-235, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) to monitor protein loading among samples. Multiple autoradiographs were obtained using different exposure times, and immunoreactive bands within the linear range of detection were quantified by densitometric scanning using Scion image software (Scion Corp., Frederick, MD, USA).

RNA isolation and mRNA microarray

Brain tissues (~30 mg) were placed in 1ml of RNAlater (Ambion, Grand Island, NY, USA) whereby total RNA isolation was performed using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality

was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and RNA Integrity Numbers (RIN) ranged from 8.1 to 9.4. RIN is a measure of RNA quality which ranges from 0 (completely degraded RNA) to 10 (intact RNA).

For gene expression experiments, total RNA samples from the PFC were amplified and labeled using Agilent's Low Input Quick Amp Labeling Kit (Cat. # 5190-2306) according to the manufacturer recommendations. Briefly, mRNA contained in 200 ng of total RNA was converted into cDNA using a poly-dT primer that also contained the T7 RNA polymerase promoter sequence. Subsequently, T7 RNA polymerase was added to cDNA samples to amplify original mRNA molecules and to simultaneously incorporate cyanine 3-labeled CTP (cRNA) into the amplification products. In addition, Agilent RNA spike-in controls (Cat. # 5188-5282) were added to samples prior cDNA synthesis and were used as experimental quality control. In the next step, labeled RNA molecules were purified using Qiagen's RNeasy Mini Kit (Cat. # 74104). After spectrophotometric assessment of dye incorporation and cRNA yield, samples were stored at -80°C until hybridization. Labeled cRNA samples were hybridized to SurePrint G3 Rat GE 8x60K microarrays (G4853A, Agilent, Santa Clara, CA, USA) at 65°C for 17 hours using Agilent's Gene Expression Hybridization Kit (Cat. # 5188-5242) according to the manufacturer's recommendations. After washes, arrays were scanned using an Agilent Microarray Scanner System (G2565CA) and the data were extracted from images with the Feature Extractor Software version 10.7.3.1 (Agilent, Santa Clara, CA, USA). In this process, background correction using detrending algorithms was performed. Subsequently, background-corrected data was uploaded into GeneSpring GX version 11.5.1 for analysis. In this process, data was log₂ transformed, quantile normalized and

base line transformed using the median of all samples. Then, data was filtered by flags in a way that at least 3 out of the 4 biological replicates have a “detected” flag in at least one of the two treatment groups. Differentially expressed genes were determined by analysis of the data using the Mann-Whitney unpaired statistics. A cutoff p-value of 0.05 was used. Additionally, a fold change cutoff value of 1.5 was used to filter the data.

Data analyses

Data are presented as mean \pm standard error of the mean (SEM), and n represents the number of subjects for each group. Mixed-factors ANOVAs were conducted on the sucrose self-administration and nicotine self-administration data from the final week of testing. Housing was the between-subjects factor (EC and IC). Testing day (7 days) and lever (active and inactive) were the within-subjects factors. Simple effect comparisons were conducted between the EC and IC active lever responding where appropriate. To evaluate the effects of repeated nicotine administration on the activity of signaling proteins (pERK1/2, and ERK1/2); mixed-factor ANOVAs (housing condition \times treatment) were performed on PFC with environment and treatment as between-group factors. Simple effect comparisons were made for post hoc analyses. To determine whether a relationship existed between nicotine infusions and immunoreactivity of pERK1 and pERK2 separate Pearson correlations were conducted. Differentially expressed genes were determined by analysis of the data using the Mann-Whitney unpaired statistics. A cutoff p-value of 0.05 was used. Additionally, a fold change cutoff value of 1.5 was used to filter the data. Identified genes were then evaluated by mixed-factor ANOVAs (housing condition \times treatment). All statistical analyses were performed using IBM SPSS Statistics version 20, and α level was set at $p < 0.05$ for all analyses.

4.3 RESULTS

EC rats respond less to the reinforcing effects of nicotine compared to IC rats

In the current study, we determined the effect of environmental enrichment on nicotine self-administration. Rats were first trained to respond for sucrose whereby the last two days of training consisted of two 30 min FR-1 sessions consisting of both an active and inactive lever. A housing condition \times lever ANOVA revealed a main effect for lever ($F_{(1,52)} = 721.1, p < 0.001$), where both EC ($t_{(26)} = 17.8, p < 0.001$) and IC ($t_{(26)} = 21.1, p < 0.001$) rats were found to significantly press the active lever for sucrose compared to the inactive lever (no sucrose). Interestingly, it was also found that IC rats significantly pressed the inactive lever more than the EC rats did ($t_{(26)} = 2.8, p < 0.05$). Regarding Day 2 for sucrose responding, a housing condition \times lever ANOVA revealed a main effect for housing condition ($F_{(1,52)} = 6.6, p < 0.05$), lever ($F_{(1,52)} = 534.5, p < 0.001$), and a significant housing condition \times lever interaction ($F_{(1,52)} = 5.5, p < 0.05$). EC ($t_{(26)} = 13.6, p < 0.001$) and IC ($t_{(26)} = 19.69, p < 0.001$) rats were both found to significantly lever press (active) for sucrose compared to the inactive lever (no sucrose). However, in contrast to day 1 where a significant difference between EC and IC rats were found on the inactive lever; a significant difference with respect to the active lever ($t_{(26)} = 2.5, p < 0.05$) was found between EC and IC rats.

Rats were then given the opportunity to respond for nicotine (FR-1), via the active lever or with no consequence on the inactive lever, daily for 21 consecutive days. Regarding active lever responding (i.e., nicotine infusion), EC and IC rats exhibited 20 and 34 nicotine infusions, respectively, on day 1 of testing and the number of infusions

decreased across the first week (data not shown). During the second week, active lever responding began to stabilize: IC rats continued to show greater responding on the active lever, relative to the EC rats, e.g., ~ 13 and 3 infusions, respectively. In terms of the inactive lever responding, the pattern differed for the EC and IC rats during the first and second weeks. EC rats exhibited low, but equal responding on the active and inactive levers. In contrast, the IC animals exhibited variable inactive lever responding during the first week, but interestingly, inactive lever responding stabilized to approximately half of active lever responding during the second week, (data not shown). For the third week, active and inactive lever responding remained stable, as shown in Figure 4.2. The housing \times day \times lever ANOVA revealed a significant main effect of housing ($F_{(1,10)} = 33.7, p < 0.001$) and a housing \times lever interaction ($F_{(1,10)} = 4.6, p = 0.057$). Simple effect comparisons between EC and IC rats on active lever responding show that IC rats consistently responded more for nicotine than EC animals (housing: $F_{(1,10)} = 16.2, p < 0.01$; housing \times day: $F_{(1,10)} = 0.1, p > 0.05$).

Nicotine infusion was positively correlated with the nicotine-induced phosphorylated ERK1/2 levels in the PFC

We next determined whether activation of the ERK pathway is associated with nicotine self-administration behavior. Twenty-four hours after completion of the last operant session, we examined the levels of pERK1/2 and total ERK1/2 in the PFC of rats that self-administered nicotine or saline (Fig. 4.3A). No differences in total ERK1/2 were found among saline or nicotine EC and IC groups (data not shown). A two-way ANOVA on the level of pERK1 revealed a main effect for treatment ($F_{(1,20)} = 6.9, p < 0.05$). Neither a main effect of housing nor a housing \times treatment interaction was determined to be

significant. Similarly, with regard to the level of pERK2, a two-way ANOVA revealed a significant main effect of treatment ($F_{(1, 20)} = 11.0, p < 0.01$); however, neither main effect of housing nor housing \times treatment interaction were significant. In the saline control groups, no differences in pERK1/2 were found between EC and IC rats (Fig. 4.3B). Compared to saline controls, IC rats that self-administered nicotine exhibited increased pERK1 ($F_{(1, 10)} = 5.7, p < 0.05$) and pERK2 ($F_{(1, 10)} = 5.9, p < 0.05$), whereas the levels of pERK1/2 were not altered in EC rats that self-administered nicotine (Fig. 4.3B). This suggests that an enriched environment attenuates nicotine-mediated increased ERK1/2 activity.

To explore the contribution of nicotine-mediated ERK activation in the nicotine reinforced behavior within EC and IC rats, we examined the correlations of immunoblot densities of the ratios of pERK1/2 to total ERK1/2 in the PFC of rats that self-administered nicotine with their respective active lever responding collected from operant sessions conducted on days 15-21 of the experiment. The results reveal that the ratios of pERK1/2 to total ERK1/2 were positively correlated with total number of nicotine infusions (Fig. 4.3C and D, $p < 0.05$, Pearson $r = 0.75$ and 0.55 for pERK1 and pERK2, respectively). Therefore, these findings indicate that activation of ERK1/2 may be responsible for enriched environment-induced reductions in nicotine self-administration behavior.

Enrichment attenuates orexin receptor-1 (OX1R) upregulation within the PFC in response to nicotine self-administration

We next determined the potential regulatory mechanism(s) underlying the EC-induced attenuation in pERK1/2 in the PFC in response to nicotine self-administration by

using microarrays to examine prefrontal mRNA expression in EC and IC rats. After analyzing mRNAs that were differentially regulated by 1.5-fold difference between EC and IC rats that underwent nicotine self-administration, we identified the OXR1 as a potential mediator of ERK1/2 signaling. Microarray data showed that IC rats have decreased basal levels of OXR1 expression in PFC relative to EC rats in saline control group ($F_{(1,6)} = 7.6, p < 0.05$). However, OXR1 expression was significantly increased in IC rats ($F_{(1,12)} = 7.7, p < 0.05$) but not in EC rats following nicotine self-administration. Furthermore, we found a significant difference when we examined orexin mRNA levels ($F_{(1,12)} = 61.0, p < 0.05$). There were no differences between EC and IC groups when assessing saline-treated and nicotine-treated groups. However, within rearing conditions, we found a significant decrease in orexin levels within the PFC in response to nicotine in both EC ($F_{(1,6)} = 8.7, p < 0.05$) and IC ($F_{(1,6)} = 10.7, p < 0.05$) rats when compared to baseline. These findings suggest that an enriched environment attenuates nicotine-mediated increases in OXR1 gene expression within the PFC which may underlie the attenuated pERK1/2 response within the PFC of EC rats undergoing nicotine self-administration.

4.4 DISCUSSION

In this study, we report that EC rats self-administered less nicotine than IC rats. In agreement with our findings from repeated systemic nicotine administration (as reported in Chapter 2), the levels of pERK1/2 in the PFC were significantly increased in IC rats, but not in EC rats that self-administered nicotine. Further, the pERK1/2 levels in EC and IC rats were positively correlated with their total number of nicotine infusions. Nicotine-

induced pERK1/2 increases in IC rats were also accompanied by an increase in OX1R mRNA strictly in IC rats within the PFC. These findings suggest that an enrichment mediated attenuation in nicotine-induced increases in prefrontal ERK1/2 activity may be modulated by increased OX1R mRNA in the PFC; and potentially may be responsible for the altered nicotine-intake between differentially reared rats.

In the current study, we evaluated the effects of an enriched environment in response to intravenous nicotine using the drug self-administration procedure, which is considered the most reliable and predictive experimental model for evaluating the reinforcing effects of drugs in animals (Panlilio et al., 2008). Rearing rats in an enriched environment has been shown to reduce amphetamine, methylphenidate, and cocaine self-administration (Bardo et al., 2001, Green et al., 2010, Green et al., 2002, Alvers et al., 2012). The present experiment is the first to our knowledge, to investigate the effects of an enriched environment on intravenous nicotine self-administration. We found that in accordance with other psychostimulants, EC rats self-administered less nicotine than IC rats across the daily three-week test sessions, suggesting that EC rats have a reduced sensitivity to the reinforcing effects of nicotine. This phenomenon, however, is not due to the inability of EC rats to respond for reinforcers as lever presses for sucrose in our training phase indicated robust active lever responses closely resembling that of the IC rats. Interestingly, it has been noted that food-restricted EC rats have increased responding for sucrose in the initial training phase of an incremental increasing FR schedule (Bardo et al., 2001). In our case, EC rats were shown to have decreased sucrose responding on day 2 of training. Due to the short nature of the training period in our study, it is difficult to speculate if decreased EC responding for sucrose would have

continued after day 2 of FR-1 sucrose responding, but this study, along with other reports, suggest EC rats do have differential responding for non-drug rewards compared to IC rats (Bardo et al., 2001, Green et al., 2010). It is also worth mentioning that although EC rats respond less for nicotine, EC rats did not discriminate between active and inactive levers. Whether this is a function of the drastically low levels of active responding in EC rats, the current dose of nicotine used, or the schedule of reinforcement remains to be determined. In support of this view, enrichment-induced protective effects in psychostimulant intake have been abundantly clear at low unit doses where as the difference between drug intake in EC and IC rats seem to diminish at higher doses of psychostimulants (Bardo et al., 2001, Green et al., 2002, Alvers et al., 2012, Gipson et al., 2011). Therefore, examination of dose-response curves for nicotine, in addition to increasing the FR schedule of reinforcement in EC and IC rats undergoing nicotine self-administration is an intriguing study for the future.

Given the relationship between drug-taking behavior and impulsivity, it is also possible that the attenuation of nicotine self-administration in EC rats reflects an enrichment-induced reduction in impulsivity. High impulsive rats show increases in both the initiation and maintenance of nicotine self-administration compared with low impulsive rats (Diergaarde et al., 2008). Indeed, previous research suggests that EC rats are less impulsive compared to IC rats (Wood et al., 2006, Perry et al., 2008, Kirkpatrick et al., 2013). Additionally, acute methylphenidate and amphetamine decreases impulsive choice in IC rats, but not EC rats (Perry et al., 2008), suggesting psychostimulants alter impulsive behaviors in IC rats only. Interestingly, the PFC dopaminergic system is thought to play a critical role in not only drug self-administration, but impulsivity (Perry

et al., 2011). Prefrontal DA-depletion within the mPFC of rats show increased impulsivity in rats (Sokolowski et al., 1994); in addition, expression levels of DA receptors are associated with impulsive choice (Loos et al., 2010). In particular raclopride, a D2 antagonist, into the mPFC increased impulsive choice in rats (Pardey et al., 2013). Previous research has determined that EC rats have a lower dopaminergic tone in the PFC compared to IC rats (Zhu et al., 2005b, Del Arco et al., 2007a, Gomez et al., 2012, Zhu et al., 2004). We also have preliminary data demonstrating that EC rats have increased basal levels of D2 mRNA compared to IC rats (data not shown). Therefore, the current findings imply that DAergic-dependent alterations within the PFC may provide a potential molecular mechanism underlying enriched environment-induced decreases in impulsivity and vulnerability to nicotine dependence.

Another explanation for the differential rearing-mediated disparities in nicotine-taking behavior may be due to enrichment-mediated alterations in the hypothalamic-pituitary-adrenal (HPA) axis-mediated stress response system. Multiple studies have demonstrated that elevations in stress hormones increase psychostimulant self-administration (Deroche et al., 1997, Goeders and Guerin, 1996, Ambroggi et al., 2009). In fact, EC rats have been shown to have decreased basal levels of corticosterone and adrenocorticotrophic hormone (ACTH) (Stairs et al., 2011, Skwara et al., 2012). Moreover, EC rats have a trend for increased glucocorticoid receptor expression (Garrido et al., 2013). Administration of RU-486, a glucocorticoid receptor antagonist, has been previously shown to decrease amphetamine self-administration in EC and IC rats, however the decrease in IC rats showed more of a blunted response (Stairs et al., 2011); suggesting that IC rats may have an augmented HPA response to psychostimulants. In

fact, nicotine increases stress hormone levels (Armario, 2010, Lutfy et al., 2012) and augments the “normal” HPA response to stressors (Yu et al., 2008, Yu and Sharp, 2010, Yu and Sharp, 2012). Moreover, enriched rats have a reduced ACTH response to acute nicotine compared to standard rats (Skwara et al., 2012), and decreased corticosterone levels in response to acute stress (Garrido et al., 2013). Thus, these findings support a possible role whereby EC rats have reductions in a sensitized stress response to nicotine, thereby potentially reducing nicotine self-administration.

The current results are also in line with the repeated nicotine sensitization model, which also suggests that environmental enrichment attenuates nicotine-mediated pERK1/2 levels in the PFC and likely impacts the differences in nicotine-associated behaviors: nicotine-mediated locomotor sensitization and nicotine self-administration. Contrary to the basal pERK1/2 changes in our model of repeated saline administration (chapter 2), the basal levels of pERK1/2 in the PFC were not significantly different between control EC and IC rats that underwent saline self-administration. One possible explanation is that EC and IC rats experienced different aspects between the behavioral paradigms. For instance, EC and IC rats that experienced saline self-administration also underwent sucrose dipper training for lever discrimination prior to saline self-administration. It has been shown that ERK activity is subject to modification after operant behavior for non-drug reinforcers (Guegan et al., 2013). Furthermore, due to the extended timeframe of the self-administration paradigm compared to the locomotor paradigm, the postnatal day of age of EC and IC rats when they were sacrificed differed. Along these lines, studies have indicated that basal levels of pERK1/2 in the cortex and associated mesocorticolimbic brain regions of rodents can vary in an age-dependent

manner, particularly with regard to the adolescent versus adult brain (Zhen et al., 1999, Spanos et al., 2012). Nevertheless, it is likely that enrichment-dependent prefrontal plasticity may contribute to the behavioral and neurochemical manifestations to nicotine exposure.

Passive nicotine administration has been shown to elevate the phosphorylation levels of ERK1/2 both *in vitro* and *in vivo* (Nakayama et al., 2001, Valjent et al., 2004b). However, the effect of nicotine self-administration on pERK1/2 levels remains lesser known. The present results demonstrate that a significant increase in nicotine-mediated pERK1/2 in the PFC was observed in IC rats, but not in EC rats in a self-administration paradigm. This finding also corresponds with our previous finding from a repeated nicotine model. This suggests that an environmental enrichment-induced attenuation in nicotine-mediated pERK1/2 levels is universal to both nicotine models. Interestingly, the levels of pERK1/2 in the PFC of nicotine self-administered EC and IC rats were positively correlated with their respective total number of nicotine infusions during the last week (15-21 days), whereas no nicotine-mediated differences in pERK1/2 in the PFC between EC and IC rats was observed in our repeated model. One possible explanation for the discrepancy of the correlation of pERK1/2 with the behavioral response to nicotine between sensitization and self-administration paradigms may be due to animals being treated with nicotine via different routes of administration. Evidence shows that systemic nicotine produces profound enhancements of ERK in the PFC compared to other brain regions (Valjent et al., 2004b), indicating that the PFC plays a crucial role in nicotine-mediated ERK activity and the consequence of its behavioral effects. In accordance, the current findings show that an enriched environment produces

neuroadaptations in the prefrontal ERK pathway, which may alter nicotine-enhanced activation of ERK1/2 and the relevant nicotine-mediated behaviors.

We previously found that EC rats had increased miR-221 levels in the PFC in response to repeated nicotine; and that LV-miR-221 overexpression in PC12 cells and in the PFC of IC rats attenuates nicotine-mediated pERK1/2 increases. Therefore, to assess the possible role of miR-221 in EC-induced pERK1/2 attenuation in the nicotine self-administration paradigm, we used qPCR to examine miR-221 levels in EC and IC rats after undergoing nicotine self-administration. In preliminary studies, we observed a slight increase in miR-221 levels in EC nicotine self-administering rats, although not nearly as robust as seen within our repeated nicotine model (chapter 3). This suggests that miR-221 may not completely mediate the nicotine-induced attenuation in pERK1/2 levels across behavioral models. One explanation for the miR-221 differences across models could be due to the amount of nicotine received by EC rats in the two models. Whereas EC and IC rats received the same experimenter-delivered dose of daily nicotine in the sensitization model, the intake of nicotine by EC rats in the self-administration model was drastically lower to that of IC rats. In fact, studies have shown that miR-212 levels are dependent upon cocaine-intake (Hollander et al., 2010). Additionally, miR expression patterns are altered in response to differing doses of chronic nicotine administration (Taki et al., 2014), suggesting the dose of nicotine can dramatically alter miR expression. Of interest, would be to alter the dose of nicotine in self-administration to: 1) try to increase the levels of nicotine intake within the EC group, and 2) observe if miR-221 expression levels alter in response to nicotine self-administration in a dose-response manner in EC rats.

To identify potential targets regulating the EC-induced attenuation in pERK1/2 in response to nicotine self-administration, we used microarray analyses to examine mRNA expression between EC and IC rats undergoing nicotine self-administration. We determined that EC rats have an attenuation in OX1R upregulation in response to nicotine self-administration, which could potentially underlie the nicotine-mediated behavioral and neurochemical differences between EC and IC rats. The neuropeptide orexin and its receptor system have been deeply implicated in modulating drug reward and reinforcement (Mahler et al., 2012). Acute nicotine activates orexin-containing neurons (Pasumarthi et al., 2006), and chronic nicotine upregulates both the orexin peptide and orexin receptor expression (Kane et al., 2000). Moreover administration of SB-334867, an OX1R receptor antagonist, decreases nicotine-intake in a self-administration paradigm (Hollander et al., 2008, LeSage et al., 2010). This implies that the EC attenuated response in OX1R upregulation may be responsible for the blunted nicotine-intake observed in EC rats. In addition to the contributions of the orexin system to nicotine self-administration, OX1Rs have the ability to signal to ERK1/2. OX1R receptor activates mitogen-activated kinases (MAPKs) and couples to intracellular calcium stores to activate ERK1/2 (Milasta et al., 2005, Ammoun et al., 2006, Ekholm et al., 2007). Although the detailed mechanism(s) of OX1R-mediated ERK1/2 activation is still unknown, these data support the possible role that the EC-and IC-induced differences in nicotine-intake are a manifestation of differential rearing-mediated differences in OX1R-mediated ERK1/2 activation. However, further experiments involving prefrontal SB334867 infusions in EC and IC rats undergoing nicotine self-administration would be needed to confirm if OX1R is mediating environment-mediated differences in nicotine-taking behavior.

In conclusion, these findings have important implications for preclinical studies involving the role of enrichment in individual differences in vulnerability to nicotine abuse. The aftermath of these findings advocate that manipulations of prefrontal cortical ERK activity or the upstream mechanisms, such as targeting OXR1, responsible for ERK activation may represent an approach in the treatment of drug addiction and other addictive behaviors.

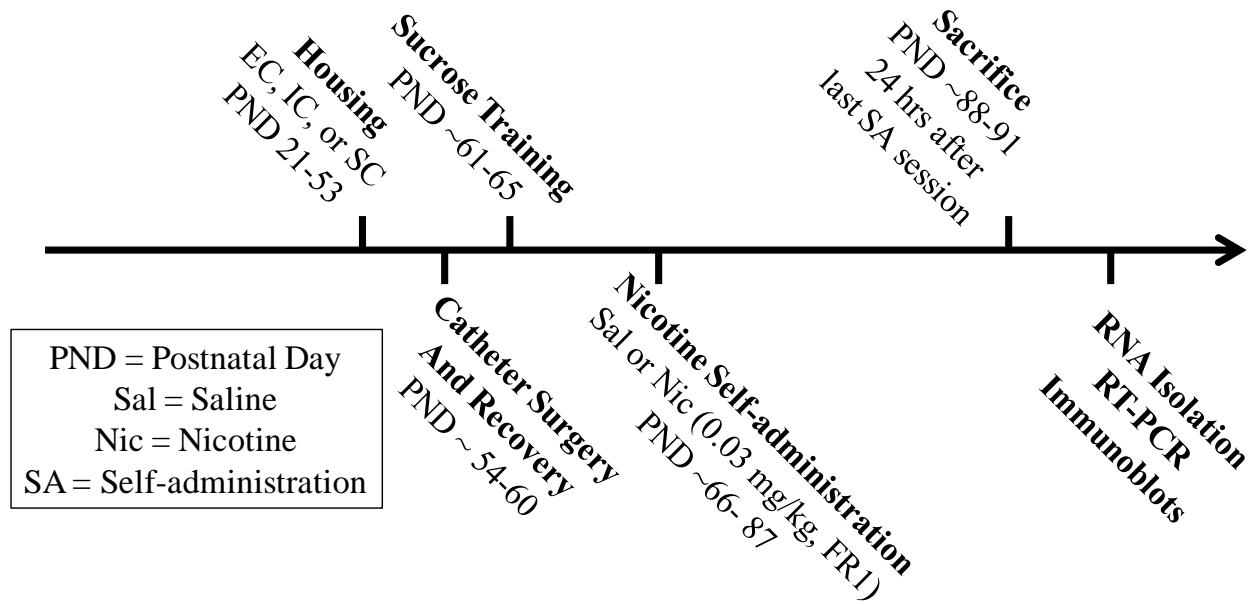


Figure 4.1. Timeline of the experimental paradigm starting on postnatal day 21 of EC and IC rats used for the nicotine self-administration experiments involving self-administration behavior and assessment of intracellular signaling proteins.

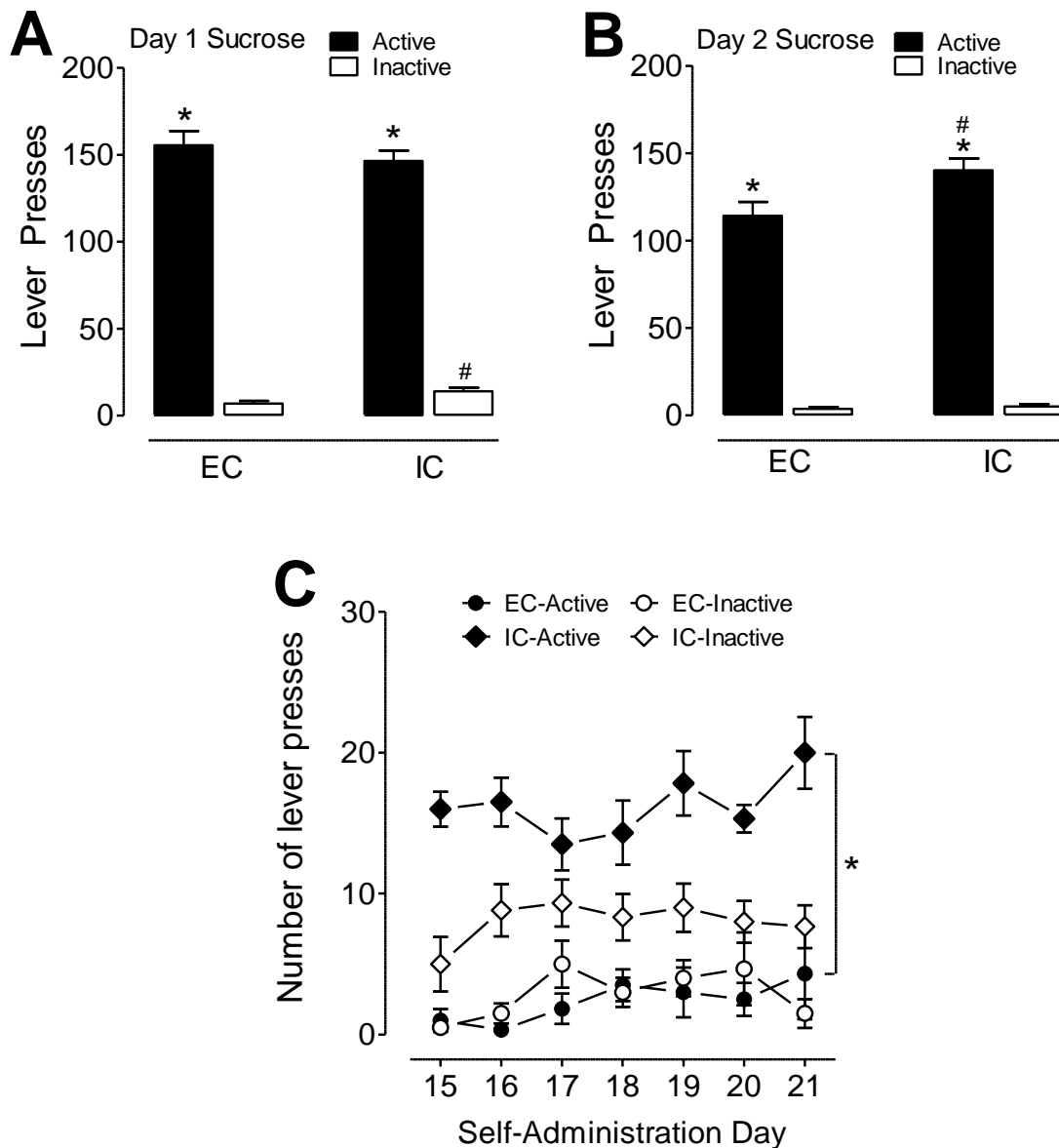


Figure 4.2. Nicotine self-administration in EC and IC rats. Number of active and inactive lever presses (mean \pm SEM) for day 1 (A) and day 2 (B) of the training phase for sucrose presentation (30 min/session, FR1). * $p < 0.05$ denotes difference between active and inactive levers within housing group. # $p < 0.05$ denotes difference between EC and IC groups. $n=14$ rats/group. Rats were then allowed to self-administer nicotine (3 h/day) for 21 consecutive days. (C) Number of active and inactive lever presses (mean \pm SEM) is plotted for the last week (15-21 days) of nicotine self-administration for EC and IC rats. Nicotine (0.03 mg/kg) was infused (60 μ l, 3.3 s) following depression of the active lever response, while responding on the inactive lever resulted in no nicotine infusion, but was recorded. Self-administration was conducted under a fixed ratio-1 schedule. * $p < 0.05$ denotes difference between IC-active and EC-active. $n=6$ rats/group.

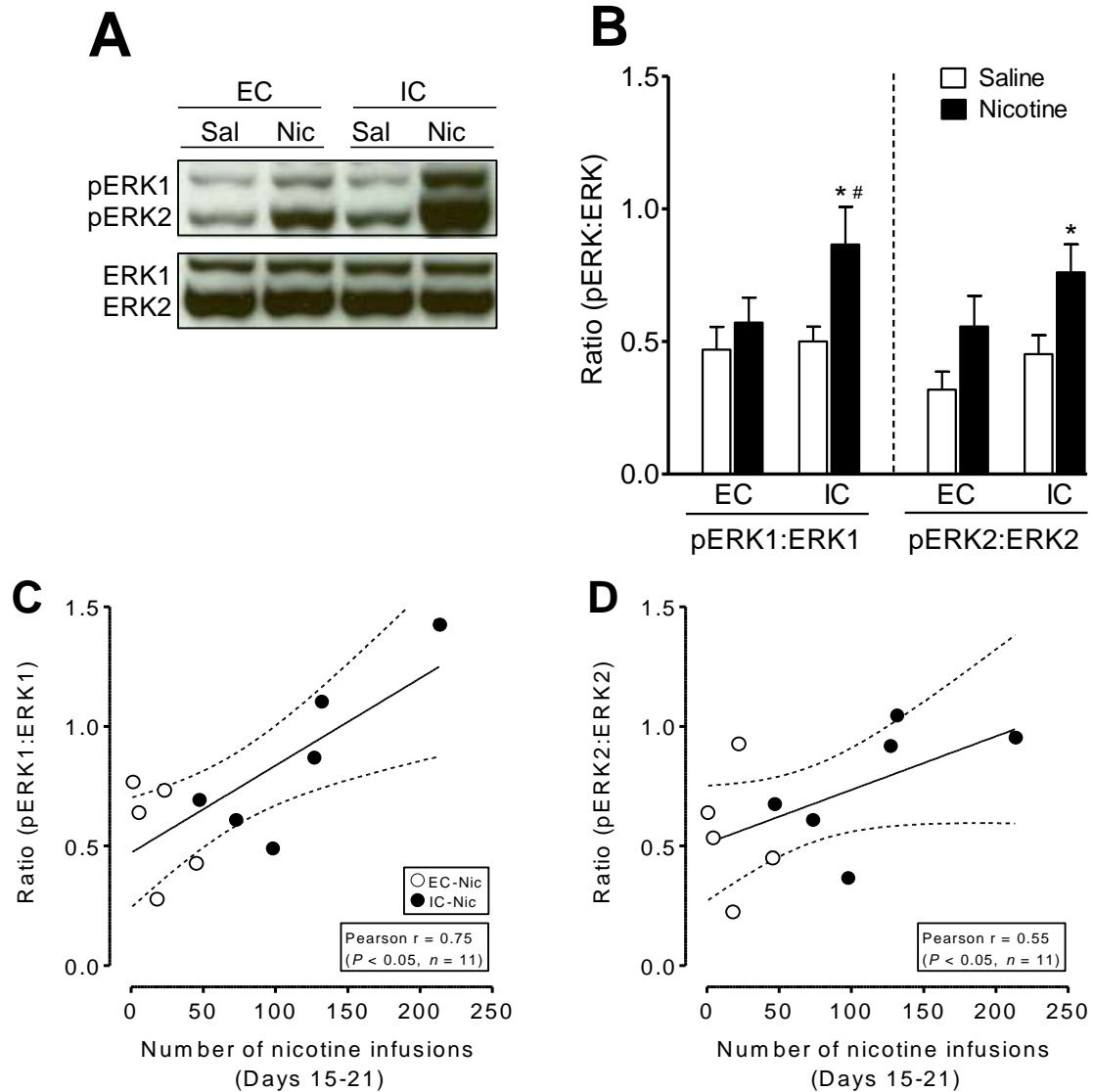


Figure 4.3. (A) Representative immunoblots of pERK1/2 and total ERK1/2 immunoreactivity (Band 1 = 44 kDa, Band 2 = 42 kDa) and (B) the ratio of pERK1/2 level to total ERK1/2 in the PFC of EC and IC rats that self-administered nicotine (Nic; 0.03 mg/kg/infusion) or saline (Sal) for 21 days. Data are presented as the percentage of pERK1/2 to total ERK1/2 densitometry values of immunoreactivity. Histograms represent means and error bars represent SEM. Total ERK1/2 and pERK1/2 were measured at the same time with the same loading volume of protein. $*p < 0.05$ denotes difference within the same housing condition between self-administered nicotine and saline groups. $\#p < 0.05$ denotes difference between nicotine self-administered EC and IC rats. $n = 6$ rats/group. (C) Correlations of the values of pERK1:ERK1 and (D) pERK2:ERK2 collected from panel B with total number of nicotine infusions for nicotine self-administered EC and IC rats during the last week (15-21 days) of nicotine self-administration. Dashed lines represent the 95% confidence interval of the linear regression fit (solid line). $n = 5$ rats/EC group, 6 rats/IC

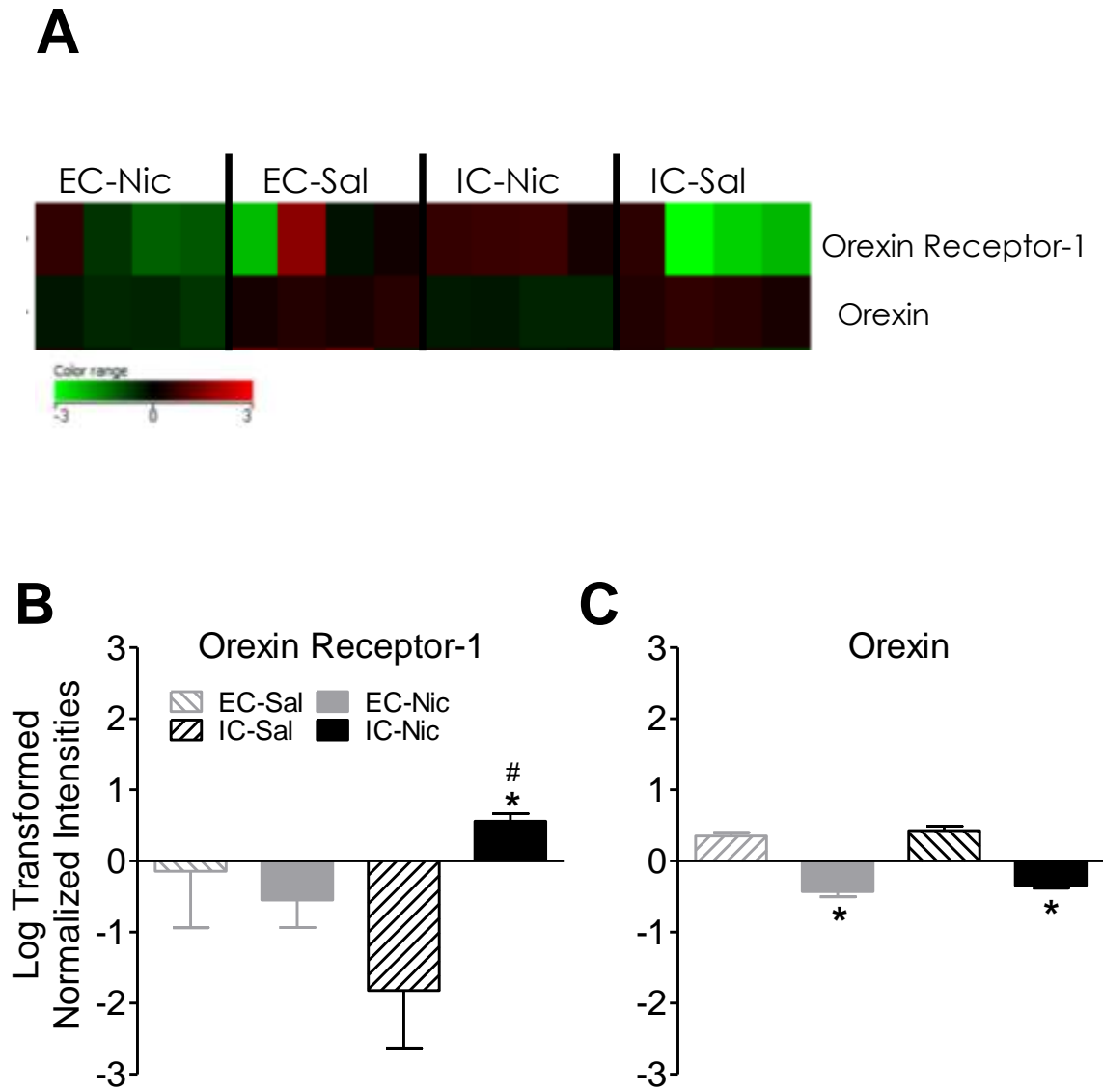


Figure 4.4. (A) Expression analysis using Agilent GeneSpring software showing significantly differentiated mRNAs within the PFC of EC and IC rats that underwent nicotine (Nic; 0.03 mg/kg/infusion) or saline (Sal) self-administration where the color transition from green-black-red represents low-medium-high mRNA expression. (B) Corresponding bar graphs showing values from expression analysis. Data are expressed as mean \pm SEM of log₂ normalized intensity values. * p < 0.05, vs saline control. # p < 0.05, vs EC rats. $n=4$ rats/group.

CHAPTER 6

GENERAL DISCUSSION

5.1 SUMMARY

The studies outlined here implicate novel molecular substrates to explain the way in which an enriched environment engenders a neuroprotective-like phenotype in nicotine-mediated behaviors. In summary, rats raised in an enriched environment exhibit increased locomotor sensitivity to acute and repeated nicotine administration, in comparison to rats raised in an impoverished or standard condition. Results support the possibility that this may be due to enrichment-mediated regulatory processes within the PFC involved in attenuating the nicotine-induced pERK1/2 response. In response to repeated nicotine, enriched rats exhibit altered miR expression patterns, in particular upregulation of miR-221. Upregulation of miR-221 can then exert control of the ERK1/2 pathway by preventing nicotine-induced enhancements in pERK1/2 levels, and thus allowing for enrichment-induced increases in nicotine-mediated locomotor sensitivity. However, these studies do not yet clarify as to what target miR-221 is pinpointing to elicit the reversal of nicotine-induced pERK1/2 activation.

Representing a more complete behavioral rodent model of addiction, rats raised in an enriched environment exhibit reductions in self-administration behavior to the reinforcing properties of nicotine. Enrichment-induced attenuation in prefrontal nicotine-mediated ERK1/2 activation was observed as well, although the mechanism may

not be completely mediated by miR-221, but by an enrichment-induced attenuation in OX1R expression in response to nicotine self-administration. However, although not as robustly as in the repeated model of repeated nicotine administration, miR-221 was increased significantly within the PFC of EC rats that underwent nicotine self-administration, suggesting miR-221 may be a universal mediator of the enrichment-induced protective-like phenotype in response to nicotine exposure. Combined, these studies identify ERK1/2, potentially regulated by miR-221, within the PFC as a promising nexus point in integrating the molecular mechanisms of enrichment-mediated neuroadaptations with the behavioral manifestations of a neuroprotective-like phenotype in response to nicotine.

The experiments outlined above are the first to examine the underlying molecular mechanisms of environmental enrichment-mediated neuroprotection in nicotine addiction. Ultimately, the studies have potentially identified a single miR, miR-221, which could mediate the protective-like phenotype of environmental enrichment across two behavioral paradigms that address nicotine addiction preclinically: nicotine-mediated locomotor sensitization and nicotine self-administration. These studies have greatly advanced research into how environmental factors influence nicotine addiction susceptibility both behaviorally and biochemically. Additionally by employing standard house rats within our testing, these studies contribute to a growing body of literature into how nicotine mediates neuroadaptations that potentially underlie the behavioral manifestations of nicotine addiction preclinically.

5.2 FUTURE DIRECTIONS

These experiments performed here have advanced the understanding of the molecular mechanisms in the impact of environment to nicotine addiction vulnerability. However, critical future studies will be needed to further solidify the findings presented here. Of highest importance is striving to find the molecular target of miR-221. As we have shown, miR-221 targets the ERK1/2 pathway in response to nicotine, but bioinformatics and pathway analyses have identified at least 12 potential targets where miR-221 could exert its control over the ERK1/2 signaling cascade. Determining the exact target will help us better understand both how and why enrichment upregulates miR-221 in response to nicotine. Another important line of work will involve determining why miR-221 was not upregulated as robustly in the nicotine self-administration paradigm, despite observing an enrichment-induced attenuation in nicotine-mediated pERK1/2 levels as well (figure 5.3). Upregulation of miR-221 may be a function of the nicotine dose as previous studies have shown that drug-induced miRs may be expressed in a dose-specific manner (Hollander et al., 2010, Taki et al., 2014). This notion would correspond to why we also did not observe a miR-221 increase in EC rats administered a higher dose of nicotine (0.6 mg/kg, s.c.) repeatedly in our preliminary studies. Examining miR-221 levels after adjusting the schedule of reinforcement or nicotine dose may reveal a potential role of miR-221 in our self-administration paradigm.

Along these lines, modifying the reinforcement schedule or dose may also expose whether the enrichment-induced neuroprotective-like phenotype only exists at 0.03 mg/kg/infusion in our nicotine self-administration model. Enrichment-mediated neuroprotection in psychostimulant self-administration has been shown to diminish in a

as the dose of psychostimulants increases. In fact, EC rats appear to have steeper increases in responding at higher doses compared to IC rats and the disparity between psychostimulant-taking behavior starts to shrink (Bardo et al., 2001, Green et al., 2002, Alvers et al., 2012, Gipson et al., 2011). If these same findings extend to nicotine self-administration, it would support the view that the protective-like effects of enrichment exist only for low unit doses of psychostimulants.

In the event that the regulation of ERK1/2 across both nicotine models may not be mediated by a common molecular mechanism, we have begun to examine large scale gene expression using microarrays of the PFC in EC and IC rats that have undergone both repeated nicotine administration and nicotine self-administration (see figures 5.1, 5.2 below). We have begun to utilize pathway analyses to identify potential commonalities into how ERK1/2 might be influenced between the two different behavioral paradigms. As shown in the figures, ERK1/2 appears to play a central role in both models, although the commonalities between the two models need further investigation.

Lastly, the complexity of the enriched environment paradigm comprises multiple components: a large space, physical exercise, novel objects, and social cohorts. Although it appears that each factor may contribute to the enrichment-induced protective effect in response to drugs of abuse (Kanarek et al., 1995, Meeusen and De Meirleir, 1995, Lynch et al., 2010, Solinas et al., 2008, Gipson et al., 2011), isolating and determining which factor contributes to the neuroprotective-like phenotype is necessary for further understanding the role of environmental factors in nicotine abuse vulnerability.

5.3 CONCLUSIONS

Here, we have begun to lay the framework in establishing how environmental factors impact nicotine abuse vulnerability within preclinical models. We have potentially identified a universal miR, miR-221, mediated enrichment-induced neuroprotection in nicotine-mediated behaviors. Future studies examining the upstream and downstream mechanisms by which miR-221 is mediating its effects will better clarify the exact mechanism of miR-221. Additionally, by identifying the molecular mechanism(s) of enrichment-mediated neuroprotection in response to nicotine, we can better provide a foundation for understanding vulnerability to nicotine addiction and also, in the process, aid in identifying potential targets for therapeutics in the treatment of nicotine addiction.

EC-Nic vs. IC-Nic (Repeated nicotine 0.35 mg/kg; s.c.)

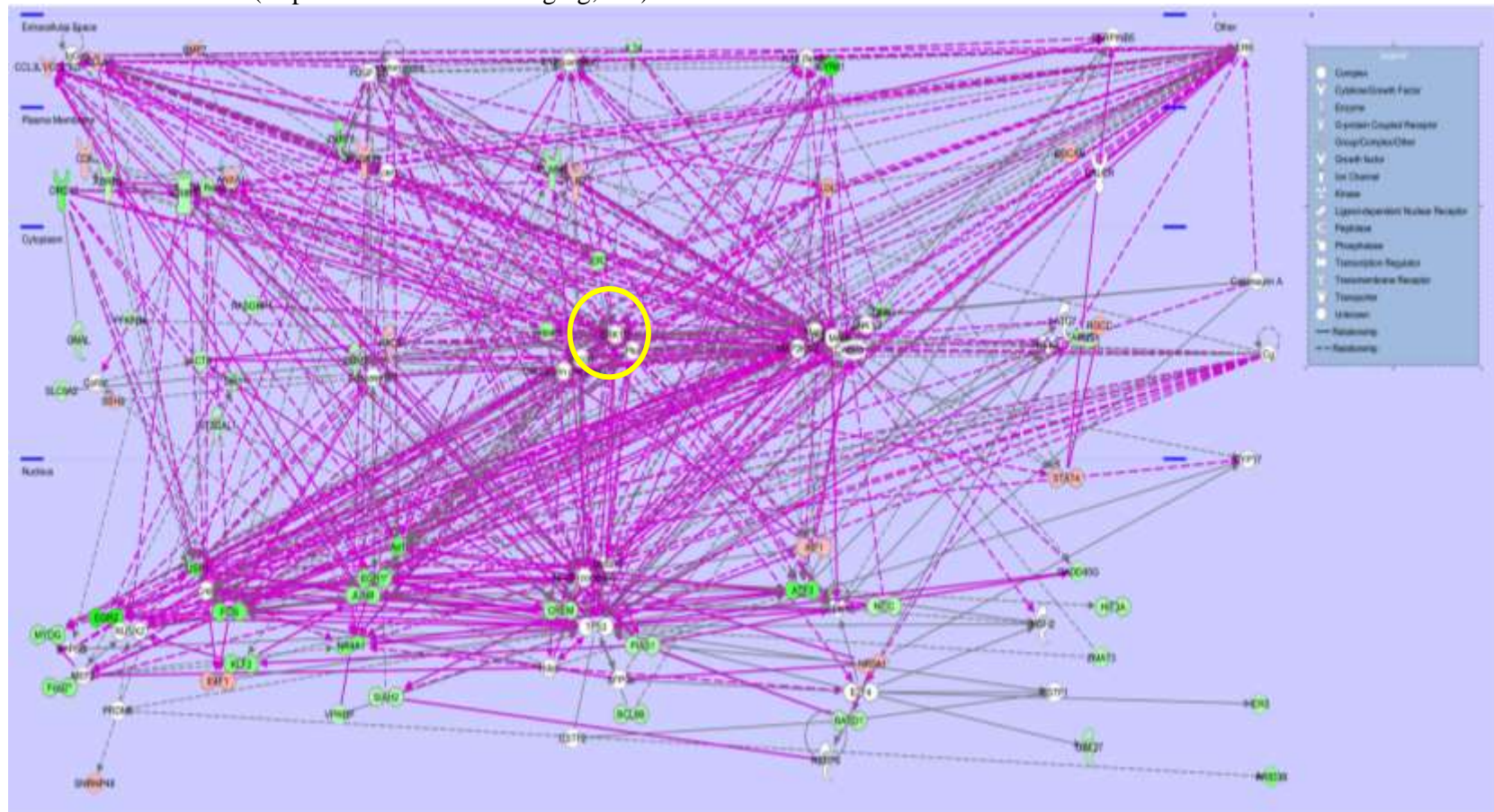


Figure 5.1. Ingenuity pathway analysis from microarray findings depicting differentially expressed mRNAs within the PFC of EC and IC rats that underwent repeated nicotine administration (0.35 mg/kg, s.c., 16 days). Data are represented as differentially expressed genes from the cellular level to the subcellular level where green symbols denote decreased mRNA levels and red symbols denote increased mRNA levels in EC rats compared to IC rats. Intensity of the color denotes greater fold change. Yellow circle highlights ERK1/2 signaling within the networks of the differentially expressed mRNA genes. n=4 rats/group.

EC-Nic vs. IC-Nic (Nicotine self-administration 0.03 mg/kg/infusion)

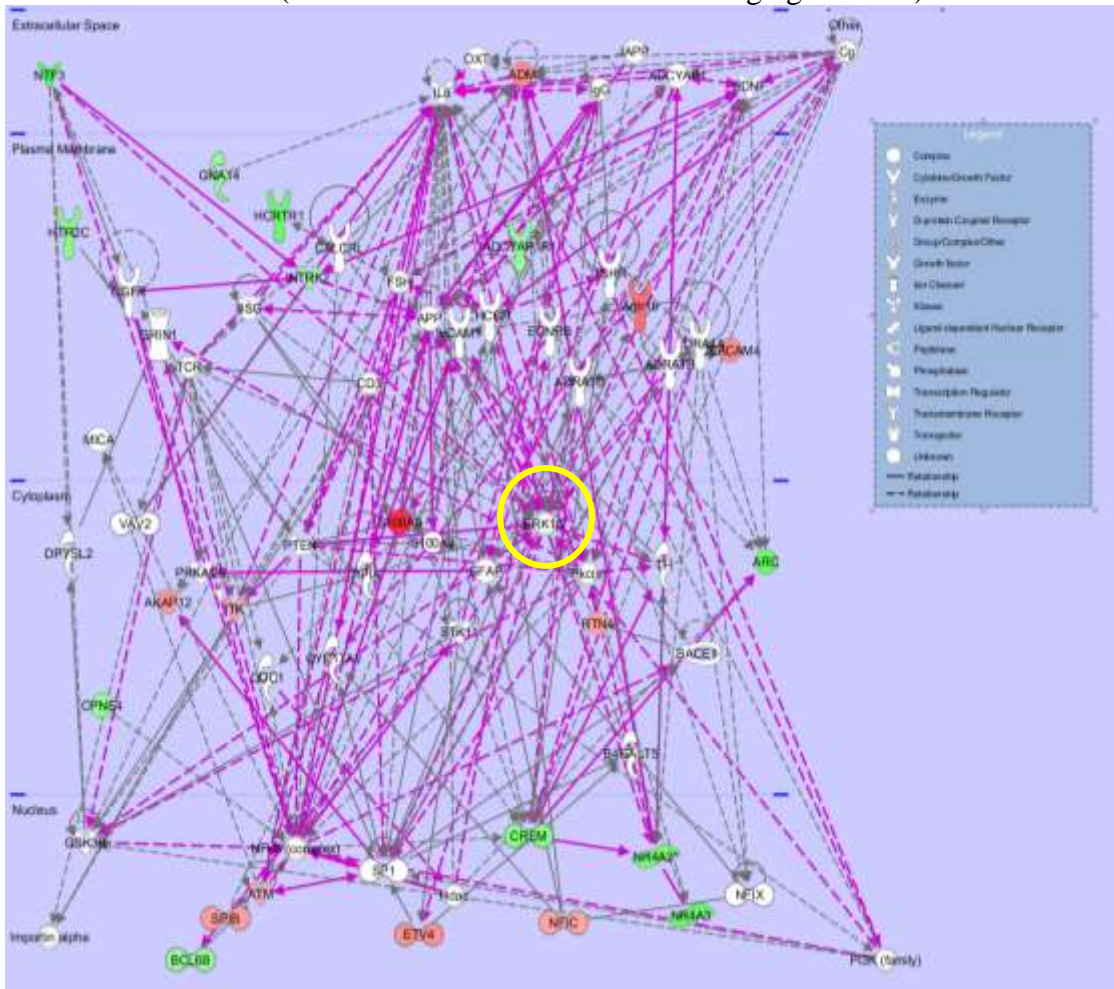


Figure 5.2. Ingenuity pathway analysis from microarray findings depicting differentially expressed mRNAs within the PFC of EC and IC rats that underwent nicotine self-administration (0.03 mg/kg/infusion, 21 days, FR1). Data are represented as differentially expressed genes from the cellular level to the subcellular level where green symbols denote decreased mRNA levels and red symbols denote increased mRNA levels in EC rats compared to IC rats. Intensity of the color denotes greater fold change. Yellow circle highlights ERK1/2 signaling within the networks of the differentially expressed mRNA genes. n=4 rats/group.

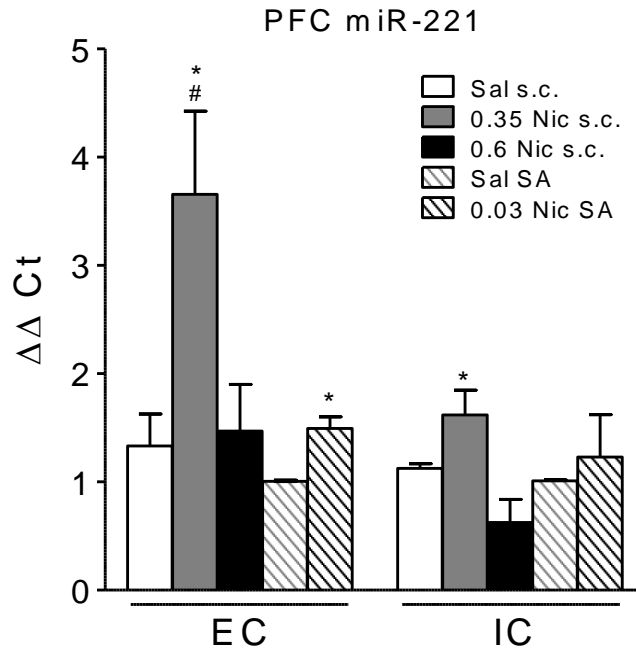


Figure 5.3. Preliminary studies involving qPCR validation of miR-221 from the PFC of EC and IC rats undergoing repeated saline or nicotine injections (0.35 mg/kg or 0.6 mg/kg, s.c.), and undergoing saline or nicotine self-administration (0.03 mg/kg/infusion). $\Delta\Delta\text{Ct}$ value is calculated by subtracting the ΔCt value of the control sample (miR-423-3p) from the ΔCt of miR-221. Data expressed as mean \pm SEM. * $p < 0.05$ denotes difference between the nicotine- and saline-treated groups. # $p < 0.05$ denotes difference in nicotine-treated groups among EC, IC and SC rats. $n=2-6$ rats/group. s.c.=subcutaneous administration, SA = self-administration.

REFERENCES

- ABDOLAH, A., ACOSTA, G., BRESLIN, F. J., HEMBY, S. E. & LYNCH, W. J. 2010. Incubation of nicotine seeking is associated with enhanced protein kinase A-regulated signaling of dopamine- and cAMP-regulated phosphoprotein of 32 kDa in the insular cortex. *Eur J Neurosci*, 31, 733-41.
- ADDY, N. A., FORNASIERO, E. F., STEVENS, T. R., TAYLOR, J. R. & PICCIOTTO, M. R. 2007. Role of calcineurin in nicotine-mediated locomotor sensitization. *J Neurosci*, 27, 8571-80.
- AHMED, S. H. & KOOB, G. F. 1998. Transition from moderate to excessive drug intake: change in hedonic set point. *Science*, 282, 298-300.
- ALVERS, K. M., MARUSICH, J. A., GIPSON, C. D., BECKMANN, J. S. & BARDO, M. T. 2012. Environmental enrichment during development decreases intravenous self-administration of methylphenidate at low unit doses in rats. *Behavioural Pharmacology*, 23, 650-7.
- AMBROGGI, F., TURIAULT, M., MILET, A., DEROCHE-GAMONET, V., PARNAUDEAU, S., BALADO, E., BARIK, J., VAN DER VEEN, R., MAROTEAUX, G., LEMBERGER, T., SCHUTZ, G., LAZAR, M., MARINELLI, M., PIAZZA, P. V. & TRONCHE, F. 2009. Stress and addiction: glucocorticoid receptor in dopaminergic neurons facilitates cocaine seeking. *Nat Neurosci*, 12, 247-9.
- AMMOUN, S., JOHANSSON, L., EKHOLM, M. E., HOLMQVIST, T., DANIS, A. S., KORHONEN, L., SERGEEVA, O. A., HAAS, H. L., AKERMAN, K. E. & KUKKONEN, J. P. 2006. OX1 orexin receptors activate extracellular signal-regulated kinase in Chinese hamster ovary cells via multiple mechanisms: the role of Ca²⁺ influx in OX1 receptor signaling. *Mol Endocrinol*, 20, 80-99.
- ARMARIO, A. 2010. Activation of the hypothalamic-pituitary-adrenal axis by addictive drugs: different pathways, common outcome. *Trends Pharmacol Sci*, 31, 318-25.
- BAHI, A. & DREYER, J. L. 2013. Striatal modulation of BDNF expression using microRNA124a-expressing lentiviral vectors impairs ethanol-induced conditioned-place preference and voluntary alcohol consumption. *European Journal of Neuroscience*, 38, 2328-37.

- BARDO, M. T., BOWLING, S. L., ROWLETT, J. K., MANDERSCHIED, P., BUXTON, S. T. & DWOSKIN, L. P. 1995. Environmental enrichment attenuates locomotor sensitization, but not in vitro dopamine release, induced by amphetamine. *Pharmacol Biochem Behav*, 51, 397-405.
- BARDO, M. T., KLEBAUR, J. E., VALONE, J. M. & DEATON, C. 2001. Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. *Psychopharmacology (Berl)*, 155, 278-84.
- BARDO, M. T., ROBINET, P. M. & HAMMER, R. F., JR. 1997. Effect of differential rearing environments on morphine-induced behaviors, opioid receptors and dopamine synthesis. *Neuropharmacology*, 36, 251-9.
- BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-97.
- BEAULIEU, J. M. & GAINETDINOV, R. R. 2011. The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev*, 63, 182-217.
- BENOWITZ, N. L. 2008. Clinical pharmacology of nicotine: implications for understanding, preventing, and treating tobacco addiction. *Clin Pharmacol Ther*, 83, 531-41.
- BENOWITZ, N. L. 2010. Nicotine addiction. *N Engl J Med*, 362, 2295-303.
- BERRIDGE, K. C. & ROBINSON, T. E. 1998. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Research Reviews*, 28, 309-369.
- BIBB, J. A., SNYDER, G. L., NISHI, A., YAN, Z., MEIJER, L., FIENBERG, A. A., TSAI, L. H., KWON, Y. T., GIRAULT, J. A., CZERNIK, A. J., HUGANIR, R. L., HEMMINGS, H. C., JR., NAIRN, A. C. & GREENGARD, P. 1999. Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature*, 402, 669-71.
- BOILEAU, I., DAGHER, A., LEYTON, M., GUNN, R. N., BAKER, G. B., DIKSIC, M. & BENKELFAT, C. 2006. Modeling sensitization to stimulants in humans: an [11C]raclopride/positron emission tomography study in healthy men. *Arch Gen Psychiatry*, 63, 1386-95.
- BOWLING, S. L. & BARDO, M. T. 1994. Locomotor and rewarding effects of amphetamine in enriched, social, and isolate reared rats. *Pharmacol Biochem Behav*, 48, 459-64.

- BOWLING, S. L., ROWLETT, J. K. & BARDO, M. T. 1993. The effect of environmental enrichment on amphetamine-stimulated locomotor activity, dopamine synthesis and dopamine release. *Neuropharmacology*, 32, 885-93.
- BRENES, J. C., RODRIGUEZ, O. & FORNAGUERA, J. 2008. Differential effect of environment enrichment and social isolation on depressive-like behavior, spontaneous activity and serotonin and norepinephrine concentration in prefrontal cortex and ventral striatum. *Pharmacol Biochem Behav*, 89, 85-93.
- BROOKS, A. M. & BERNS, G. S. 2013. Aversive stimuli and loss in the mesocorticolimbic dopamine system. *Trends Cogn Sci*, 17, 281-6.
- CARDINALI, B., CASTELLANI, L., FASANARO, P., BASSO, A., ALEMA, S., MARTELLI, F. & FALCONE, G. 2009. MicroRNA-221 and microRNA-222 modulate differentiation and maturation of skeletal muscle cells. *PLoS One*, 4, e7607.
- CARR, K. D., DE VACA, S. C., SUN, Y., CHAU, L. S., PAN, Y. & DELA CRUZ, J. 2009. Effects of the MEK inhibitor, SL-327, on rewarding, motor- and cellular-activating effects of D-amphetamine and SKF-82958, and their augmentation by food restriction in rat. *Psychopharmacology (Berl)*, 201, 495-506.
- CHANDRASEKAR, V. & DREYER, J. L. 2009. microRNAs miR-124, let-7d and miR-181a regulate Cocaine-induced Plasticity. *Molecular and Cellular Neuroscience*, 42, 350-362.
- CHANDRASEKAR, V. & DREYER, J. L. 2011. Regulation of MiR-124, Let-7d, and MiR-181a in the accumbens affects the expression, extinction, and reinstatement of cocaine-induced conditioned place preference. *Neuropsychopharmacology*, 36, 1149-64.
- CHAUVET, C., LARDEUX, V., GOLDBERG, S. R., JABER, M. & SOLINAS, M. 2009. Environmental enrichment reduces cocaine seeking and reinstatement induced by cues and stress but not by cocaine. *Neuropsychopharmacology*, 34, 2767-78.
- CHEN, X., LI, Y., KLINE, A. E., DIXON, C. E., ZAFONTE, R. D. & WAGNER, A. K. 2005. Gender and environmental effects on regional brain-derived neurotrophic factor expression after experimental traumatic brain injury. *Neuroscience*, 135, 11-7.
- CLARKE, P. B. & KUMAR, R. 1983a. Characterization of the locomotor stimulant action of nicotine in tolerant rats. *Br J Pharmacol*, 80, 587-94.
- CLARKE, P. B. & KUMAR, R. 1983b. The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. *Br J Pharmacol*, 78, 329-37.

- COMPTON, W. M., THOMAS, Y. F., CONWAY, K. P. & COLLIVER, J. D. 2005. Developments in the epidemiology of drug use and drug use disorders. *Am J Psychiatry*, 162, 1494-502.
- COOLON, R. A. & CAIN, M. E. 2009. Effects of mecamylamine on nicotine-induced conditioned hyperactivity and sensitization in differentially reared rats. *Pharmacol Biochem Behav*, 93, 59-66.
- DAJAS-BAILADOR, F. & WONNACOTT, S. 2004. Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol Sci*, 25, 317-24.
- DASH, P. K., KARL, K. A., COLICOS, M. A., PRYWES, R. & KANDEL, E. R. 1991. cAMP response element-binding protein is activated by Ca²⁺/calmodulin- as well as cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A*, 88, 5061-5.
- DAVIS, S., VANHOUTTE, P., PAGES, C., CABOCHE, J. & LAROCHE, S. 2000. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J Neurosci*, 20, 4563-72.
- DEEHAN, G. A., JR., PALMATIER, M. I., CAIN, M. E. & KIEFER, S. W. 2011. Differential rearing conditions and alcohol-preferring rats: consumption of and operant responding for ethanol. *Behav Neurosci*, 125, 184-93.
- DEL ARCO, A., SEGOVIA, G., CANALES, J. J., GARRIDO, P., DE BLAS, M., GARCIA-VERDUGO, J. M. & MORA, F. 2007a. Environmental enrichment reduces the function of D1 dopamine receptors in the prefrontal cortex of the rat. *J Neural Transm*, 114, 43-8.
- DEL ARCO, A., SEGOVIA, G., GARRIDO, P., DE BLAS, M. & MORA, F. 2007b. Stress, prefrontal cortex and environmental enrichment: studies on dopamine and acetylcholine release and working memory performance in rats. *Behav Brain Res*, 176, 267-73.
- DEROCHE, V., MARINELLI, M., LE MOAL, M. & PIAZZA, P. V. 1997. Glucocorticoids and behavioral effects of psychostimulants. II: cocaine intravenous self-administration and reinstatement depend on glucocorticoid levels. *J Pharmacol Exp Ther*, 281, 1401-7.
- DIERGAARDE, L., PATTIJ, T., POORTVLIET, I., HOGENBOOM, F., DE VRIES, W., SCHOFFELMEER, A. N. & DE VRIES, T. J. 2008. Impulsive choice and impulsive action predict vulnerability to distinct stages of nicotine seeking in rats. *Biol Psychiatry*, 63, 301-8.

- EKHOLM, M. E., JOHANSSON, L. & KUKKONEN, J. P. 2007. IP3-independent signalling of OX1 orexin/hypocretin receptors to Ca²⁺ influx and ERK. *Biochem Biophys Res Commun*, 353, 475-80.
- EL RAWAS, R., THIRIET, N., LARDEUX, V., JABER, M. & SOLINAS, M. 2009. Environmental enrichment decreases the rewarding but not the activating effects of heroin. *Psychopharmacology (Berl)*, 203, 561-70.
- ELCHEVA, I., GOSWAMI, S., NOUBISSI, F. K. & SPIEGELMAN, V. S. 2009. CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. *Mol Cell*, 35, 240-6.
- ESTELLER, M. 2011. Non-coding RNAs in human disease. *Nat Rev Genet*, 12, 861-74.
- EZZATI, M. & LOPEZ, A. D. 2003. Estimates of global mortality attributable to smoking in 2000. *Lancet*, 362, 847-52.
- FANG, Y. & SVOBODA, K. K. 2005. Nicotine inhibits human gingival fibroblast migration via modulation of Rac signalling pathways. *J Clin Periodontol*, 32, 1200-7.
- FERGUSON, S. M., FASANO, S., YANG, P., BRAMBILLA, R. & ROBINSON, T. E. 2006. Knockout of ERK1 enhances cocaine-evoked immediate early gene expression and behavioral plasticity. *Neuropsychopharmacology*, 31, 2660-8.
- FITZGERALD, M. L., MACKIE, K. & PICKEL, V. M. 2013. The impact of adolescent social isolation on dopamine D2 and cannabinoid CB1 receptors in the adult rat prefrontal cortex. *Neuroscience*, 235, 40-50.
- GAROFALO, M., DI LEVA, G., ROMANO, G., NUOVO, G., SUH, S. S., NGANKEU, A., TACCIOLI, C., PICHIORRI, F., ALDER, H., SECCHIERO, P., GASPARINI, P., GONELLI, A., COSTINEAN, S., ACUNZO, M., CONDORELLI, G. & CROCE, C. M. 2009. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. *Cancer Cell*, 16, 498-509.
- GARRIDO, P., DE BLAS, M., RONZONI, G., CORDERO, I., ANTON, M., GINE, E., SANTOS, A., DEL ARCO, A., SEGOVIA, G. & MORA, F. 2013. Differential effects of environmental enrichment and isolation housing on the hormonal and neurochemical responses to stress in the prefrontal cortex of the adult rat: relationship to working and emotional memories. *J Neural Transm*, 120, 829-43.
- GILL, K. E., BEVERIDGE, T. J., SMITH, H. R. & PORRINO, L. J. 2013. The effects of rearing environment and chronic methylphenidate administration on behavior and dopamine receptors in adolescent rats. *Brain Res*, 1527, 67-78.

- GIPSON, C. D., BECKMANN, J. S., EL-MARAGHI, S., MARUSICH, J. A. & BARDO, M. T. 2011. Effect of environmental enrichment on escalation of cocaine self-administration in rats. *Psychopharmacology (Berl)*, 214, 557-66.
- GIROD, R., BARAZANGI, N., MCGEHEE, D. & ROLE, L. W. 2000. Facilitation of glutamatergic neurotransmission by presynaptic nicotinic acetylcholine receptors. *Neuropharmacology*, 39, 2715-25.
- GOEDERS, N. E. & GUERIN, G. F. 1996. Role of corticosterone in intravenous cocaine self-administration in rats. *Neuroendocrinology*, 64, 337-48.
- GOMEZ, A. M., MIDDE, N. M., MACTUTUS, C. F., BOOZE, R. M. & ZHU, J. 2012. Environmental Enrichment Alters Nicotine-Mediated Locomotor Sensitization and Phosphorylation of DARPP-32 and CREB in Rat Prefrontal Cortex. *PLoS One*, 7, e44149.
- GRACE, A. A. 2000. The tonic/phasic model of dopamine system regulation and its implications for understanding alcohol and psychostimulant craving. *Addiction*, 95 Suppl 2, S119-28.
- GRACE, A. A., FLORESCO, S. B., GOTO, Y. & LODGE, D. J. 2007. Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci*, 30, 220-7.
- GREEN, T. A., ALIBHAI, I. N., ROYBAL, C. N., WINSTANLEY, C. A., THEOBALD, D. E., BIRNBAUM, S. G., GRAHAM, A. R., UNTERBERG, S., GRAHAM, D. L., VIALOU, V., BASS, C. E., TERWILLIGER, E. F., BARDO, M. T. & NESTLER, E. J. 2010. Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. *Biol Psychiatry*, 67, 28-35.
- GREEN, T. A., CAIN, M. E., THOMPSON, M. & BARDO, M. T. 2003a. Environmental enrichment decreases nicotine-induced hyperactivity in rats. *Psychopharmacology (Berl)*.
- GREEN, T. A., CAIN, M. E., THOMPSON, M. & BARDO, M. T. 2003b. Environmental enrichment decreases nicotine-induced hyperactivity in rats. *Psychopharmacology (Berl)*, 170, 235-41.
- GREEN, T. A., GEHRKE, B. J. & BARDO, M. T. 2002. Environmental enrichment decreases intravenous amphetamine self-administration in rats: dose-response functions for fixed- and progressive-ratio schedules. *Psychopharmacology (Berl)*, 162, 373-8.
- GREENGARD, P., ALLEN, P. B. & NAIRN, A. C. 1999. Beyond the Dopamine Receptor: the DARPP-32/Protein Phosphatase-1 Cascade. *Neuron*, 23, 435-447.

- GROBLEWSKI, P. A., FRANKEN, F. H. & CUNNINGHAM, C. L. 2011. Inhibition of extracellular signal-regulated kinase (ERK) activity with SL327 does not prevent acquisition, expression, and extinction of ethanol-seeking behavior in mice. *Behav Brain Res*, 217, 399-407.
- GUEGAN, T., CUTANDO, L., GANGAROSSA, G., SANTINI, E., FISONE, G., MARTINEZ, A., VALJENT, E., MALDONADO, R. & MARTIN, M. 2013. Operant behavior to obtain palatable food modifies ERK activity in the brain reward circuit. *Eur Neuropsychopharmacol*, 23, 240-52.
- HALL, F. S., WILKINSON, L. S., HUMBY, T., INGLIS, W., KENDALL, D. A., MARSDEN, C. A. & ROBBINS, T. W. 1998. Isolation rearing in rats: pre- and postsynaptic changes in striatal dopaminergic systems. *Pharmacol Biochem Behav*, 59, 859-72.
- HAMADA, M., HENDRICK, J. P., RYAN, G. R., KUROIWA, M., HIGASHI, H., TANAKA, M., NAIRN, A. C., GREENGARD, P. & NISHI, A. 2005. Nicotine regulates DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) phosphorylation at multiple sites in neostriatal neurons. *J Pharmacol Exp Ther*, 315, 872-8.
- HAMADA, M., HIGASHI, H., NAIRN, A. C., GREENGARD, P. & NISHI, A. 2004. Differential regulation of dopamine D1 and D2 signaling by nicotine in neostriatal neurons. *J Neurochem*, 90, 1094-103.
- HAMADA, N., FUJITA, Y., KOJIMA, T., KITAMOTO, A., AKAO, Y., NOZAWA, Y. & ITO, M. 2012. MicroRNA expression profiling of NGF-treated PC12 cells revealed a critical role for miR-221 in neuronal differentiation. *Neurochem Int*, 60, 743-50.
- HARROD, S. B., LACY, R. T. & MORGAN, A. J. 2012. Offspring of Prenatal IV Nicotine Exposure Exhibit Increased Sensitivity to the Reinforcing Effects of Methamphetamine. *Front Pharmacol*, 3, 116.
- HARROD, S. B., MACTUTUS, C. F., FITTING, S., HASSELROT, U. & BOOZE, R. M. 2008. Intra-accumbal Tat1-72 alters acute and sensitized responses to cocaine. *Pharmacol Biochem Behav*, 90, 723-9.
- HARROD, S. B. & VAN HORN, M. L. 2009. Sex differences in tolerance to the locomotor depressant effects of lobeline in periadolescent rats. *Pharmacol Biochem Behav*, 94, 296-304.
- HE, L. & HANNON, G. J. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*, 5, 522-31.

- HEMMINGS, H. C., JR., GREENGARD, P., TUNG, H. Y. & COHEN, P. 1984. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature*, 310, 503-5.
- HIROI, N., FIENBERG, A. A., HAILE, C. N., ALBURGES, M., HANSON, G. R., GREENGARD, P. & NESTLER, E. J. 1999. Neuronal and behavioural abnormalities in striatal function in DARPP-32-mutant mice. *Eur J Neurosci*, 11, 1114-8.
- HOLLANDER, J. A., IM, H. I., AMELIO, A. L., KOCERHA, J., BALI, P., LU, Q., WILLOUGHBY, D., WAHLESTEDT, C., CONKRIGHT, M. D. & KENNY, P. J. 2010. Striatal microRNA controls cocaine intake through CREB signalling. *Nature*, 466, 197-202.
- HOLLANDER, J. A., LU, Q., CAMERON, M. D., KAMENECKA, T. M. & KENNY, P. J. 2008. Insular hypocretin transmission regulates nicotine reward. *Proc Natl Acad Sci U S A*, 105, 19480-5.
- HOPFER, C. J., CROWLEY, T. J. & HEWITT, J. K. 2003. Review of twin and adoption studies of adolescent substance use. *J Am Acad Child Adolesc Psychiatry*, 42, 710-9.
- HORVITZ, J. C. 2000. Mesolimbocortical and nigrostriatal dopamine responses to salient non-reward events. *Neuroscience*, 96, 651-6.
- HOTTE, M., THUAULT, S., LACHAISE, F., DINELEY, K. T., HEMMINGS, H. C., NAIRN, A. C. & JAY, T. M. 2006. D1 receptor modulation of memory retrieval performance is associated with changes in pCREB and pDARPP-32 in rat prefrontal cortex. *Behav Brain Res*, 171, 127-33.
- HUANG, W. & LI, M. D. 2009. Nicotine modulates expression of miR-140*, which targets the 3'-untranslated region of dynamin 1 gene (Dnm1). *Int J Neuropsychopharmacol*, 12, 537-46.
- IM, H. I., HOLLANDER, J. A., BALI, P. & KENNY, P. J. 2010. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nat Neurosci*, 13, 1120-7.
- KALIVAS, P. W. 1995. Interactions between dopamine and excitatory amino acids in behavioral sensitization to psychostimulants. *Drug Alcohol Depend*, 37, 95-100.
- KANAREK, R. B., MARKS-KAUFMAN, R., D'ANCI, K. E. & PRZYPEK, J. 1995. Exercise attenuates oral intake of amphetamine in rats. *Pharmacol Biochem Behav*, 51, 725-9.

- KANE, J. K., PARKER, S. L., MATTA, S. G., FU, Y., SHARP, B. M. & LI, M. D. 2000. Nicotine up-regulates expression of orexin and its receptors in rat brain. *Endocrinology*, 141, 3623-9.
- KIRKPATRICK, K., MARSHALL, A. T., CLARKE, J. & CAIN, M. E. 2013. Environmental rearing effects on impulsivity and reward sensitivity. *Behav Neurosci*, 127, 712-24.
- KOOB, G. F. 1992. Neural mechanisms of drug reinforcement. *Ann N Y Acad Sci*, 654, 171-91.
- KOOB, G. F. 2004. Allostatic view of motivation: implications for psychopathology. *Nebr Symp Motiv*, 50, 1-18.
- KOOB, G. F., AHMED, S. H., BOUTREL, B., CHEN, S. A., KENNY, P. J., MARKOU, A., O'DELL, L. E., PARSONS, L. H. & SANNA, P. P. 2004. Neurobiological mechanisms in the transition from drug use to drug dependence. *Neurosci Biobehav Rev*, 27, 739-49.
- KOOB, G. F. & LE MOAL, M. 1997. Drug abuse: hedonic homeostatic dysregulation. *Science*, 278, 52-8.
- KOOB, G. F. & VOLKOW, N. D. 2010. Neurocircuitry of addiction. *Neuropsychopharmacology*, 35, 217-38.
- KOSIK, K. S. 2006. The neuronal microRNA system. *Nat Rev Neurosci*, 7, 911-20.
- KOZOROVITSKIY, Y., GROSS, C. G., KOPIL, C., BATTAGLIA, L., MCBREEN, M., STRANAHAN, A. M. & GOULD, E. 2005. Experience induces structural and biochemical changes in the adult primate brain. *Proc Natl Acad Sci U S A*, 102, 17478-82.
- LACY, R. T., HORD, L. L., MORGAN, A. J. & HARROD, S. B. 2012. Intravenous gestational nicotine exposure results in increased motivation for sucrose reward in adult rat offspring. *Drug Alcohol Depend*, 124, 299-306.
- LAGOS-QUINTANA, M., RAUHUT, R., YALCIN, A., MEYER, J., LENDECKEL, W. & TUSCHL, T. 2002. Identification of tissue-specific microRNAs from mouse. *Curr Biol*, 12, 735-9.
- LAVIOLETTE, S. R. & VAN DER KOOY, D. 2004. The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci*, 5, 55-65.
- LEE, R. C. & AMBROS, V. 2001. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*, 294, 862-4.

- LEE, R. C., FEINBAUM, R. L. & AMBROS, V. 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 75, 843-54.
- LEE, Y., AHN, C., HAN, J., CHOI, H., KIM, J., YIM, J., LEE, J., PROVOST, P., RADMARK, O., KIM, S. & KIM, V. N. 2003. The nuclear RNase III Droscha initiates microRNA processing. *Nature*, 425, 415-9.
- LEE, Y., JEON, K., LEE, J. T., KIM, S. & KIM, V. N. 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, 21, 4663-70.
- LEHMANN, M. L. & HERKENHAM, M. 2011. Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. *J Neurosci*, 31, 6159-73.
- LESAGE, M. G., PERRY, J. L., KOTZ, C. M., SHELLEY, D. & CORRIGALL, W. A. 2010. Nicotine self-administration in the rat: effects of hypocretin antagonists and changes in hypocretin mRNA. *Psychopharmacology (Berl)*, 209, 203-12.
- LESHNER, A. I. 2000. Vulnerability to addiction: new research opportunities. *Am J Med Genet*, 96, 590-1.
- LI, M. D. & VAN DER VAART, A. D. 2011. MicroRNAs in addiction: adaptation's middlemen? *Mol Psychiatry*, 16, 1159-68.
- LINDSKOG, M., SVENNINGSSON, P., FREDHOLM, B. B., GREENGARD, P. & FISONE, G. 1999. Activation of dopamine D2 receptors decreases DARPP-32 phosphorylation in striatonigral and striatopallidal projection neurons via different mechanisms. *Neuroscience*, 88, 1005-8.
- LIPPI, G., STEINERT, J. R., MARCZYLO, E. L., D'ORO, S., FIORE, R., FORSYTHE, I. D., SCHRATT, G., ZOLI, M., NICOTERA, P. & YOUNG, K. W. 2011. Targeting of the Arpc3 actin nucleation factor by miR-29a/b regulates dendritic spine morphology. *J Cell Biol*, 194, 889-904.
- LOOS, M., PATTIJ, T., JANSSEN, M. C., COUNOTTE, D. S., SCHOFFELMEER, A. N., SMIT, A. B., SPIJKER, S. & VAN GAALEN, M. M. 2010. Dopamine receptor D1/D5 gene expression in the medial prefrontal cortex predicts impulsive choice in rats. *Cereb Cortex*, 20, 1064-70.
- LUTFY, K., AIMIUWU, O., MANGUBAT, M., SHIN, C. S., NERIO, N., GOMEZ, R., LIU, Y. & FRIEDMAN, T. C. 2012. Nicotine stimulates secretion of corticosterone via both CRH and AVP receptors. *J Neurochem*, 120, 1108-16.

- LYNCH, W. J., PIEHL, K. B., ACOSTA, G., PETERSON, A. B. & HEMBY, S. E. 2010. Aerobic exercise attenuates reinstatement of cocaine-seeking behavior and associated neuroadaptations in the prefrontal cortex. *Biol Psychiatry*, 68, 774-7.
- MAHLER, S. V., SMITH, R. J., MOORMAN, D. E., SARTOR, G. C. & ASTON-JONES, G. 2012. Multiple roles for orexin/hypocretin in addiction. *Prog Brain Res*, 198, 79-121.
- MANSVELDER, H. D., KEATH, J. R. & MCGEHEE, D. S. 2002. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron*, 33, 905-19.
- MANSVELDER, H. D. & MCGEHEE, D. S. 2002. Cellular and synaptic mechanisms of nicotine addiction. *J Neurobiol*, 53, 606-17.
- MATHERS, C. D. & LONCAR, D. 2006. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med*, 3, e442.
- MAZZUCHELLI, C., VANTAGGIATO, C., CIAMEI, A., FASANO, S., PAKHOTIN, P., KREZEL, W., WELZL, H., WOLFER, D. P., PAGES, G., VALVERDE, O., MAROWSKY, A., PORRAZZO, A., ORBAN, P. C., MALDONADO, R., EHRENGRUBER, M. U., CESTARI, V., LIPP, H. P., CHAPMAN, P. F., POUYSSEGUR, J. & BRAMBILLA, R. 2002. Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. *Neuron*, 34, 807-20.
- MCGEHEE, D. S., HEATH, M. J., GELBER, S., DEVAY, P. & ROLE, L. W. 1995. Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science*, 269, 1692-6.
- MCGEHEE, D. S. & ROLE, L. W. 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol*, 57, 521-46.
- MCGUE, M., LYKKEN, D. T. & IACONO, W. G. 1996. Genotype-environment correlations and interactions in the etiology of substance abuse and related behaviors. *NIDA Res Monogr*, 159, 49-72; discussion 73-80.
- MEEUSEN, R. & DE MEIRLEIR, K. 1995. Exercise and brain neurotransmission. *Sports Med*, 20, 160-88.
- MELLENDEZ, R. I., GREGORY, M. L., BARDO, M. T. & KALIVAS, P. W. 2004. Impoverished rearing environment alters metabotropic glutamate receptor expression and function in the prefrontal cortex. *Neuropsychopharmacology*, 29, 1980-7.

- MIDDE, N. M., GOMEZ, A. M., HARROD, S. B. & ZHU, J. 2011. Genetically expressed HIV-1 viral proteins attenuate nicotine-induced behavioral sensitization and alter mesocorticolimbic ERK and CREB signaling in rats. *Pharmacol Biochem Behav*, 98, 587-97.
- MILASTA, S., EVANS, N. A., ORMISTON, L., WILSON, S., LEFKOWITZ, R. J. & MILLIGAN, G. 2005. The sustainability of interactions between the orexin-1 receptor and beta-arrestin-2 is defined by a single C-terminal cluster of hydroxy amino acids and modulates the kinetics of ERK MAPK regulation. *Biochem J*, 387, 573-84.
- MILLER, C. A. & MARSHALL, J. F. 2005. Molecular substrates for retrieval and reconsolidation of cocaine-associated contextual memory. *Neuron*, 47, 873-84.
- MISHIMA, T., MIZUGUCHI, Y., KAWAHIGASHI, Y., TAKIZAWA, T. & TAKIZAWA, T. 2007. RT-PCR-based analysis of microRNA (miR-1 and -124) expression in mouse CNS. *Brain Res*, 1131, 37-43.
- MYCHASIUK, R., MUHAMMAD, A. & KOLB, B. 2014. Environmental Enrichment Alters Structural Plasticity of the Adolescent Brain But Does Not Remediate the Effects of Prenatal Nicotine Exposure. *Synapse*.
- NAIRN, A. C., SVENNINGSSON, P., NISHI, A., FISONE, G., GIRAULT, J. A. & GREENGARD, P. 2004. The role of DARPP-32 in the actions of drugs of abuse. *Neuropharmacology*, 47 Suppl 1, 14-23.
- NAKA, F., SHIGA, T., YAGUCHI, M. & OKADO, N. 2002. An enriched environment increases noradrenaline concentration in the mouse brain. *Brain Res*, 924, 124-6.
- NAKAYAMA, H., NUMAKAWA, T., IKEUCHI, T. & HATANAKA, H. 2001. Nicotine-induced phosphorylation of extracellular signal-regulated protein kinase and CREB in PC12h cells. *J Neurochem*, 79, 489-98.
- NEDDENS, J., BRANDENBURG, K., TEUCHERT-NOODT, G. & DAWIRS, R. R. 2001. Differential environment alters ontogeny of dopamine innervation of the orbital prefrontal cortex in gerbils. *J Neurosci Res*, 63, 209-13.
- NEUGEBAUER, N. M., CUNNINGHAM, S. T., ZHU, J., BRYANT, R. I., MIDDLETON, L. S. & DWOSKIN, L. P. 2004. Effects of environmental enrichment on behavior and dopamine transporter function in medial prefrontal cortex in adult rats prenatally treated with cocaine. *Brain Res Dev Brain Res*, 153, 213-23.
- NICHOLS, J. A., JAKKAMSETTI, V. P., SALGADO, H., DINH, L., KILGARD, M. P. & ATZORI, M. 2007. Environmental enrichment selectively increases

- glutamatergic responses in layer II/III of the auditory cortex of the rat. *Neuroscience*, 145, 832-40.
- NISHI, A., BIBB, J. A., SNYDER, G. L., HIGASHI, H., NAIRN, A. C. & GREENGARD, P. 2000. Amplification of dopaminergic signaling by a positive feedback loop. *Proc Natl Acad Sci U S A*, 97, 12840-5.
- NITHIANANTHARAJAH, J., BARKUS, C., MURPHY, M. & HANNAN, A. J. 2008. Gene-environment interactions modulating cognitive function and molecular correlates of synaptic plasticity in Huntington's disease transgenic mice. *Neurobiology of Disease*, 29, 490-504.
- NOCJAR, C. & PANKSEPP, J. 2002. Chronic intermittent amphetamine pretreatment enhances future appetitive behavior for drug- and natural-reward: interaction with environmental variables. *Behav Brain Res*, 128, 189-203.
- NOCJAR, C. & PANKSEPP, J. 2007. Prior morphine experience induces long-term increases in social interest and in appetitive behavior for natural reward. *Behav Brain Res*, 181, 191-9.
- OROM, U. A., NIELSEN, F. C. & LUND, A. H. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell*, 30, 460-71.
- PANLILIO, L. V., THORNDIKE, E. B. & SCHINDLER, C. W. 2008. A stimulus-control account of regulated drug intake in rats. *Psychopharmacology (Berl)*, 196, 441-50.
- PARDEY, M. C., KUMAR, N. N., GOODCHILD, A. K. & CORNISH, J. L. 2013. Catecholamine receptors differentially mediate impulsive choice in the medial prefrontal and orbitofrontal cortex. *J Psychopharmacol*, 27, 203-12.
- PASUMARTHI, R. K., REZNIKOV, L. R. & FADEL, J. 2006. Activation of orexin neurons by acute nicotine. *Eur J Pharmacol*, 535, 172-6.
- PAULSON, P. E., CAMP, D. M. & ROBINSON, T. E. 1991. Time course of transient behavioral depression and persistent behavioral sensitization in relation to regional brain monoamine concentrations during amphetamine withdrawal in rats. *Psychopharmacology (Berl)*, 103, 480-92.
- PAXINOS, G. & WATSON, C. 2007. The rat brain in stereotaxic coordinates. 6th Edition.
- PERRY, J. L., JOSEPH, J. E., JIANG, Y., ZIMMERMAN, R. S., KELLY, T. H., DARNA, M., HUETTL, P., DWOSKIN, L. P. & BARDO, M. T. 2011. Prefrontal cortex and drug abuse vulnerability: translation to prevention and treatment interventions. *Brain Res Rev*, 65, 124-49.

- PERRY, J. L., STAIRS, D. J. & BARDO, M. T. 2008. Impulsive choice and environmental enrichment: effects of d-amphetamine and methylphenidate. *Behav Brain Res*, 193, 48-54.
- PETO, R., LOPEZ, A. D., BOREHAM, J., THUN, M., HEATH, C., JR. & DOLL, R. 1996. Mortality from smoking worldwide. *Br Med Bull*, 52, 12-21.
- PINAUD, R., PENNER, M. R., ROBERTSON, H. A. & CURRIE, R. W. 2001. Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res Mol Brain Res*, 91, 50-6.
- REICHEL, C. M., LINKUGEL, J. D. & BEVINS, R. A. 2008. Bupropion differentially impacts acquisition of methamphetamine self-administration and sucrose-maintained behavior. *Pharmacol Biochem Behav*, 89, 463-72.
- REZVANI, A. H., EDDINS, D., SLADE, S., HAMPTON, D. S., CHRISTOPHER, N. C., PETRO, A., HORTON, K., JOHNSON, M. & LEVIN, E. D. 2008. Neonatal 6-hydroxydopamine lesions of the frontal cortex in rats: persisting effects on locomotor activity, learning and nicotine self-administration. *Neuroscience*, 154, 885-97.
- RHEE, S. H., HEWITT, J. K., YOUNG, S. E., CORLEY, R. P., CROWLEY, T. J. & STALLINGS, M. C. 2003. Genetic and environmental influences on substance initiation, use, and problem use in adolescents. *Arch Gen Psychiatry*, 60, 1256-64.
- RICHARDSON, N. R. & ROBERTS, D. C. 1996. Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods*, 66, 1-11.
- ROBINSON, T. E. & BECKER, J. B. 1986. Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Res*, 396, 157-98.
- ROBINSON, T. E. & BERRIDGE, K. C. 1993. The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev*, 18, 247-91.
- ROBINSON, T. E. & BERRIDGE, K. C. 2008. Review. The incentive sensitization theory of addiction: some current issues. *Philos Trans R Soc Lond B Biol Sci*, 363, 3137-46.
- ROLE, L. W. & BERG, D. K. 1996. Nicotinic receptors in the development and modulation of CNS synapses. *Neuron*, 16, 1077-85.

- ROSENZWEIG, M. R., BENNETT, E. L., HEBERT, M. & MORIMOTO, H. 1978. Social grouping cannot account for cerebral effects of enriched environments. *Brain Res*, 153, 563-76.
- RUEDA, A. V., TEIXEIRA, A. M., YONAMINE, M. & CAMARINI, R. 2012. Environmental enrichment blocks ethanol-induced locomotor sensitization and decreases BDNF levels in the prefrontal cortex in mice. *Addiction Biology*, 17, 736-45.
- SALAMONE, J. D. & CORREA, M. 2012. The mysterious motivational functions of mesolimbic dopamine. *Neuron*, 76, 470-85.
- SCHMITT, J. M., GUIRE, E. S., SANEYOSHI, T. & SODERLING, T. R. 2005. Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. *J Neurosci*, 25, 1281-90.
- SEGOVIA, G., DEL ARCO, A., GARRIDO, P., DE BLAS, M. & MORA, F. 2008. Environmental enrichment reduces the response to stress of the cholinergic system in the prefrontal cortex during aging. *Neurochem Int*, 52, 1198-203.
- SEMPERE, L. F., FREEMANTLE, S., PITHA-ROWE, I., MOSS, E., DMITROVSKY, E. & AMBROS, V. 2004. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol*, 5, R13.
- SHI, X. & MCGINTY, J. F. 2006. Extracellular signal-regulated mitogen-activated protein kinase inhibitors decrease amphetamine-induced behavior and neuropeptide gene expression in the striatum. *Neuroscience*, 138, 1289-98.
- SHTUTMAN, M., MALIYEKKEKEL, A., SHAO, Y., CARMACK, C. S., BAIG, M., WARHOLIC, N., COLE, K., BROUDE, E. V., HARKINS, T. T., DING, Y. & RONINSON, I. B. 2010. Function-based gene identification using enzymatically generated normalized shRNA library and massive parallel sequencing. *Proc Natl Acad Sci U S A*, 107, 7377-82.
- SHUM, F. W., WU, L. J., ZHAO, M. G., TOYODA, H., XU, H., REN, M., PINAUD, R., KO, S. W., LEE, Y. S., KAANG, B. K. & ZHUO, M. 2007. Alteration of cingulate long-term plasticity and behavioral sensitization to inflammation by environmental enrichment. *Learn Mem*, 14, 304-12.
- SIMPSON, J. & KELLY, J. P. 2011. The impact of environmental enrichment in laboratory rats--behavioural and neurochemical aspects. *Behav Brain Res*, 222, 246-64.

- SKWARA, A. J., KARWOSKI, T. E., CZAMBEL, R. K., RUBIN, R. T. & RHODES, M. E. 2012. Influence of environmental enrichment on hypothalamic-pituitary-adrenal (HPA) responses to single-dose nicotine, continuous nicotine by osmotic mini-pumps, and nicotine withdrawal by mecamylamine in male and female rats. *Behav Brain Res*, 234, 1-10.
- SMITH, J. K., NEILL, J. C. & COSTALL, B. 1997. Post-weaning housing conditions influence the behavioural effects of cocaine and d-amphetamine. *Psychopharmacology (Berl)*, 131, 23-33.
- SOKOLOWSKI, J. D., MCCULLOUGH, L. D. & SALAMONE, J. D. 1994. Effects of dopamine depletions in the medial prefrontal cortex on active avoidance and escape in the rat. *Brain Res*, 651, 293-9.
- SOLINAS, M., CHAUVET, C., THIRIET, N., EL RAWAS, R. & JABER, M. 2008. Reversal of cocaine addiction by environmental enrichment. *Proc Natl Acad Sci U S A*, 105, 17145-50.
- SOLINAS, M., THIRIET, N., CHAUVET, C. & JABER, M. 2010. Prevention and treatment of drug addiction by environmental enrichment. *Prog Neurobiol*, 92, 572-92.
- SPANOS, M., BESHEER, J. & HODGE, C. W. 2012. Increased sensitivity to alcohol induced changes in ERK Map kinase phosphorylation and memory disruption in adolescent as compared to adult C57BL/6J mice. *Behav Brain Res*, 230, 158-66.
- STAIRS, D. J. & BARDO, M. T. 2009. Neurobehavioral effects of environmental enrichment and drug abuse vulnerability. *Pharmacol Biochem Behav*, 92, 377-82.
- STAIRS, D. J., PRENDERGAST, M. A. & BARDO, M. T. 2011. Environmental-induced differences in corticosterone and glucocorticoid receptor blockade of amphetamine self-administration in rats. *Psychopharmacology (Berl)*, 218, 293-301.
- STANWOOD, G. D. 2008. Protein-protein interactions and dopamine D2 receptor signaling: a calcium connection. *Mol Pharmacol*, 74, 317-9.
- STEINER, R. C., HEATH, C. J. & PICCIOTTO, M. R. 2007. Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII. *J Neurochem*, 103, 666-78.
- STOLERMAN, I. P. & JARVIS, M. J. 1995. The scientific case that nicotine is addictive. *Psychopharmacology (Berl)*, 117, 2-10; discussion 14-20.
- STRAKOWSKI, S. M., SAX, K. W., ROSENBERG, H. L., DELBELLO, M. P. & ADLER, C. M. 2001. Human response to repeated low-dose d-amphetamine:

- evidence for behavioral enhancement and tolerance. *Neuropsychopharmacology*, 25, 548-54.
- STRAKOWSKI, S. M., SAX, K. W., SETTERS, M. J. & KECK, P. E., JR. 1996. Enhanced response to repeated d-amphetamine challenge: evidence for behavioral sensitization in humans. *Biol Psychiatry*, 40, 872-80.
- SVENNINGSSON, P., LINDSKOG, M., ROGNONI, F., FREDHOLM, B. B., GREENGARD, P. & FISONE, G. 1998. Activation of adenosine A2A and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. *Neuroscience*, 84, 223-8.
- SVENNINGSSON, P., NAIRN, A. C. & GREENGARD, P. 2005. DARPP-32 mediates the actions of multiple drugs of abuse. *AAPS J*, 7, E353-60.
- SVENNINGSSON, P., NISHI, A., FISONE, G., GIRAULT, J. A., NAIRN, A. C. & GREENGARD, P. 2004. DARPP-32: an integrator of neurotransmission. *Annu Rev Pharmacol Toxicol*, 44, 269-96.
- TAKI, F. A., PAN, X. & ZHANG, B. 2014. Chronic nicotine exposure systemically alters microRNA expression profiles during post-embryonic stages in *Caenorhabditis elegans*. *J Cell Physiol*, 229, 79-89.
- TANG, Y. P., WANG, H., FENG, R., KYIN, M. & TSIEN, J. Z. 2001. Differential effects of enrichment on learning and memory function in NR2B transgenic mice. *Neuropharmacology*, 41, 779-90.
- TAPOCIK, J. D., BARBIER, E., FLANIGAN, M., SOLOMON, M., PINCUS, A., PILLING, A., SUN, H., SCHANK, J. R., KING, C. & HEILIG, M. 2014. microRNA-206 in Rat Medial Prefrontal Cortex Regulates BDNF Expression and Alcohol Drinking. *J Neurosci*, 34, 4581-8.
- TERASAWA, K., ICHIMURA, A., SATO, F., SHIMIZU, K. & TSUJIMOTO, G. 2009. Sustained activation of ERK1/2 by NGF induces microRNA-221 and 222 in PC12 cells. *FEBS J*, 276, 3269-76.
- THIEL, K. J., ENGELHARDT, B., HOOD, L. E., PEARTREE, N. A. & NEISEWANDER, J. L. 2011. The interactive effects of environmental enrichment and extinction interventions in attenuating cue-elicited cocaine-seeking behavior in rats. *Pharmacol Biochem Behav*, 97, 595-602.
- THIEL, K. J., PENTKOWSKI, N. S., PEARTREE, N. A., PAINTER, M. R. & NEISEWANDER, J. L. 2010. Environmental living conditions introduced during forced abstinence alter cocaine-seeking behavior and Fos protein expression. *Neuroscience*, 171, 1187-96.

- THIRIET, N., GENNEQUIN, B., LARDEUX, V., CHAUVET, C., DECRESSAC, M., JANET, T., JABER, M. & SOLINAS, M. 2011. Environmental enrichment does not reduce the rewarding and neurotoxic effects of methamphetamine. *Neurotox Res*, 19, 172-82.
- TZSCHENTKE, T. M. 2001. Pharmacology and behavioral pharmacology of the mesocortical dopamine system. *Prog Neurobiol*, 63, 241-320.
- VALJENT, E., BERTRAN-GONZALEZ, J., AUBIER, B., GREENGARD, P., HERVE, D. & GIRAULT, J. A. 2010. Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. *Neuropsychopharmacology*, 35, 401-15.
- VALJENT, E., CORBILLE, A. G., BERTRAN-GONZALEZ, J., HERVE, D. & GIRAULT, J. A. 2006a. Inhibition of ERK pathway or protein synthesis during reexposure to drugs of abuse erases previously learned place preference. *Proc Natl Acad Sci U S A*, 103, 2932-7.
- VALJENT, E., CORVOL, J. C., PAGES, C., BESSON, M. J., MALDONADO, R. & CABOCHE, J. 2000. Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J Neurosci*, 20, 8701-9.
- VALJENT, E., CORVOL, J. C., TRZASKOS, J. M., GIRAULT, J. A. & HERVE, D. 2006b. Role of the ERK pathway in psychostimulant-induced locomotor sensitization. *BMC Neurosci*, 7, 20.
- VALJENT, E., PAGES, C., HERVE, D., GIRAULT, J. A. & CABOCHE, J. 2004a. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. *European Journal of Neuroscience*, 19, 1826-36.
- VALJENT, E., PAGES, C., HERVE, D., GIRAULT, J. A. & CABOCHE, J. 2004b. Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. *Eur J Neurosci*, 19, 1826-36.
- VALJENT, E., PASCOLI, V., SVENNINGSSON, P., PAUL, S., ENSLEN, H., CORVOL, J. C., STIPANOVICH, A., CABOCHE, J., LOMBROSO, P. J., NAIRN, A. C., GREENGARD, P., HERVE, D. & GIRAULT, J. A. 2005. Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci U S A*, 102, 491-6.
- VENEBRA-MUNOZ, A., CORONA-MORALES, A., SANTIAGO-GARCIA, J., MELGAREJO-GUTIERREZ, M., CABA, M. & GARCIA-GARCIA, F. 2014. Enriched environment attenuates nicotine self-administration and induces changes in DeltaFosB expression in the rat prefrontal cortex and nucleus accumbens. *Neuroreport*.

- VICKERY, R. G. & VON ZASTROW, M. 1999. Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J Cell Biol*, 144, 31-43.
- VOLMAN, S. F., LAMMEL, S., MARGOLIS, E. B., KIM, Y., RICHARD, J. M., ROITMAN, M. F. & LOBO, M. K. 2013. New insights into the specificity and plasticity of reward and aversion encoding in the mesolimbic system. *J Neurosci*, 33, 17569-76.
- WINTERFELD, K. T., TEUCHERT-NOODT, G. & DAWIRS, R. R. 1998. Social environment alters both ontogeny of dopamine innervation of the medial prefrontal cortex and maturation of working memory in gerbils (*Meriones unguiculatus*). *J Neurosci Res*, 52, 201-9.
- WISE, R. A. & BOZARTH, M. A. 1987. A psychomotor stimulant theory of addiction. *Psychol Rev*, 94, 469-92.
- WOOD, D. A., SIEGEL, A. K. & REBEC, G. V. 2006. Environmental enrichment reduces impulsivity during appetitive conditioning. *Physiol Behav*, 88, 132-7.
- WOOTERS, T. E., BARDO, M. T., DWOSKIN, L. P., MIDDE, N. M., GOMEZ, A. M., MACTUTUS, C. F., BOOZE, R. M. & ZHU, J. 2011. Effect of environmental enrichment on methylphenidate-induced locomotion and dopamine transporter dynamics. *Behav Brain Res*, 219, 98-107.
- YI, R., QIN, Y., MACARA, I. G. & CULLEN, B. R. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, 17, 3011-6.
- YU, G., CHEN, H., ZHAO, W., MATTA, S. G. & SHARP, B. M. 2008. Nicotine self-administration differentially regulates hypothalamic corticotropin-releasing factor and arginine vasopressin mRNAs and facilitates stress-induced neuronal activation. *J Neurosci*, 28, 2773-82.
- YU, G. & SHARP, B. M. 2010. Nicotine self-administration diminishes stress-induced norepinephrine secretion but augments adrenergic-responsiveness in the hypothalamic paraventricular nucleus and enhances adrenocorticotrophic hormone and corticosterone release. *J Neurochem*, 112, 1327-37.
- YU, G. & SHARP, B. M. 2012. Nicotine modulates multiple regions in the limbic stress network regulating activation of hypophysiotrophic neurons in hypothalamic paraventricular nucleus. *J Neurochem*, 122, 628-40.
- ZACHARIOU, V., SGAMBATO-FAURE, V., SASAKI, T., SVENNINGSSON, P., BERTON, O., FIENBERG, A. A., NAIRN, A. C., GREENGARD, P. &

- NESTLER, E. J. 2006. Phosphorylation of DARPP-32 at Threonine-34 is required for cocaine action. *Neuropsychopharmacology*, 31, 555-62.
- ZHAI, H., LI, Y., WANG, X. & LU, L. 2008. Drug-induced alterations in the extracellular signal-regulated kinase (ERK) signalling pathway: implications for reinforcement and reinstatement. *Cell Mol Neurobiol*, 28, 157-72.
- ZHANG, X., MAO, H., CHEN, J. Y., WEN, S., LI, D., YE, M. & LV, Z. 2013. Increased expression of microRNA-221 inhibits PAK1 in endothelial progenitor cells and impairs its function via c-Raf/MEK/ERK pathway. *Biochem Biophys Res Commun*, 431, 404-8.
- ZHANG, Y., SVENNINGSSON, P., PICETTI, R., SCHLUSSMAN, S. D., NAIRN, A. C., HO, A., GREENGARD, P. & KREEK, M. J. 2006. Cocaine self-administration in mice is inversely related to phosphorylation at Thr34 (protein kinase A site) and Ser130 (kinase CK1 site) of DARPP-32. *J Neurosci*, 26, 2645-51.
- ZHEN, X., URYU, K., CAI, G., JOHNSON, G. P. & FRIEDMAN, E. 1999. Age-associated impairment in brain MAPK signal pathways and the effect of caloric restriction in Fischer 344 rats. *J Gerontol A Biol Sci Med Sci*, 54, B539-48.
- ZHU, H., LEE, M., GUAN, F., AGATSUMA, S., SCOTT, D., FABRIZIO, K., FIENBERG, A. A. & HIROI, N. 2005a. DARPP-32 phosphorylation opposes the behavioral effects of nicotine. *Biol Psychiatry*, 58, 981-9.
- ZHU, J., APPARSUNDARAM, S., BARDO, M. T. & DWOSKIN, L. P. 2005b. Environmental enrichment decreases cell surface expression of the dopamine transporter in rat medial prefrontal cortex. *J Neurochem*, 93, 1434-43.
- ZHU, J., BARDO, M. T. & DWOSKIN, L. P. 2013. Distinct effects of enriched environment on dopamine clearance in nucleus accumbens shell and core following systemic nicotine administration. *Synapse*, 67, 57-67.
- ZHU, J., BARDO, M. T., GREEN, T. A., WEDLUND, P. J. & DWOSKIN, L. P. 2007a. Nicotine increases dopamine clearance in medial prefrontal cortex in rats raised in an enriched environment. *J Neurochem*, 103, 2575-88.
- ZHU, J., BARDO, M. T., GREEN, T. A., WEDLUND, P. J. & DWOSKIN, L. P. 2007b. Nicotine increases dopamine clearance in medial prefrontal cortex in rats raised in an enriched environment. *J Neurochem*.
- ZHU, J., GREEN, T., BARDO, M. T. & DWOSKIN, L. P. 2004. Environmental enrichment enhances sensitization to GBR 12935-induced activity and decreases dopamine transporter function in the medial prefrontal cortex. *Behav Brain Res*, 148, 107-17.