Intracellular Signaling Contributions to Behaviors Relevant to Nicotine Addiction

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Intracellular Signaling Contributions to Behaviors Relevant to Nicotine Addiction

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

Intracellular Signaling Contributions to Behaviors Relevant to Nicotine Addiction

By Lauren Elizabeth Thompson, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, at Virginia Commonwealth University.

Virginia Commonwealth University, 2011.

Thesis Director: Darlene H. Brunzell, Ph.D., Department of Pharmacology and Toxicology

Nicotine is the primary addictive substance in tobacco, and most smokers who quit will relapse within a year. Evidence shows that cigarette craving increases over time, termed “incubation.” The purpose of these studies was to see if protracted abstinence from chronic nicotine increases rat self-administration, an animal model with good face validity for human tobacco use, and if nicotine self-administration during daily exposure/after 8+ days of abstinence is regulated by extracellular signal-regulated kinase (ERK) signaling in the nucleus accumbens (NAc) shell or anterior cingulate cortex (PFC). ERK kinase inhibitor U0126 was infused in the NAc shell or PFC of Long Evans rats immediately prior to daily self-administration sessions and following 8+ days of abstinence. U0126 in the PFC decreased responding for nicotine during daily sessions. Following 8+ days of abstinence, animals showed a robust increase in responding for nicotine, blocked by U0126 in the NAc shell, but not the PFC. Western blots revealed that nicotine treatment decreased levels of a substrate of ERK, ribosomal s6 kinase (RSK), in the NAc shell and increased it in the PFC, which occurred independent of abstinence period. In contrast, levels of RSK were increased in the NAc shell following a nicotine challenge during the abstinence period. In summary, our data show that the ERK signaling pathway plays a vital role in nicotine addiction during daily nicotine exposure and following periods of abstinence.
INTRODUCTION

Neurotransmitter Systems Affected by Nicotine

Nicotine is a major addictive substance of tobacco that activates a variety of nicotinic acetylcholine receptor (nAChR) subtypes in animals and humans. nAChRs are composed of α and β subunits (α2-10 and β2-4) (Corringer et al., 2006) and form homopentamers and heteropentamers in both the central and peripheral nervous systems (Dani and Bertrand, 2007; Gotti et al., 2009). nAChRs reside on neurons where they affect neurotransmitter release. These receptors are ligand-gated so that upon activation by nicotine, or the endogenous neurotransmitter acetylcholine (ACh), nAChRs allow cations (Na\(^+\), K\(^+\) and Ca\(^{2+}\)) to cross the cell membrane via their ion channels. The homomeric receptors have five agonist binding sites (Gotti et al., 2006) and heteromeric receptors have two (Elgoyhen et al., 2001). This agonist binding activity can lead to depolarization of the membrane, activation of voltage-gated ion channels and release of neurotransmitters. When located at the axon terminals, nAChRs are thought to promote vesicle docking and neurotransmitter release via calcium entry into the cell. Many subtypes are expressed in the brain; however the homomeric α7 receptors and heteromeric receptors containing α4 and β2 subunits occur most frequently (Gotti and Clementi, 2004; Albuquerque et al., 2009). The α4β2 receptors account for 90% of the high affinity neuronal nAChRs throughout the mammalian brain. The α3β4 subtype is predominant in certain sub regions of the brain, including the interpeduncularis and dorsal medulla (in Gotti et al., 2009). Receptors with multiple types of α subunits are also present in the brain, including the α4β2* nAChRs (* is conventionally used to denote the possible assembly with other subunits). These α4β2* nAChRs can also contain the α5 (Brown et al., 2007) or α6 subunit. Subtypes which
contain α6β2 without α4 are highly enriched in catecholaminergic nuclei, including the ventral tegmental area (VTA) and on VTA terminals in the dorsal striatum and nucleus accumbens (NAc) (Cui et al., 2003; Salminen et al., 2005; Salminen et al., 2007; Pons et al., 2008; Yang et al. 2011). Dopamine (DA), norepinephrine (NE), serotonin(5-HT), gamma-aminobutyric acid (GABA) glutamate and ACh neurons are modulated by α4β2, α4β2*, α6β2(β3), α3β4 and α7 nAChRs on their soma, dendrites and primarily on their axons where nicotinic receptors act as neuromodulators of transmitter release (Picciotto et al., 2001; Gotti and Clementi, 2004; Changeux, 2010). Following the binding of nicotine, the nAChRs desensitize rapidly and remain in this state for prolonged periods of time. Receptor composition and neuronal location determine the specific function of the individual receptors, upon activation by ACh or nicotine.

Nicotine has many acute and chronic effects in the brain, including reward, aversion, craving, angiogenesis, anxiolysis and locomotor effects. As with other addictive psychomotor stimulants, the rewarding and addictive effects of nicotine are mediated in part via the mesocorticolimbic dopaminergic pathway (Robinson and Berridge, 1993; Nestler et al., 1996). In this pathway, neurons from the VTA in the midbrain project to the NAc (Ferrari et al., 2002; Picciotto and Corrigall, 2002) and the prefrontal cortex (PFC). The VTA is responsible for reinforcement, the NAc for satiety, and motivational valence for rewarding and aversive stimuli (Barrot et al., 2002), and the PFC is important for inhibitory control and associative learning (Brunzell et al., 2003). Differences in states of reward and aversion are modulated in the NAc by DA receptors from the VTA (Laviolette et al., 2008). DA neurons in the VTA are modulated by α4β2* nAChRs on the soma and on GABA terminals, by α7 nAChRs on glutamate terminals as well as by α6β2β3, and α3β4 nAChRs in this region (Mansvelder and McGehee, 2002; Wooltorton et al., 2003). DA projections to the NAc and dorsal striatum contain α4β2* and
α6β2* nAChRs that support DA release (Champtiaux et al., 2003; Salminen et al., 2004; Salminen et al., 2007) with α6β2* nAChR-dependent DA release predominating in the NAc (Exley et al., 2008; Changeux, 2010).

The posterior VTA and the NAc shell are critically involved in reward and motivational processes related to drug dependence and abuse, which occur via regulation of the medium spiny neurons in the NAc (Carlezon and Wise, 1996; Rodd-Henricks et al., 2000; Zangen et al., 2002). These processes have been measured by self-administration studies in rats, where microinfusions of the selective α4β2* nicotinic antagonist dihydro-β-erythroidine (DHβE) into the VTA led to a decrease in nicotine self-administration (Corrigall et al., 1994). Genetic studies confirm that α4β2* and α6β2* nAChRs in the VTA or on terminals in VTA projection regions are sufficient for acquisition of nicotine self-administration (Pons et al., 2008). Additionally, 6-hydroxy DA lesions of DA projections to the NAc greatly attenuate nicotine self-administration (Corrigall et al., 1992). Furthermore, nicotine is self-administered in the posterior, but not the anterior VTA (Ikemoto et al., 2006), where expression of α4* nAChRs is necessary and sufficient for nicotine-mediated DA release (Zhao- Shea et al., 2011). Posterior, but not anterior VTA self-administration has also been shown with other drugs of abuse (Zangen et al., 2002).

The NAc, composed largely of GABA-ergic medium spiny neurons, is also functionally and morphologically heterogeneous. This structure is divided into the dorsolateral core and the ventromedial shell, and nicotine can affect these areas differently. For example, nicotine self-administration preferentially increases DA in the NAc shell compared to the core. This increase in DA can remain elevated at least 30 minutes after exposure to nicotine, however levels return to baseline following one week of extinction (Lecca et al., 2006). The posterior VTA is
topographically mapped onto the NAc shell, which is consistent with the results seen from the self-administration studies (Ikemoto et al., 2006), showing that the pVTA-NAc shell projection is an important mediator of nicotine self-administration. Whereas both α4β2* or α6β2* nAChRs appear to play a prevalent role in nicotine ingestion at the level of the VTA, activation of the NAc shell α6β2* nAChRs and not α4β2* nAChRs appear to be essential for motivation to self-administer nicotine (Corrigall et al., 1994; Brunzell et al., 2010).

The DA system is modified by repeated nicotine exposure. Although questions still remain regarding the neurochemical systems responsible for nicotine addiction, it is well established that nicotine affects DA levels in the mesolimbic system. Intra-VTA application of nicotine increases levels of extracellular DA in the NAc (Di Chiara and Imperato, 1988) and specifically in the NAc shell (in Di Chiara et al., 2004). The increased DA levels in the VTA and NAc lead to locomotor activation, as well as long-lasting sensitization to nicotine as demonstrated by increased locomotor activity in rats after repeated exposures to the same dose of systemic nicotine injections (Robinson and Berridge, 1993; Nisell et al., 1996). This behavioral sensitization is mediated via the DA pathways of the NAc (Clarke et al., 1988) suggesting that drugs such as nicotine exert their behavioral effects via the DA projections to the NAc. Systemic, intra-NAc and intra-VTA nicotine administration all increase in DA levels in the NAc, but only intra-VTA (and not intra-NAc) administration is sufficient to increase locomotor activity (Ferrari et al., 2002). The α4*, α6*, β2* and α7* containing receptors appear to be most important for modulating locomotor activity and sensitization in these areas (le Novere et al., 1999; Cui et al., 2003; King et al., 2004; Young et al., 2004; Avale et al., 2008; Drenan et al., 2010). Although DA levels remain high and DA neurons remain excitable for a sustained period of time following nicotine exposure, the nAChRs on the DA neurons desensitize in
seconds to minutes after exposure to physiologically relevant nicotine concentrations (Pidoplichko et al., 1997; Dani et al., 2000; Dani and Bertrand, 2007). This suggests that signaling events downstream of the nAChRs and DA receptors may be responsible for nicotine’s longer-lasting effects.

VTA DA neurons and DA release in the NAc are also influenced by laterodorsal tegmental nucleus (LDTg) cholinergic cells (Forster and Blaha, 2000), as well as the central linear nucleus and supramammillary nucleus. Rats self-administer nicotine into the posterior VTA, central linear nucleus, and supramammillary nucleus, and this is blocked with co-administration of the nAChR antagonist mecamylamine (Ikemoto et al., 2006). It is also probable that additional neurochemical systems also mediate the addictive properties of nicotine, including ACh, glutamate, GABA, 5-HT, and opioid peptide projections (Watkins et al., 2000).

The actions of these neurotransmitters are determined by the receptors to which they bind. DA receptors are metabotropic G-protein coupled receptors (GPCRs) and hence DA is classified as a slow-acting neurotransmitter that necessarily exerts its effects through cascades of biochemical reactions that take place within the cell (Kebabian and Greengard, 1971; Greengard, 2001). Therefore, the DA-mediated addictive and behavioral effects of nicotine must be occurring via intracellular signaling cascades, which lead to long-lasting changes to the affected neuron and its projections, in the form of transcriptional regulation and synaptic plasticity. In addition to DA, nicotine also affects the levels of glutamate and GABA, which are fast acting neurotransmitters that bind to ionotropic receptors; this activation (opening) of the channel leads to immediate changes in membrane potential that affect neurotransmitter release. Glutamate, which allows influx of Na⁺ and Ca²⁺ into the neuron, is generally involved in excitatory
neurotransmission. Glutamate can also bind to metabotropic glutamate receptors, which are a class of GPCRs and hence act more slowly, altering intracellular signaling pathways to indirectly affect ion channels, transporters, and cellular transcription. In contrast, ionotropic GABA<sub>A</sub> receptors are permeable to chloride so that GABA action on the neuron is inhibitory. Metabotropic GABA<sub>B</sub> receptors are GPCRs, which when activated, promote efflux of K<sup>+</sup>, also leading to inhibition of the neuron. In the VTA, nicotine inhibits GABA-ergic transmission (following an initial period of enhancement) and enhances glutamatergic transmission, leading to a net effect of sustained increases in DA neuron excitability, suggesting that nAChRs on glutamate neurons desensitize less rapidly than the receptors on GABA neurons (Mansvelder and McGehee, 2002).

Nicotine Effects on Intracellular Signaling

Nicotine leads to short and long-term changes in intracellular signaling. Alterations in nicotine-associated ion permeability occur through action at the nicotinic receptors and voltage-gated ion channels. Transient changes, such as increases in intracellular Ca<sup>2+</sup>, can ultimately promote neuroplasticity as measured physiologically by long term depression (LTD) and long term potentiation (LTP). Long-term changes from nicotine take place via its effects on DA and glutamate (Mansvelder and McGehee, 2002), which act at GPCRs. These effects lead to intracellular changes in neurochemistry that are promoted by the opening of ion channels reviewed (Dajas-Bailador and Wonnacott, 2004). Activation of these intracellular signaling pathways leads to changes in receptor and transporter sensitivity at the membrane and chromatin remodeling and transcriptional activation in the nucleus. These changes in cellular sensitivity and
gene production ultimately affect processes including learning and memory, drug craving and reward, and locomotor sensitization (in Kelley, 2004).

A signaling pathway critical to the expression of drug-induced behavioral and cognitive effects is the extracellular signal regulated kinase (ERK) pathway. ERK, a serine/threonine protein kinase, is expressed throughout the brain (Ortiz et al., 1995) and importantly, is present in the neurons of the mesocorticolimbic DA system. Its activation in this pathway is responsive to drugs of abuse, including nicotine (Valjent et al., 2004; Corbille et al., 2007). ERK is a member of the mitogen-activated protein kinase (MAPK) family, which is a family of kinases important for cell growth and death, as well as proliferation and differentiation (Kyosseva, 2004). The ERK pathway plays a key role in neuroplasticity in adults, (in Zhai et al., 2008) and thus an important contributor to the neuroadaptations that occur with drug addiction. Despite strong evidence that ERK regulates the addictive behaviors involved with other drugs of abuse, its role in the behaviors of nicotine addiction is unknown.

There are two forms of ERK in mammalian nerve cells, including human and rodent, ERK1 (p44 MAPK) and ERK2 (p42 MAPK), with close structural homology. ERK can be regulated by the neurotransmitters DA and glutamate, and its activation occurs via several different mechanisms. These processes include GPCR activation of Ras, increased Ca\(^{2+}\) levels through the activation of ligand-gated ion channels, such as those formed by nAChRs, and tyrosine kinase receptor (Trk) activation by neurotrophins such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor (FGF) (Zhai et al., 2008). BDNF is released into the blood via platelet degranulation (Radka et al., 1996) and BDNF genes are expressed in rat tissues including the brain (Falkenberg et al., 1993; Kokaia et al., 1994; Nakayama et al., 1994), lungs
and heart (Ohara et al., 1992; Timmusk et al., 1993). BDNF levels are regulated by nicotine in the serum of human smokers (Kim et al., 2007) as well as in the NAc and VTA of mice following 24 hours and 29 days of withdrawal (Kivinummi et al. 2011). While the relationship of serum and brain levels of BDNF is not completely known, one study demonstrated that positive correlations exist between plasma and brain levels in rats and pigs, but not mice (Klein et al., 2011). ERK can also be activated via second messenger systems, such as intracellular Ca$^{2+}$ stores, cyclic adenosine monophosphate (cAMP) with protein kinase A (PKA) (Morozov et al., 2003), protein kinase C (PKC) (Goldin and Segal, 2003), and Ca$^{2+}$/calmodulin-dependent kinase (CaMK) (Schmitt et al., 2005). Activation of these Ca$^{2+}$-mediated systems are important for both acute behavioral effects as well as for states of withdrawal (Damaj, 1997; Jackson and Damaj, 2009; Jackson et al., 2009b). All of the events upstream of ERK converge to activate it via a common mechanism, activation of MAPK/ERK kinase (MEK). MEK is the only known kinase to phosphorylate ERK to its active form, (pERK). As mentioned above, ERK activation can also be regulated by glutamate and DA release, and may act as a coincidence detector of these independent transmissions (Valjent et al., 2005a; Valjent et al., 2005b). ERK is dephosphorylated, and thus inactivated, by protein phosphatases, such as protein phosphatase-1 (PP-1), protein phosphatase-2a (PP-2a) (Mansuy and Shenolikar, 2006), and striatal-enriched protein phosphatase (STEP) (Valjent et al., 2005b). PP-1 and STEP are inactivated by DA- and cAMP-regulated neuronal phosphoprotein 32 kDa (DARPP-32). DARPP-32 can be dephosphorylated by calcineurin, a serine/threonine phosphatase which targets Ca$^{2+}$ signaling pathways that modulate neuroplasticity (Nishi et al., 1999; Winder and Sweatt, 2001). Blockade of calcineurin in the VTA, but not the NAc, of rats attenuates nicotine-mediated locomotor
sensitization, but not acute locomotor activation (Addy et al., 2007). Calcineurin has additional targets in the ERK pathway, including STEP (Paul et al., 2003; Paul et al., 2007).

Once activated, ERK has both cytoplasmic and nuclear targets. In the cytoplasm, ERK regulates cytoskeletal proteins, regulatory enzymes, and membrane ion channels (Frodin and Gammeltoft, 1999). It can translocate to the nucleus and regulate transcription factors, including Elk-1 and cyclic-AMP response element binding protein (CREB) which is essential for nicotine reward (Walters et al., 2003; Brunzell et al., 2009; Jackson et al., 2009a). Via its action on other substrates with nuclear activity, ERK can also affect gene regulation through chromatin remodeling. For example, ERK phosphorylates mitogen and stress-activated protein kinase (MSK), which then phosphorylates histone H3 at the promoter region of genes such as c-fos (Brami-Cherrier et al., 2009), enhancing gene transcription. ERK also activates ribosomal s6 kinase (RSK), which promotes gene transcription via activation of CREB. ERK activity leads to long-term alterations, such as locomotor sensitization and synaptic plasticity. However, ERK appears to mediate only the induction, and not the expression of increased locomotor responsiveness to challenge injections of psychostimulants. Responsiveness is reduced in mice pretreated with the MEK inhibitor SL327, but this compound has no effect on the expression of sensitization, since it does not decrease the enhanced locomotor response when administered just before the challenge injection (Valjent et al., 2005a).

There have been studies conducted on the effects of nicotine, cocaine, amphetamine, morphine and ethanol on ERK signaling (Ortiz et al., 1995; Berhow et al., 1996; Brunzell et al., 2003; Valjent et al., 2004; Lu et al., 2005; Kivinummi et al. 2011). Acute nicotine treatment in mice increases levels of pERK in the NAc, lateral bed nucleus of stria terminalis (BnstL), central
amygdala (CeA), lateral septum and deep PFC through a D1 receptor-dependent mechanism (Valjent et al., 2004). A study using synaptosomes demonstrated that ERK activation is necessary for presynaptic α7 nAChR-induced glutamate release in the PFC (Dickinson et al., 2008). These patterns of activation are not observed with non-addictive psychoactive drugs, suggesting that these effects may be relevant for drug reward. Administration of a D1 receptor antagonist blocks the nicotine-dependent ERK activation in areas including the dorsal striatum, NAc shell, central amygdala, and PFC, suggesting that regulation of ERK by nicotine is DA-mediated. Administration of the MEK inhibitor SL327 attenuates addictive psychostimulant-induced locomotor activity, demonstrating the importance of the ERK pathway in acquisition of locomotor sensitization and psychostimulant-conditioned locomotor response (Valjent et al., 2004). The role of ERK in nicotine locomotor activation and sensitization is unknown. Other studies have compared the acute and chronic effects of nicotine exposure. Following chronic, but not acute nicotine exposure in mice, both total ERK and pERK decrease in the amygdala, but increase in the PFC. After withdrawal from chronic nicotine, ERK2 decreases in the VTA (Brunzell et al., 2003). This suggests that ERK has long-term effects on neuronal transmission and may play a role in nicotine dependence.

CREB activity in response to these substances has also been studied in great detail. CREB is a transcription factor that binds to the CRE element in DNA, leading to immediate early gene (IEG) activation and transcription of genes such as BDNF, which binds to TrkB receptors, and tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis. CREB also leads to activation of IEGs including c-fos, Jun-B, Zif 268 and FosB (Sgambato et al., 1998). Each of these IEGs respond to inhibitors of the ERK pathway (Radwanska et al., 2005), suggesting that their activity, and thus transcription is ERK-dependent. CREB is phosphorylated in the nucleus
on serine 133 (and thus activated) by ERK indirectly, via RSK, and is also activated directly by PKA and CaMKII.

These additional activators of CREB are highly sensitive to nicotine administration. Acute nicotine treatment in mice leads to increases in CaMKII activity in the VTA, NAc, and amygdala, and decreases in activity in the PFC. Blockade of this CaMKII activity attenuates the nicotine-induced changes in pCREB in those same areas (Jackson et al., 2009a). CREB activity is thought to increase drug reinforcement and is required for nicotine-conditioned reward (Walters and Blendy, 2001; Walters et al., 2005). Like ERK, CREB is affected by acute and chronic nicotine administration, as well as withdrawal (Brunzell et al., 2003). Acute nicotine results in elevated levels of CREB and pCREB in the NAc and VTA of C57Bl/6J mice (Walters and Blendy, 2001; Brunzell et al., 2009) but chronic exposure leads to reductions of pCREB in the NAc (Brunzell et al., 2003) suggesting that this effect tolerates over time. pCREB reductions are accompanied by an increase in total CREB, perhaps due to homeostasis. In the PFC, chronic oral nicotine increases pCREB, and there are no effects in the VTA (Brunzell et al., 2003). Upon 24 hours withdrawal, the Brunzell study found that CREB is increased in the NAc and pCREB is increased in the VTA (Brunzell et al., 2003), however another chronic oral study in a different strain of mice (NMRI) found that pCREB levels were increased in the NAc after 29 days of abstinence and decreased in the VTA following 24 hours of withdrawal (Kivinummi et al. 2011). Pluzarev and Pandey (2004) conducted additional studies with rats (Sprague-Dawley) in states of nicotine withdrawal. After chronic treatment of twice daily injections of nicotine for 10 days, followed by 18 hours of natural withdrawal, animals show significant reductions in pCREB and CREB in the NAc shell. However when nicotine is present, there is no reduction of pCREB in the shell. This study found no effects of nicotine on CREB function in the NAc core. Decreases
in pCREB are consistent with the Brunzell mouse study, but the Pandey study found a decrease, rather than an increase, in total CREB levels in the NAc. An earlier study by Pandey looked at CREB activity in other brain regions during withdrawal and found that nicotine withdrawal decreases CREB and pCREB levels in the parietal, piriform and cingulate cortices, as well as the medial and basolateral amygdala (Pandey et al., 2001). This was accompanied by decreases in CRE-DNA binding of CREB in both the cortex and amygdala (Pandey et al., 2001).

Accumulating evidence has demonstrated the importance of the ERK pathway in nicotine addiction and implicates it as a key player affected by the mesocorticolimbic dopaminergic pathway. Elucidating its activity during the different states of drug acquisition, addiction, withdrawal and relapse will provide a key piece to the puzzle of understanding the neurochemical mechanisms of drug addiction.

Nicotine Self-Administration

Drug self-administration is an operant conditioning paradigm in which a drug serves as a reinforcer, i.e. an operant response results in delivery or availability of the drug. Self-administration experiments have high face, construct and predictive validities, making them valuable to understanding addictive behavior (Perkins, 1999). Parameters such as session length and frequency, reward frequency, cue type, dose of drug, and route of administration are manipulated to test different aspects related to the animal’s motivation to take the drug. This motivation can be measured by the breakpoint in progressive ratio reinforcement (Arnold and Roberts, 1997; Deroche-Gamonet et al., 2002). While exposure lengths can vary, studies have shown that 1-2 hours per day of limited access leads to faster acquisition and higher, more stable
rates of drug-maintained behavior (Goldberg and Spealman, 1983; Goldberg et al., 1983; Henningfield and Goldberg, 1983; Carroll et al., 1989).

Addictive substances, including nicotine are routinely used in self-administration studies. Nicotine reinforces operant responding in a variety of species, including rats (Corrigall and Coen, 1989; Corrigall et al., 1992; Donny et al., 1995; Smith and Roberts, 1995; Tessari et al., 1995; Chiamulera et al., 1996), and rats are commonly used as reliable test subjects (Weeks and Collins, 1978). Nicotine has been shown to be reinforcing from the very first drug exposure in naive animals (Donny et al., 1998).

Smokers are highly responsive to cigarette associated cues (Due et al., 2002). A key factor in rodent self-administration studies is the pairing of nicotine administration with cues that predict or coincide with the drug’s availability. Such cues become conditioned reinforcers. Other studies show that a light stimulus can have primary reinforcing properties (Caggiula et al., 2002) that like nicotine are dependent upon DA release (Olsen and Winder, 2009), making administration of cues in the absence of drug a relevant control for behavioral and neurochemical studies that assess the effects of nicotine self-administration.

Neuroplasticity can occur following chronic exposure to drugs, such as nicotine. Following repeated exposure to nicotine, a state of locomotor sensitivity develops so that the same dose of nicotine leads to increases in locomotor activity (Clarke et al., 1988; Di Chiara and Imperato, 1988; Benwell and Balfour, 1992; Robinson and Berridge, 1993; Nisell et al., 1996). When rats are allowed extended access to nicotine, an increase in responding and a dose-dependent increase in intake occurs (O'Dell et al., 2007). Chronic exposure followed by a period of abstinence can lead to an increase in drug-seeking in animals, termed “incubation” (Shaham et
al., 2003). This incubation period leads to increases in drug craving in both animals and humans and can occur following only seven days of abstinence and lasts for up to 90 days post-withdrawal (Gawin et al., 1986; Grimm et al., 2001; Grimm et al., 2003; Bedi et al. 2011). In rats, this increase in drug-seeking coincides with an increase in brain-derived neurotrophic factor (BDNF) in the mesocorticolimbic pathway (VTA, NAc and amygdala) following both 30 and 90 days of withdrawal (Grimm, et al. 2003). Incubation effects are also associated with enhanced PKA signaling in the insular cortex through phosphorylation of DARPP-32 (Abdolahi et al. 2010). BDNF and PKA are upstream effectors of the ERK signaling pathway, suggesting that ERK may also contribute to these incubation effects. Studies with cocaine have implicated increases in ERK activity in the drug craving effects caused by protracted abstinence (Lu et al., 2005). This activity is specifically increased in the NAc, and blocked upon microinjection of a MEK inhibitor U0126 into the NAc (Schumann and Yaka, 2009). It is unknown if ERK activity might also affect states of craving caused by nicotine incubation during protracted abstinence.

The purpose of this study was to test if ERK in the PFC (anterior cingulate cortex) or NAc shell regulates nicotine reinforcement during daily administration or following an “incubation period” of protracted abstinence. Additionally we tested how ERK signaling mediates locomotor activity. Since locomotor sensitization occurs with repeated exposures to the same dose of nicotine (Robinson and Berridge, 1993; Nisell et al., 1996) and because ERK activity can mediate the locomotor effects of other psychostimulant drugs (Valjent et al., 2006), we wanted to explore how ERK signaling mediates locomotor activity at different periods of abstinence from nicotine. Finally, we determined the neurochemical effects of ERK signaling in these and other brain areas following protracted abstinence, in order to identify possible
connections in the ERK signaling pathway and the behavioral effects of nicotine, particularly following periods of abstinence.
MATERIALS AND METHODS

Subjects

For these studies, 65 adult, male, Long Evans rats (Harlan Laboratories, Dublin, VA) were used. Animals were housed individually in a humidity- and temperature-controlled vivarium on a 12/12h light/dark cycle (lights on 0600 h). Behavioral tests occurred during 1300 and 1900 h. Upon arrival, rats weighed approximately 300 g and after reaching 320 g, testing was started. Restricted diets ensured that the rats’ weights remained consistent throughout testing. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals, as set forth by the National Institutes of Health.

Drug Dosing and Administration

Rats were infused intravenously during self-administration procedures with 0.03 mg/kg of nicotine (by weight of freebase) in 0.0533 ml, which occurred over a 1 s period of time. Using 0.9% sterile saline as a solvent, nicotine hydrogen tartrate salt was dissolved and stored in the dark to avoid degradation. MEK inhibitor, U0126, was infused into the NAc shell and anterior cingulate cortex (PFC) at concentrations ranging from 0.1-4.0 μg/hemisphere. During locomotor testing, rats were administered subcutaneous (s.c.) injections of 0.175 mg/kg nicotine (by weight of freebase) in sterile saline at a concentration of 1.0 mg/ml.

Intrajugular Catheter Implantation

Aseptic procedures were used throughout all surgeries. 3.5% isoflurane gas and 3.5 liter/minute of oxygen was used to induce anesthesia and anesthesia was maintained at 2.0%
isoflurane and 2.5 liter/minute of oxygen. Preparation of surgical areas occurred by shaving and cleaning with 70% reagent alcohol and 7.5% povidone–iodine. Above the atrium, into the right jugular vein, a tapered polyurethane catheter (3.5 French; Access Technologies) was implanted. The catheter was then passed through a subcutaneous route to the rat's back where it was connected to a back-mounted pedestal (Plastics One, Roanoke, VA). All rats received s.c. injections of 75000 U penicillin G and 0.1 ml injections into the catheter of 0.031 mg/ml ticarcillin/clavulanate in 25% glycerol/heparinized saline (catheter lock) in order to prevent infection. Rats were given 5 mg/kg, intraperitoneal, carprofen during surgery for preventative analgesia. Rats were also given 64 mg acetaminophen mixed in wet chow for 3 days post-surgery and were given at least 5 days to recover prior to self-administration training. Catheters were irrigated with 0.1 ml heparinized saline (5 USP units per ml heparin) both before and after training sessions. Animals received 0.1 ml of the catheter lock following the post-session flushing. Rapid loss of balance following 1.6 mg, i.v., ketamine injection confirmed catheter patency. If catheter failure of the right jugular vein occurred, the left jugular vein was catheterized before returning the animal to the study.

Intracranial Guide Cannula Implants

Following anesthesia, animals were prepared for surgery, and then received postoperative care as described above. A stereotaxic device was used to hold rats’ skulls secure and level bregma and lambda to within 0.05 mm. A 22-gauge bilateral guide cannula (Plastics One) targeting the NAc shell (+1.6 mm anterior, ±0.75 mm from midline, −6.5 mm ventral from bregma) or anterior cingulate cortex (+1.8 anterior, ±0.75 from midline, −2.75 ventral from bregma) were implanted into each animal. Dental cement anchored with jeweler’s screws held
guide cannulae into place. Patency was maintained with dummy cannulae. Brains were harvested to confirm cannulae placement following the behavioral testing.

Nicotine Self-Administration

For nicotine self-administration, animals were placed into two groups, cue self-administration (CUE SA) or nicotine self-administration (NIC SA). Operant chambers inside of sound-attenuating boxes (MED Associates, St Albans, VT) were used for all self-administration tests. Sessions ran for 2 hours, at the beginning of which rats were placed in the operant chambers and attached via an implanted pedestal to stainless-steel-encased infusion tubing hung from the chamber ceiling (Plastics One). Use of these tethers allowed rats freedom to move throughout the chamber while training and testing. Throughout all behavioral testing, levers were extended and a 5W houselight remained on. A fixed ratio 1 (FR1) schedule of reinforcement followed by a 20 second timeout period was used with all rats during their acquisition period. A Model PHS-100 syringe pump placed on the outside each sound-attenuating box was used to administer the nicotine infusions. Compound cues consisting of light and sound were delivered by a panel light above the active (right-side) lever and a Sonalert tone generator at the back of the chamber. When NIC SA rats depressed the active lever, a 1 s, 0.03 mg/kg, intravenous nicotine bolus and a 20 s light+tone cue was delivered to them. CUE SA rats received identical cues without nicotine infusion after pressing the active lever in order to control for possible effects of the MEK inhibitor on locomotor activity and effects on the primary reinforcing properties of the cues (Caggiulla et al., 2002) that like nicotine are dependent upon DA release (Olsen and Winder, 2009). Depression of the inactive (left) lever did not lead to any consequences. Rats were trained on the FR1 schedule 10+ days and were required to reach 3
consecutive days of >70% accuracy, which was measured by the ratio of active/total (left+right) lever presses. If animals had cannula implanted following training, they were given a refresher session using FR1 conditions prior to progressive ratio (PR) training. MED-PC IV software (MED Associates, St. Albans, VT) controlled behavioral programs and data collection.

Nicotine Maintenance of Progressive Ratio Responding

Rats were reinforced using a PR schedule after their FR training. For PR, rats obtained each subsequent cue presentation and/or nicotine infusion (according to the algorithm in Arnold and Roberts, 1997) by depressing the active lever an increasing number of times. Sessions lasted until rats did not respond for 20 consecutive min or for 2 h. The “break point” was defined as the highest lever depression ratio reached within 2 h. Under PR schedules of reinforcement the animal must work harder and longer to obtain the same amount of infusions as in FR1 responding (eg, Donny et al, 2000) so that the break point is thought to measure motivation to work for reinforcers like nicotine. Active lever pressing and number of infusions/cue deliveries were used as secondary measures of nicotine and cue reinforcement. Nonspecific lever-pressing activity was assessed by measuring the inactive lever responding. Steady levels of responding are maintained over days of nicotine self-administration by using a 2 h time limit for PR sessions (Donny et al, 2000). Following acquisition, animals were intracerebrally infused with 0, 0.1, 0.4 µg/hemisphere U0126 using a within-subject Latin square design immediately prior to PR testing. During the protracted abstinence period, animals remained in their homecages, where they did not receive treatment, however they were fed daily and cannula were flushed regularly to maintain patency.
Locomotor Assay

The locomotor effects of prior nicotine exposure were assessed in Med Associates locomotor chambers as a measure of distance traveled in cm. Chambers measured 30 cm H x 44 cm W x 44 cm L with infrared photo-sensor beams spaced 2.5 cm apart and 2 cm above the chamber floor. Treatment Group A (no infusion) was tested using a 2 x 2 ANOVA (Behavioral Treatment x Acute Challenge) design. Rats on prior chronic self-administration schedules maintained by cue or nicotine (CUE SA or NIC SA) received a challenge s.c. dose of saline or 0.175 mg/kg nicotine (SAL or NIC), which has been shown to be locomotor activating in rats (Brunzell et al., 2010) and is approximate to the amount of nicotine intake during a daily PR session (Table 4), prior to locomotor testing. This dosing paradigm produced 4 groups of animals (CUE SA-SAL, CUE SA-NIC, NIC SA-SAL, NIC SA-NIC; Table 1). To test if the MEK inhibitor U0126 affects locomotor activity, treatment Group B compared locomotor behavior of NIC SA rats that received infusion of MEK inhibitor U0126 (U0126) or DMSO vehicle (VEH) into the PFC or NAc, to CUE SA subjects that received VEH or no infusion (Table 1). At least one day prior to testing, rats were habituated to handling. Results are expressed as distance traveled in cm and time ambulatory in s in the locomotor testing chamber. Data for Group B rats were analyzed using a one-way ANOVA.

Group A – Rats were introduced to a novel open field chamber for a 20 min habituation period during which locomotor activity was measured. After rats were habituated to the testing chambers, they received a 0.175 mg/kg s.c. injection of nicotine or saline, and were replaced in the chambers for an additional 20 min, during which time locomotor activity measures resumed. Immediately following the 20 min activity phase, brains were harvested via rapid decapitation
and immediately sectioned on a chilled brain block and placed in PBS for harvest of the NAc shell, PFC, and VTA for Western blotting. All PFC samples were obtained from the anterior cingulate cortex.

Group B – Prior to the 20 min habituation phase, rats received a 0.5 uL/min infusion of 0.4 ug total U0126 or VEH into the PFC or NAc shell for 1 min followed by a 2 min wait period. Rats were then placed into the locomotor chamber as described for Group A.

A timeline for the locomotor testing procedures is shown in Table 2.

Western Blotting

Brains were harvested via rapid decapitation, and following removal placed in chilled 1Xphosphate-buffered saline (PBS), (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄ dissolved in 1L ddH₂O and pH adjusted to 7.3) and sectioned with razor blades in a stainless steel brain block chilled on ice. Coronal slices were obtained and submerged in chilled PBS then core punches were taken of the NAc shell, PFC, and VTA. Dry ice was used to rapidly freeze tissue samples until time to process them for western blot procedures. Samples were placed on ice for sonification in Homogenization Buffer A with 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged for 15 min at 13,000 g. The supernatant liquid was stored at -80°C after removal. The Lowry Protein Detection Assay was used to determine sample concentrations, run in duplicate. Protein samples (10 µg per lane) was loaded for detection of phospho-P90RSK (pP90RSK), total P90RSK, p-ERK, total ERK, GAPDH, striatal-enriched protein phosphatase (STEP), BDNF, histone H3 (H3) immunoreactivity. After loading, samples were run on a 10% SDS-polyacrylamide gel at 120 V for 1 h. Chilled transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) was used to transfer gels to nitrocellulose
membranes at 100 V for 55 m. After rinsing in 1X TBS, membranes were blocked for 1.5 h at room temperature in 5% non-fat milk block in TBS with 0.1% Tween (TBS-T). Remaining incubations were done at 4°C. Following a brief rinse in TBS-T, membranes were incubated overnight in primary antibody diluted in 3% BSA in TBS-T. Antibody against pP90RSK and H3 were used at a dilution of 1:500, ERK and pERK (threonine 183/tyrosine 185) were used at a dilution of 1:2000 and GAPDH at a dilution of 1:20,000 (Cell Signaling Technology, Beverly, MA, USA). Anti-BDNF (Millipore, Billerica, MA, USA) and STEP (Cell Signaling) were diluted at 1:1000. Following a 30 min wash in TBS-T, blots were incubated for 1h, 15 min in 1:1000 affinity-purified, peroxidase-labeled, anti-rabbit IgG (for pP90RSK, H3, STEP, BDNF and H4 antisera), 1:2000 anti-rabbit IgG (for pERK and GAPDH antisera), (Vector Labs, Burlingame, CA, USA) secondary antibody in 3% non-fat milk in TBS-T. Blots were then washed for 30 min in TBS-T followed by a final TBS wash for 10 m. Following a 1 min wash in enhanced chemiluminescence detection reagents, blots were exposed to x-ray film and developed.

For Western blots, Band densities were quantified using NIH Image software, Image J. Density of bands corresponding to proteins of interest and GAPDH were quantified on each blot. Within each blot, values for all proteins were normalized to GAPDH values as a loading control, and then normalized to CUE SA control rats so that values could be compared across blots. Data were then combined across blots for statistical analysis. Data are represented as percent of CUE SA control values. Arrangement of treatment groups are listed in Table 3.
Data Analysis and Statistics

Nicotine self-administration studies were analyzed using a 2 x 3 (Behavioral Treatment x Dose of U0126) ANOVA with Behavioral treatment as a between subjects factor and dose of U0126 as a within-subject repeated measure. The NAc and PFC infusions were performed in discrete groups of animals and were analyzed separately. Locomotor studies were analyzed using a 2 x 2 between-subject design (Behavioral Treatment x Nicotine Challenge) for Group A. One-way ANOVA was used to analyze locomotor measures for Group B subjects and for Western blots. Post-hoc t-tests were performed to determine the nature of significant interactions and main effects of factors with greater than 2 levels. Values of p < 0.05 were considered to be statistically significant for all groups.
RESULTS

Self-Administration Studies

To assess the potential influence of PFC and NAc ERK signaling on motivation to self-administer nicotine, rats were infused with U0126 immediately before daily progressive ratio sessions or after a period of at least 8 days of abstinence from nicotine. Placement of the cannulae into the NAc shell and PFC was confirmed in Nissl stained 40 μm coronal sections of the respective brain areas. Blockade of ERK in the PFC dose-dependently decreased responding for nicotine during daily PR sessions. There was a significant interaction of behavioral treatment with U0126 dose for the measures of active lever pressing ($F_{1,8} = 6.0, p = 0.04$), breakpoint ($F_{1,8} = 5.6, p = 0.045$), number of infusions earned ($F_{1,8} = 7.0, p = 0.03$) and amount of nicotine ingested ($F_{1,8} = 8.4, p = 0.018$). NIC SA animals showed dose-dependent decreases in breakpoint, active lever presses and nicotine infusions following local infusion of U0126, with maximal effect at 0.4 μg/hemisphere U0126 (Figure 1 and Table 4). There was no effect of U0126 on any measure in CUE SA animals, suggesting that motivation to respond for nicotine, but not cues, is regulated by ERK signaling in the PFC. This control group was essential for ensuring that inhibition of ERK did not affect responding for the cues alone. There was also no effect of ERK blockade in the NAc on PR responding (Figure 1) during daily administration of nicotine. Rats showed a similar level of breakpoint, active lever pressing and earned infusions, regardless of U0126 infusion ($p$’s $> 0.1$). There was no effect of % active lever responding in PFC or NAc rats, suggesting that infusion of MEK inhibitor did not affect accuracy in lever pressing. These results suggest that ERK activity in the PFC, but not the NAc is necessary for the motivation to respond for nicotine during daily nicotine exposure.
Following daily PR sessions, animals went through a period of at least 8 days of protracted abstinence and were returned to test chambers where nicotine-self administration was reassessed in comparison to daily responding following infusion of VEH or 0.4 μg U0124. There was a significant interaction of abstinence period and drug infusion for measures of active lever pressing ($F_{1,8} = 11.74, p = 0.009$) breakpoint ($F_{1,8} = 7.46, p = 0.02$), and nicotine deliveries earned ($F_{1,8} = 7.289, p = 0.02$) in NAc-infused rats. Following a period of protracted abstinence, VEH-infused NIC SA rats showed a significant increase in nicotine-self administration behavior compared to their daily levels as measured by active lever pressing ($t_4 = 3.22, p = 0.03$) and nicotine deliveries ($t_4 = 5.009, p = 0.007$) with a nearly significant effect of breakpoint ($t_4 = 2.54, p = 0.06$). This abstinence-associated increase in responding was not observed following infusions of MEK inhibitor into the NAc shell ($p$’s > 0.05) (Figure 2). Post hoc t tests showed that rats that received NAc shell infusions of U0126 showed significantly fewer active lever presses ($t_4 = 2.34, p < 0.05$) than rats that received vehicle infusions following protracted abstinence from nicotine but did not differ from rats receiving daily infusions of the drug. There was no effect of % active lever responding, indicating that abstinence-associated increases in responding were directed to the lever that resulted in delivery of nicotine. Together with findings from the daily sessions of nicotine self-administration, these data suggest that ERK activation in the NAc shell is necessary for observation of elevated responding following protracted abstinence from nicotine. In contrast, there was no elevation of responding observed following a period of abstinence in VEH-infused PFC rats, suggesting that this effect may have been blocked by prior inhibition of ERK in this brain area. There were no main effects or significant interactions of time point or dose of U0126 for the measures of active lever pressing, breakpoint, infusions, or nicotine ingested when rats received U0126 infusions in the PFC following
protracted abstinence from nicotine (Table 4). The increase in responding for nicotine following protracted abstinence may be due to an “incubation” effect, which appears to be mediated by ERK activity in the NAc, but not the PFC.

**Locomotor Assay**

To test if protracted abstinence from nicotine might affect nicotine locomotor activation, rats with (NIC SA) or without a prior history of nicotine (CUE SA) received an acute injection of saline (SAL) or a nicotine challenge (NIC) dose that was similar to nicotine levels achieved during self-administration (Table 4) and shown previously to result in locomotor activation in Long Evans rats (Brunzell et al., 2010). When measuring raw data, there was a main effect of nicotine challenge on distance traveled ($F_{1,26} = 6.349, p = 0.018$) and a significant interaction of time bin with prior treatment ($F_{1,26} = 4.224, p = 0.05$). There was no interaction of prior nicotine exposure with nicotine challenge on distance traveled ($F_{1,26} = 1.87, p = 0.183$) during the habituation period, however there was a trend for a main effect of prior exposure on time ambulatory ($F_{1,26} = 3.12, p = 0.089$) during the habituation period. There were no significant interactions or main effects of prior exposure with challenge s.c. injection of 0.175 mg/kg nicotine or saline in CUE SA-NIC, NIC SA-SAL and NIC SA-NIC vs. CUE SA-SAL control rats. When rat test behavior was normalized to their baseline activity, there was a significant main effect of prior chronic nicotine treatment on distance traveled ($F_{1,26} = 4.28, p = 0.049$) and time ambulatory ($F_{1,26} = 7.36, p = 0.012$), revealing that NIC SA-SAL and NIC SA-NIC rats habituated less to the open field environment compared to CUE SA subjects (Figure 3). This result suggests that chronic administration of nicotine may be sufficient to increase locomotor activity, or to decrease the ability to habituate to a new environment.
To determine if the decrease in responding seen following MEK inhibition during PR may be due in part to locomotor depressant effects of U0126, we next tested if a dose of U0126 that was capable of attenuating nicotine self-administration would also lead to a depression of locomotor activity in an open field. Our results indicate that locomotor activity was not affected by infusion of MEK inhibitor U0126 into either the PFC or NAc. There was no significant interaction or main effect of prior treatment or MEK inhibitor challenge on distance traveled or time ambulatory (p’s > 0.05) (Figure 4).

Western Blot Analysis

We next wanted to measure if nicotine exposure affects levels of ERK signaling proteins in brain areas relevant to nicotine addiction. We measured levels of total and phosphorylated ERK, downstream substrates P90RSK, and histone H3, a phosphatase of ERK, STEP, and BDNF, a neurotrophin that binds to TrkB receptors upstream of ERK and that is downstream of the transcription factor CREB. Using a 2 x 2 ANOVA we assessed the effects of behavioral treatment (CUE SA vs. NIC SA) on ERK signaling protein expression during protracted abstinence or following 24 hours of withdrawal. CUE SA and NIC SA groups were composed of animals with either NAc or PFC cannula that had previously received infusions of U0126, however these groups were not broken down in the analysis due to small n sizes. Animals had not received infusions within 3 days prior to brain harvest. A two-way ANOVA analyzing treatment group and time of harvest revealed a main effect of behavioral treatment on levels of RSK in the anterior NAc shell (F_{1,20} = 6.897, p = 0.018). Compared to CUE SA subjects, NIC SA rats showed a reduction in total RSK mainly following protracted abstinence (t_{7} = 2.168, p = 0.067) although there was no significant interaction of behavioral treatment with time point for this measure. There were no other main effects and no significant interactions of behavioral
treatment with time of brain harvest on pERK, ERK, pP90RSK, STEP, H3 or BDNF levels measured in the anterior or posterior NAc shell ($p > 0.1$) (Figure 5).

In the PFC there was a main effect of treatment on levels of total RSK in the PFC ($F_{1,21} = 114.81, p = 0.001$). NIC SA animals showed elevated levels of RSK in comparison to CUE SA controls regardless of duration of abstinence. There were no main effects of behavioral treatment or abstinence and no significant interactions for levels of pERK, ERK, pP90RSK, STEP, H3 or BDNF ($p$’s > 0.1). There were also no significant main effects of behavioral treatment or time point and no significant interactions for any of the total or phosphorylated signaling proteins measured in the VTA ($p$’s > 0.1) (Figure 6).

Lastly, we tested the effects of MEK inhibition in the NAc shell on ERK signaling following a nicotine challenge treatment of 0.175 mg/kg s.c. nicotine. In the anterior NAc shell there was a nearly significant effect of treatment group on levels of RSK ($F_{1,3} = 15.822, p = 0.058$). Post hoc t-tests revealed that VEH- and MEK-infused NIC SA subjects showed a significant elevation of levels of RSK compared to non-infused CUE SA rats ($t_4 = 2.762, p = 0.05$) but did not differ significantly from each other ($t < 1.0$), suggesting that this observation was due to prior chronic nicotine exposure and not due to local inhibition of MEK. There were no other effects of behavioral treatment/infusion on ERK signaling proteins in the anterior or posterior NAc shell following nicotine challenge during protracted abstinence ($p$’s > 0.1). There was also no effect of behavioral treatment/infusion on ERK signaling ($p$’s > 0.1) in the PFC or VTA following nicotine challenge during protracted abstinence from prior chronic nicotine exposure (Figure 7).
DISCUSSION

These studies have revealed that ERK signaling in the PFC and NAc shell is critical for self-administration of nicotine during PR, however, there was an abstinence-associated dichotomy in PFC and NAc shell ERK regulation of this behavior. Inhibition of ERK activity in the PFC and not the NAc shell significantly decreased responding maintained by nicotine during daily administration sessions, suggesting that ERK signaling in the PFC promotes motivation to self-administer nicotine. Following a period of protracted abstinence, responding for nicotine increased robustly, suggesting that motivation for nicotine is elevated during a period of protracted abstinence. In contrast to U0126 infusions during daily administration, inhibition of ERK activity in the NAc shell specifically decreased nicotine self administration during PR following a period of protracted abstinence. This suggests that neuronal plasticity in the ERK signaling pathway occurs during an abstinence period and that changes in ERK signaling support elevations in nicotine use. We found in our locomotor assay that ERK blockade in both the PFC and NAc shell did not decrease locomotor activity. Thus, MEK inhibitor infusions did not likely affect nicotine self-administration through locomotor effects. Western blot analysis revealed that nicotine exposure resulted in reduced levels of total RSK in the NAc shell but increased levels of this substrate of ERK in the PFC. These observations were independent of time of brain harvest, suggesting that duration of withdrawal did not regulate these effects; however, nonsignificant trends of timepoint suggest that follow-up studies with increased n sizes may reveal these changes occur specifically during protracted abstinence from chronic nicotine exposure in the NAc shell and during 24 hours of withdrawal in the PFC. In the NAc shell, nicotine associated elevations of RSK were in the opposite direction following nicotine challenge during a period of
protracted abstinence. This observation was not affected by MEK inhibition, suggesting that it was not due to ERK activation at the time of exposure. It is likely that a combination of chronic nicotine and acute nicotine challenge mediated elevations of RSK in this study.

These studies showed that protracted abstinence from nicotine results in significant elevations of motivation to self-administer nicotine. This is consistent with studies in human smokers that have demonstrated incubation effects on tobacco craving (Bedi et al., 2011), where smokers show increased craving for tobacco-related cues following periods of abstinence. Thus, events within the addiction pathways in the brain which occur during periods of abstinence are clearly important for further nicotine seeking. Since the large majority of smokers who attempt to quit are unsuccessful (National Institute of Health), it will be important to identify the neuroplastic mechanisms that occur during periods of abstinence, which lead to increases in nicotine craving. The present studies suggest that ERK signaling in the NAc shell may be critical for these incubation effects of nicotine.

In these studies we observed that infusion of a MEK inhibitor into the NAc shell reversed elevations in nicotine self-administration following protracted abstinence. The NAc shell is important for drug reward and aversion (LaViolette, 2008) and it appears from our data that ERK activity in this brain region, but not the PFC is essential for elevated nicotine intake following an incubation period. ERK signaling in the NAc is affected by drugs of abuse, including nicotine (Valjent, 2004). It is not clear why PFC-infused subjects did not show the incubation effect observed in NAc shell-infused subjects. Previous reports indicate that rats with no brain infusions show incubation effects of nicotine following 7 days of abstinence from self-administration (Abdolahi et al., 2010). ERK in the PFC regulates incubation effects following abstinence from cocaine (Lu et al., 2006). It is possible that in the PFC, where acute and
repeated exposure to nicotine lead to elevations of pERK, that MEK inhibition during daily exposure blocked incubation effects of nicotine. Studies conducted in additional brain regions have demonstrated the importance of ERK signaling in the incubation effects of drugs. Increases in cocaine seeking following 30 days of abstinence is accompanied by an increase in pERK in the amygdala (Lu et al., 2005) and incubation of opiate craving is also mediated by amygdala ERK activity (Li et al., 2008). Thus, it is likely that ERK-mediated neuroplasticity occurs during periods of abstinence from nicotine, which contributes to the increase in nicotine self-administration seen in our study. Studies in mice reveal that protracted abstinence leads to elevations in BDNF, a neurotrophin that activates receptors upstream of ERK signaling, within the mesocorticolimbic dopamine system (Kivinummi et al. 2011). Levels of BDNF are also elevated in the plasma of smokers compared to nonsmokers and further elevated in smokers by a period of protracted abstinence (Kim et al., 2007). Together with the present findings, these studies suggest that nicotine incubation effects involve changes in ERK signaling within the NAc shell and may involve the PFC.

During daily administration, ERK activity in the PFC and not the NAc shell was essential for motivation to self-administer nicotine. Since the PFC is important for cognition and executive control, it appears that the ERK pathway in this area is essential for acquiring addiction-related behaviors. It is possible that nicotine leads to ERK-mediated propagation of LTP and LTD which leads to synaptic changes responsible for the learning of these behaviors (Hotte et al., 2006) even following protracted abstinence (Penton et al. 2011). Since inhibition of ERK in the NAc did not affect responding during daily PR, its activity in this region does not appear to play a role in the maintenance of self-administration.
While blocking ERK activity in the PFC and NAc shell leads to decreases in self-administration during daily administration and following protracted abstinence, respectively, its blockade in these areas did not decrease but rather seemed to increase locomotor activity. This suggests that ERK signaling positively modulates motivation and craving for nicotine, and perhaps negatively modulates nicotine-induced locomotor activity. This finding is consistent with reports from Dong et al., (2006) showing that reductions in NAc activity results in elevated locomotor output, presumably due to disinhibition of NAc GABAergic projections to regions that modulate motoric activity.

After functionally assessing whether ERK activation is necessary for nicotine self-administration during daily administration and following protracted abstinence, we wanted to test how nicotine administration affects levels of ERK signaling proteins. Although we did not detect any direct changes in total or phosphorylated ERK at the timepoints assessed in this study, we did observe that RSK levels were regulated in both the NAc and PFC, suggesting that this substrate of ERK is sensitive to nicotine treatment and withdrawal. Our results indicate that chronic nicotine treatment leads to decreased levels of RSK in the anterior NAc shell. This decrease occurred in both abstinence groups, however the effect appeared more pronounced following protracted abstinence. This could perhaps be due to homeostatic mechanisms taking place in the NAc shell during periods of nicotine abstinence since acute challenge of nicotine led to significant increases in RSK in this brain area. There were no significant interactions of time point with self-administration condition, but non-significant trends warrant further study of a timecourse of nicotine exposure and withdrawal on this pathway. Interestingly, we did not see this effect in the posterior NAc shell. The behavioral assays above targeted the anterior NAc shell. It would be interesting to explore if the behavioral effects or MEK inhibition are specific
to the anterior NAc shell and not seen in the posterior NAc shell or in the NAc core. Previous studies have shown anatomical and functional differences in VTA projections to the NAc, and nAChR expression differs between the anterior and posterior VTA (Ikemoto et al., 2006; Zhao-Shea et al., 2011). The medioposterior VTA projects to the ventromedial striatum, including the medial NAc shell, whereas the ventrolateral VTA projects to the lateral striatum, including the lateral NAc shell and NAc core, and projections to the dorsal striatum originate in the dorsal VTA (Ikemoto, 2007). Thus it is likely that anatomical and functional differences exist within stratifications of the NAc and future studies could address these possible differences.

In our nicotine challenge/infusion study, we found that NIC SA animals given acute nicotine injections had elevated levels of RSK compared to CUE SA animals who were also acutely injected with nicotine. Since we did not see a difference between VEH and U0126 animals, our results indicate that acute ERK activation may not be essential for nicotine-induced changes in RSK following nicotine challenge. This is consistent with the observation that pP90RSK levels did not change significantly following protracted abstinence or during infusion/challenge treatment; however, we can not rule out that pP90RSK was not regulated at some time point prior to brain harvest. Since this increase in RSK was observed in the NIC SA animals given a nicotine challenge injection, it appears that chronic nicotine treatment may sensitize the NAc shell to nicotine-induced changes in RSK. This effect was in the opposite direction of the decreases in RSK observed during protracted abstinence in the absence of nicotine, suggesting that acute nicotine treatment has robust effects on RSK. Future studies comparing acute nicotine in naive animals to nicotine challenge following protracted abstinence will clarify whether RSK signaling is sensitized during the incubation period.
Chronic nicotine exposure resulted in elevated levels of RSK in the PFC. This effect was independent of abstinence period, however, small n sizes may have precluded detection of differences between groups as this affect appeared more pronounced following 24 hours of withdrawal than during protracted abstinence. This is the timepoint when animals would normally receive nicotine during daily sessions. Together with our behavioral data showing that local infusion of a MEK inhibitor into the PFC during daily sessions significantly reduced motivation to self-administer nicotine, these data suggest that RSK is an essential substrate downstream of ERK signaling that regulates this behavior. Changes in RSK signaling were not observed in the PFC of the nicotine challenge group, which is consistent with data indicating that ERK in the PFC appears to promote nicotine ingestion behaviors during daily exposure but not following protracted abstinence.

We did not see changes in levels of pERK or ERK, but changes in RSK protein suggest that ERK signaling was affected in these studies. Second messenger signaling pathways are labile and intricate. Thus, it may be difficult to pinpoint an exact timeframe where ERK or its downstream targets are activated, since changes may be rapid or transient and therefore difficult to decipher directly. Our behavioral data strongly suggest that ERK signaling is critical for nicotine self-administration. In the Western studies, brains were harvested from animals out of their homecages or a novel locomotor chamber. It is possible that the behavioral environment where nicotine was ingested (e.g. the operant chamber) contributed to the behavior findings. The PFC, VTA and NAc are activated by cigarette-associated cues in smokers (Due et al., 2002). It has been shown that introduction to a novel environment increases levels of pCREB and CREB in the hippocampus and NAc (Winograd and Viola, 2004) and nicotine-paired environments increase pCREB in the NAc shell and immediate early genes in the PFC (Kelley, 2004),
suggesting that environmental effects on ERK signaling may unmask possible nicotine-induced changes in neuroplasticity. Cigarette-associated cues can elicit strong craving in smokers. It is possible that ERK facilitates the association of cues with the primary reinforcing effects of nicotine.

While it is clear that the ERK signaling pathway in areas including the PFC, NAc and VTA plays an important role in the addictive properties of nicotine, targeting this pathway directly is not an ideal therapeutic strategy for smoking cessation. The ERK pathway has widespread effects on cellular activity throughout the entire body. Since ERK is a member of the MAPK family, which is an extensive family of kinases important for cell growth, death, proliferation and differentiation (Kyosseva, 2004), manipulating its activity in humans could lead to adverse effects throughout the body, including possible disruption of cognition, prevention of cell growth leading to tissue atrophy, or tumor growth and cancer. It would therefore be safer and more sensible to target the nAChRs, which modulate ERK activity based on the presence or absence of nicotine, without affecting important signaling events throughout the entire body. One study showed that activation of α7 and β2* nAChRs in the PFC leads to glutamate release through effects on intracellular Ca^{2+} and the α7-mediated release is ERK-dependent (Dickinson et al., 2008). Another showed that α6β2* nAChRs in the NAc shell were essential for maintaining nicotine self-administration (Brunzell et al., 2010). These data and the results from our studies implicate the α7 nAChRs in the PFC and α6β2* nAChRs in the NAc shell as possible therapeutic targets for smoking cessation. Treatments targeting the α7 and α6* receptors in combination with the β2* nAChRs would likely be more effective than those such as Varinecline, which is an α4β2 partial agonist, that does not affect the other nAChRs. Pharmacological and molecular studies need to confirm if these nAChR subtypes regulate ERK
signaling in the PFC and NAc shell during daily self-administration and following protracted abstinence from nicotine.

At the present time, manipulation of the nAChRs appears to be a promising method of inducing and maintaining abstinence from smoking. An ideal treatment would encompass multiple mechanisms that target both the physiological effects of nicotine, as well as the addictive properties of smoking-related cues, and address not only the period while a person attempts to quit, but also the abstinence and post-abstinence period where patients may be extremely vulnerable to relapse. Much remains to be learned about the neurchemical events that regulate nicotine addiction, craving and withdrawal. Our studies expand on previous data to show that the ERK signaling pathway plays a critical role in nicotine addiction behaviors. Whereas ERK may not be a viable target for smoking cessation in humans, identification of critical pathways upstream and downstream of ERK signaling may lead to novel therapeutics for smoking cessation.
References


Ikemoto S, Qin M, Liu ZH (2006) Primary reinforcing effects of nicotine are triggered from multiple regions both inside and outside the ventral tegmental area. J Neurosci 26:723-730.


Table 1. Locomotor Assay Groups

<table>
<thead>
<tr>
<th>GROUP A - NO INFUSION</th>
<th>GROUP B - U0126 OR VEH INFUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Self-Admin</strong></td>
<td><strong>Infusion</strong></td>
</tr>
<tr>
<td>CUE SA</td>
<td>SAL</td>
</tr>
<tr>
<td>CUE SA</td>
<td>NIC</td>
</tr>
<tr>
<td>NIC SA</td>
<td>SAL</td>
</tr>
<tr>
<td>NIC SA</td>
<td>NIC</td>
</tr>
</tbody>
</table>

Breakdown of locomotor groups for experiments testing the effects of prior nicotine (NIC) or cue (CUE) exposure on locomotor activity and response to 0.175 mg/kg nicotine (NIC) or saline vehicle (SAL) challenge (Group A) and on the effects of PFC- and NAc shell-infusion of 0.4 µg/hemisphere MEK inhibitor (U0126) or equal volume of vehicle (VEH) on locomotor activity following a challenge dose of 0.175 mg/kg s.c. nicotine (NIC) (Group B).
Table 2. Locomotor Assay Timeline

Locomotor Assay Timeline for Groups A and B. Note that Group A did not receive infusions of VEH or 0.4 μg U0126 immediately prior to the locomotor testing, while Group B did receive intra-NAc or VTA infusions, shown in parenthesis.
Table 3. Western Blot Groups

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Infusion</th>
<th>Challenge Tx</th>
<th>Time Abstinent</th>
<th>n size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUE SA</td>
<td>VEH</td>
<td>0.175 NIC</td>
<td>8+ DAYS</td>
<td>3</td>
</tr>
<tr>
<td>NIC SA</td>
<td>U0126</td>
<td>0.175 NIC</td>
<td>8+ DAYS</td>
<td>2</td>
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</table>
### Table 4. Nicotine Intake

<table>
<thead>
<tr>
<th></th>
<th>Daily Session (1 Day) Abstinence</th>
<th>Protracted (8+ Day) Abstinence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VEH-VEH</td>
<td>VEH-U0126</td>
</tr>
<tr>
<td>NAc Shell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine Deliveries</td>
<td>8.0 ± 2.02</td>
<td>8.8 ± 0.96</td>
</tr>
<tr>
<td>Intake mg/kg</td>
<td>0.24 ± 0.06</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>PFC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine Deliveries</td>
<td>9.25 ± 1.25</td>
<td>7.33 ± 0.85</td>
</tr>
<tr>
<td>Intake mg/kg</td>
<td>0.28 ± 0.03</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

Number of IV nicotine deliveries and total mg/kg of nicotine intake are shown for rats during a daily progressive ratio session following infusion of vehicle (VEH) into the nucleus accumbens shell (NAc shell) or anterior cingulate cortex (PFC) and in the same animals following at least 8 days protracted abstinence following an infusion of VEH or 0.4 µg U0126 in these brain areas. NAc shell-infused rats showed a significant increase in their ingestion of nicotine following protracted abstinence. This effect was blocked by local infusion of MEK inhibitor, U0126, suggesting that activation of ERK in the NAc supports incubation effects of protracted abstinence from nicotine. PFC-infused animals did not show this incubation effect. *Indicates significantly different from vehicle infused during the daily session, p< 0.05.
Table 5. % of Animals Reaching Breakpoint During Self-Administration

<table>
<thead>
<tr>
<th>Tx</th>
<th>U0126 Dose (µg/hemisphere)</th>
<th>Self-Admin</th>
<th>Brain Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>CUE SA</td>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>NIC SA</td>
<td></td>
<td>14</td>
<td>43</td>
</tr>
<tr>
<td>CUE SA</td>
<td></td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>NIC SA</td>
<td></td>
<td>29</td>
<td>33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tx</th>
<th>U0126 Dose (µg/hemisphere)</th>
<th>Self-Admin</th>
<th>Brain Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>NIC SA</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NIC SA</td>
<td></td>
<td>33</td>
<td>50</td>
</tr>
</tbody>
</table>

% of Animals Reaching Breakpoint During Self-Administration. i.e., animals who did not actively respond for the entire 2 h self-administration period. Sessions timed-out following a period of 20 minutes of no responding on the active lever.
Figure 1. Daily administration of nicotine is regulated by ERK in the PFC but not the NAc shell. Active responding and breakpoints are shown during daily progressive ratio responding maintained by nicotine (PR) for animals that received infusions of vehicle (VEH) or MEK inhibitor U0126 (0.1, 0.4 µg/hemisphere). Local infusion of U0126 into the PFC but not the NAc shell resulted in significant reductions in active lever pressing and breakpoints during PR. *Significantly different from VEH infusion, $p < 0.05$, ** $p < 0.01$. 
Figure 2. Incubation effects of nicotine are regulated by ERK signaling in the nucleus accumbens shell (NAc shell). Protracted abstinence from nicotine resulted in significant elevations in active lever pressing in vehicle-infused rats (VEH) that were blocked by local infusion of 0.4 μg of U0126 (U0126) into the NAc shell. Similar trends were observed for breakpoint. There was no effect of abstinence or infusion of U0126 on % active lever pressing, indicating that these behavioral effects were specific to the nicotine lever. *Significantly different from responding following VEH infusion during daily exposure to nicotine (1 Day Abstinent), p < 0.05; #Significantly different from VEH-infused subjects following protracted abstinence (> 8 Days Abstinent), p < 0.05.
Figure 3. Nicotine effects on locomotor activity. Challenge injections of 0.175 mg/kg nicotine did not affect locomotor activity in rats with histories of cue or nicotine self-administration, but prior nicotine exposure resulted in elevated locomotor activity in an open field chamber as measured by a significant increase distance traveled and time ambulatory in NIC compared to CUE rats. *Significantly different from CUE SA rats (p < 0.05).
Figure 4. Effects of MEK inhibition on locomotor activity. Locomotor activity as measured by % of baseline activity and % of time ambulatory is not affected by 0.4 μg/hemisphere infusions of MEK inhibitor U0126 (MEK) into the PFC or NAc shell \([p > 0.05\) compared to no infusion control (No Inf); \(p > 0.05\) compared to neuroanatomical vehicle-infused control (VEH)].
Figure 5. Effects of 24 hours and 8+ days abstinence from nicotine exposure on ERK signaling proteins in the NAc shell. Western blots analysis revealed a main effect of nicotine self-administration on levels of total RSK in the anterior NAc shell of nicotine self-administration rats (NIC SA) compared to control rats (CUE SA) independent of abstinence period ($p<0.05$), but this effect appeared to be driven by rats following protracted abstinence.
Figure 6. Effects of 24 hours and 8+ days abstinence from nicotine exposure on ERK signaling proteins in the PFC and VTA. There was main effect of prior nicotine exposure on levels of RSK in the anterior PFC of nicotine self-administration rats (NIC SA) compared to control subjects (CUE SA) ($p<0.001$). This effect was independent of abstinence period but appeared to be driven by rats following 24 h of abstinence.
Nicotine challenge effects on ERK signaling are mediated by prior nicotine exposure but not affected by local infusion of a MEK inhibitor U0126. Western blot analysis of ERK signaling proteins in the NAc shell, PFC and VTA are shown for animals during protracted abstinence following a challenge dose of 0.175 mg/kg s.c. nicotine following no infusion to cue control rats (CUE SA), or infusion of vehicle or 0.4 μg/hemisphere U0126 to NIC-VEH or NIC-SA-U0126 subjects. In the anterior NAc shell there was a nearly significant effect of treatment group on levels of RSK ($p = 0.058$) with similar trends in the posterior NAc shell. Post hoc t-tests revealed that VEH- and U0126-infused NIC SA subjects showed a significant elevation of levels of RSK compared to non-infused CUE SA rats ($p = 0.05$).
Vita

Lauren Elizabeth Thompson was born on October, 2, 1984, in Pulaski County, Virginia, and is an American citizen. She graduated from Pulaski County High School, Dublin, Virginia in 2002. She received her Bachelor of Science in Biology from Kansas State University, Manhattan, Kansas in 2007. She received a Post-Baccalaureate Certificate in Pre-Medical Health Sciences from Virginia Commonwealth University in 2010.