Glutamatergic and Neuroimmune Mechanisms of *N*-acetylcysteine-Mediated Inhibition of Cue-Induced Nicotine Seeking

By

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ABSTRACT

Nicotine self-administration is associated with decreased expression of the glial glutamate transporter 1 (GLT-1) and the cystine-glutamate exchange protein xCT in the nucleus accumbens core (NAcore). *N*-acetylcysteine (NAC), which is an antioxidant, anti-inflammatory, and glutamatergic agent, restores these proteins associated with increased relapse vulnerability. However, the specific molecular mechanisms driving NAC inhibitory effects on cue-induced nicotine reinstatement are unknown. Thus, the present study assessed NAC's effects on cue-induced nicotine reinstatement are dependent on NAcore GLT-1 expression. Here, rats were treated with NAC in combination with intra-NAcore vivo-morpholinos to examine the role of GLT-1 in NAC-mediated inhibition of cue-induced nicotine seeking. Subchronic NAC treatment attenuated cue-induced nicotine seeking in male rats and an antisense vivo-morpholino (AS) designed to selectively suppress GLT-1 expression in the NAcore blocked this effect. NAC treatment was also associated with an inhibition of pro-inflammatory tumor necrosis factor alpha (TNFα) expression in the NAcore. As well, GLT-1 AS markedly increased expression of CD40, a known marker of pro-inflammatory M1 activation of microglia and macrophages. To further examine whether NAC-induced decreases in nicotine seeking involve suppression of TNFα, we manipulated a downstream mediator of this pathway, nuclear factor kappa B (NF-kB). Considering the putative role of NF-κB in learning, memory, and synaptic plasticity, separate experiments were performed where rats were treated with herpes simplex virus (HSV) vectors designed to increase (HSV-IKKca) or decrease (HSV-IKKdn) NF-κB signaling through interactions with IκB Kinase (IKK). The goal was to examine the role of NF-κB signaling in mediating nicotine

seeking behavior and if NF-κB signaling regulates GLT-1 expression. HSV-IKKdn alone and in combination with NAC inhibited cue-induced nicotine reinstatement, while HSV-IKKca blocked the attenuating effect of NAC on reinstatement. Interestingly, both HSV-IKKdn and HSV-IKKca, regardless of NAC treatment, inhibited GLT-1 expression. Taken together, these results suggest that while GLT-1 may be a conserved neurobiological substrate underlying relapse vulnerability across drugs of abuse, immunomodulatory mechanisms may regulate drug-induced alterations in glutamatergic plasticity that mediate cue-induced drug-seeking behavior through GLT-1-independent mechanisms.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

Tobacco Use Disorder, which is characterized by clinical features such as craving, tolerance, withdrawal, and unsuccessful quit attempts, is a significant public health concern and remains the leading cause of preventable death in the United States, accounting for nearly one in five deaths in adults (Danaei et al., 2009; U.S. Department of Health and Human Services, 2014). Nearly all adults (88%) who qualify as chronic smokers began smoking prior to the age of 18, which is a critical period for the development of the brain and is a vulnerable period during which substance use disorders and psychiatric co-morbidities can form (Fuhrmann, Knoll, & Blakemore, 2015; Smith, McDonald, Bergstrom, Ehlinger, & Brielmaier, 2015; U.S. Department of Health and Human Services, 2014). While rates of smoking have continued to decline over the past decade, the rise of e-cigarettes and other forms of nicotine consumption (particularly among adolescents) highlight the pervasiveness of nicotine and tobacco products as potentials risks to public health and safety (Camenga & Klein, 2016). Nicotine, a parasympathomimetic alkaloid found in tobacco, is a nicotinic acetylcholine receptor (nAChR) agonist and is generally considered the primary psychoactive and reinforcing compound in tobacco products (Stolerman & Jarvis, 1995). Current smoking cessation treatments primarily function to replace nicotinic stimulation of nAChRs to attenuate drug craving without producing rewarding effects (Nides, 2008). For example, varenicline (Chantix®) is a partial agonist of $\alpha_4\beta_2$ nAChRs and has shown some clinical efficacy in helping individuals quit smoking (Kasza et al., 2015; McClure, Vandrey, Johnson, & Stitzer, 2013). Regardless, high rates of relapse persist even for individuals

receiving replacement therapy (Leshner & Stapleton, 1997), which highlights the need for more effective pharmacotherapies that better promote long-term abstinence from tobacco use.

The studies described herein attempt to clarify the cellular, molecular, and behavioral mechanisms underlying the pharmacotherapeutic *N*-acetylcysteine (NAC), which is an antioxidant, anti-inflammatory, and glutamatergic agent that has demonstrated some clinical efficacy in the treatment of substance use disorders and other neuropsychiatric conditions (Deepmala et al., 2015; McClure, Gipson, Malcolm, Kalivas, & Gray, 2014; Tomko et al., 2018). Below is a detailed review of the relevant literature, which examines nicotine-induced alterations in glutamatergic plasticity, the known neurobiological mechanisms underlying NAC's attenuating effects on drug-seeking behavior, neuroinflammation as a modulator of glutamatergic plasticity, the clinical and preclinical applications of NAC as a therapeutic, and the current gaps in the literature that the present studies attempt to address.

CHAPTER 2

REVIEW OF THE LITERATURE

Nicotine activates the mesolimbic reward pathway by stimulating nAChRs on dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain, which projects to the nucleus accumbens (NA), prefrontal cortex, amygdala, hippocampus, as well as several other structures (De Biasi & Dani, 2011; Laviolette & van der Kooy, 2004; Mansvelder, Keath, & McGehee, 2002; Mansvelder & McGehee, 2002; Placzek, Zhang, & Dani, 2009). Specifically, nicotine augments dopamine release through activation of $\alpha_4\beta_2$ nAChRs in the VTA (Pidoplichko et al., 2004). However, nicotine may

also stimulate the release of dopamine through activation of α_6-containing nAChRs (Liu, Zhao-Shea, McIntosh, Gardner, & Tapper, 2012), as well as α_7 nAChRs located on glutamatergic afferents in the VTA (Schilström et al., 2000; Schilström, Svensson, Svensson, & Nomikos, 1998). The neurobiological processes mediating the initiation of nicotine dependence have been fairly well studied and much has been discovered regarding genetic and environmental risk factors that may contribute to the acquisition and maintenance of nicotine addiction (Bierut et al., 2008; Changeux et al., 1998; Fowler, Lu, Johnson, Marks, & Kenny, 2011; Kuryatov, Berrettini, & Lindstrom, 2011; Stairs, Kangiser, Hickle, & Bockman, 2016). Since the discovery of the β_2 nAChR subunit's role in the acquisition of nicotine self-administration (Picciotto et al., 1998), drugs such as varenicline that stimulate cholinergic signaling have proven clinically efficacious in helping individuals quit smoking as mentioned above. Regardless, the cellular adaptations and molecular mechanisms underlying vulnerability to nicotine relapse are not as thoroughly understood.

Nicotine-Induced Alterations in Glutamatergic Signaling Underlying Relapse

Maladaptive glutamatergic plasticity has been implicated across several major drugs of abuse (Kalivas, LaLumiere, Knackstedt, & Shen, 2009; Scofield et al., 2016; van Huijstee & Mansvelder, 2014). These synaptic alterations mediate the associative learning processes that occur between environmental stimuli and drugs of abuse. For example, increased prefrontal glutamate release into the NAcore has been shown to mediate cue- and drug-induced reinstatement of drug-seeking (Gipson et al., 2013a; LaLumiere & Kalivas, 2008; McFarland, Lapish, & Kalivas, 2003; Smith et al., 2017). Specifically, glutamatergic projections from the prelimbic cortex (PL) to the NA have

been associated with rapid, transient synaptic plasticity during cue-induced reinstatement of cocaine- (Gipson et al., 2013a), heroin- (Shen, Moussawi, Zhou, Toda, & Kalivas, 2011), and nicotine-seeking (Gipson et al., 2013b). Glutamatergic stimulation of medium spiny neurons (MSNs) in the NA is critical for mediating reward seeking in response to contingent cues (Di Ciano, Cardinal, Cowell, Little, & Everitt, 2001), and chronic exposure to drugs of abuse decreases basal levels of extracellular glutamate in the NA (Baker, Shen, & Kalivas, 2002).

The catalytic subunit of the cystine-glutamate antiporter, xCT, is highly expressed in astrocytes and provides the majority of basal extracellular glutamate (Baker, Shen, & Kalivas 2002; Sato, Tamba, Ishii, & Bannai, 1999). Following self-administration of cocaine (Baker et al., 2003) and nicotine (Knackstedt et al., 2009), xCT is downregulated and is associated with a decrease in basal extracellular glutamate. This decrease in basal levels of glutamate decreases activation of group II metabotropic glutamate receptors (i.e. mGluR2/3 receptors), which provide inhibitory tone over glutamatergic afferents in the nucleus accumbens (NA). It has been suggested that this loss of tone following chronic drug exposure enhances glutamate release during both drug- and cue-induced reinstatement of drug-seeking (Baker et al., 2003; Bossert, Busch, & Gray, 2005; McFarland et al., 2003; Moran, McFarland, Melendez, Kalivas, & Seamans, 2005). In addition to down-regulated cystine-glutamate exchange, chronic drug self-administration is associated with a decrease in expression of glutamate transporter 1 (GLT-1) (Alhaddad, Das, & Sari, 2014; Gipson et al., 2013b; Knackstedt, Melendez, & Kalivas, 2010; Sari, Smith, Ali, & Rebec, 2009; Shen, Scofield, Boger, Hensley, & Kalivas, 2014), which is found predominantly in astrocytes and is responsible for 90% of

glutamate uptake in the CNS (Haugeto et al., 1996). Together with system xc^-, GLT-1 serves as a critical regulator of glutamate neurotransmission in the NA and is a putative target for treating substance use disorders (Roberts-Wolfe & Kalivas, 2015).

Neuroinflammation, Glutamatergic Plasticity, and Addiction

Neuroinflammation is a consequence of chronic drug abuse that is observed across drug classes and has been most extensively studied within alcohol abuse models (Crews & Vetreno, 2011). Clinical studies have demonstrated that chronic cigarette smoking alters normal innate immune function and can dysregulate normal immune responses to inflammatory stimuli, such as through impaired innate and adaptive immune responses that result in chronic immune cell activation and blunted immune responses to noxious stimuli such as lipopolysaccharide (LPS) (Lee, Taneja, & Vassallo, 2012). Importantly, the NF-κB pathway, which is a critical regulator of the innate immune system, is activated by cigarette smoking and can directly alter host NF-κB-dependent pro-inflammatory responses to infection (Manzel, Shi, O'Shaughnessy, Thorne, & Look, 2011). Nicotine is a known immunomodulator and, as mentioned previously, a full agonist at α_7 nAChRs that are expressed ubiquitously on immune cells like microglia and macrophages (Shytle et al., 2004). The extant literature on nicotine-induced activation of microglial α_7 nAChRs generally reports that nicotine promotes an anti-inflammatory response, such as reducing pro-inflammatory cytokine release that is triggered by inflammatory stimuli (e.g., LPS) (Egea et al., 2015; Suzuki et al., 2006). While the anti-inflammatory potential of α_7 nAChRs is well documented, there are critical gaps in the literature that warrant further investigation. For example, Thomsen and Mikkelsen (2012) attempted to clarify whether the anti-inflammatory potential of acute nicotine exposure is

due to activation or inhibition of nAChRs. In this study, Thomsen and Mikkelsen discovered that both agonists and positive allosteric modulators of α_7 nAChRs do not alter LPS-induced release of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). In contrast, this study found that the α_7 antagonist methyllycaconitine (MLA) and a weak agonist both inhibited LPS-induced TNFα release from microglia. These findings suggest that the anti-inflammatory potential of nicotine via α_7 nAChRs may not be through typical ion flux via activation of the receptor. For example, one study demonstrated that nicotinic stimulation of microglial α_7 can attenuate LPS-induced TNFα release through a phospholipase C-dependent mechanism that increases intracellular Ca^{2+} signaling in microglia. However, in the absence of LPS, nicotine alone can dose-dependently facilitate TNFα release mediated by the adenosine triphosphate receptor P2X7 (Suzuki et al., 2006). Given these findings, it is possible that chronic nicotine self-administration could facilitate TNFα release through such a mechanism. Ultimately these studies highlight the inconsistencies in the literature regarding the specific cellular and molecular mechanisms of nicotine's immunomodulatory actions. As well, the distinctions between chronic versus acute nicotine and *in vitro* versus *in vivo* effects are not fully parsed. Thus, investigation of the effects of chronic nicotine self-administration on pro-inflammatory signaling and subsequent modulation of synaptic plasticity and behavior is warranted.

TNFα signaling and the NF-κB pathway regulate synaptic plasticity (Albensi & Mattson, 2000; Kaltschmidt & Kaltschmidt, 2015; Meffert, Chang, Wiltgen, Fanselow, & Baltimore, 2003; O'Neill & Kaltschmidt, 1997), including synaptic plasticity associated with addiction-like behaviors (Clark, Wiley, & Bradberry, 2013; Crews & Vetreno, 2011;

Cui, Shurtleff, & Harris, 2014; Gilmore, 2006; Russo et al., 2009). In the canonical signaling pathway (as depicted in Figure 6), NF-κB heterodimers (e.g. p50/p65) are maintained in an inhibited state within the cytoplasm through interactions with IκB (e.g. IκBα). Through ligand binding of extracellular signals (e.g., TNFα) to their cell-surface receptors, an IκB kinase (IKK) complex phosphorylates IκBα, triggering the proteasomal degradation of IκBα and translocation NF-κB into the nucleus (Gilmore, 2006). Cocaine-induced alterations in dendritic spine morphology and the acquisition of conditioned place preference are mediated by NF-κB signaling, where inhibition of IKK activity blocks these drug-induced neurobehavioral adaptations (Russo et al., 2009). As well, chronic cocaine markedly induces NF-κB expression in the NA (Ang et al., 2008). Interestingly, the β-lactam antibiotic ceftriaxone, which restores glial glutamate transport and inhibits cue-induced drug seeking (Alhaddad et al., 2014; Knackstedt et al., 2010; Sondheimer & Knackstedt, 2011), increases NF-κB binding at the promoter of the excitatory amino acid transporter (EAAT)-2, or GLT-1), resulting in increased expression of GLT-1 and glial glutamate uptake (Lee et al., 2008). Notably, GLT-1 undergoes differential regulation by NF-κB depending on the extracellular signal that triggers its activation, where TNFα promotes transcriptional repression of GLT-1 expression through NF-κB activation (Sitcheran, Gupta, Fisher, & Baldwin, 2005). Ceftriaxone also inhibits TNFα release in a rat model of traumatic brain injury (Wei et al., 2012). Similarly, NAC, which also inhibits drug-induced alterations in synaptic plasticity and cue-induced reinstatement (Kupchik et al., 2012; Moussawi et al., 2009; Ramirez-Niño, D'Souza, & Markou, 2013; Reissner et al., 2015), has been shown *in vitro* to inhibit TNFα-induced NF-κB activation through inhibition of IKKs (Oka, Kamata, Kamata, Yagisawa, &

Hirata, 2000). Thus, drugs such as ceftriaxone and NAC may interact with specific components along the NF-κB pathway to restore glutamate homeostasis and inhibit cue-induced drug seeking. Intriguingly, TNFα rapidly increases GluA2-lacking calcium permeable (CP)-AMPAR surface expression and miniature excitatory postsynaptic currents in hippocampal slices within 15 minutes of TNFα exposure (Beattie et al., 2002), which is the same time point at which Gipson and colleagues observed transient synaptic potentiation (t-SP) of medium spiny neurons (MSNs) in the NAcore during cue-induced nicotine reinstatement (Gipson et al., 2013b). Moreover, it has been demonstrated that glia modulate activity-dependent alterations in synaptic strength through TNFα signaling (Stellwagen & Malenka, 2006) and that TNFα uniquely regulates the surface expression of both AMPA and $GABA_A$ receptors. Specifically, TNFα increases surface expression of GluA2-lacking CP-AMPARs and decreases surface expression of $GABA_A$ receptors, which enhances the excitatory strength of the synapse (Stellwagen, Beattie, Seo, & Malenka, 2005). GluA2-lacking CP-AMAPARs are increased during cocaine and nicotine withdrawal, and are associated with increased drug seeking and enhanced postsynaptic plasticity following exposure to drug-paired cues (Conrad et al., 2008; Wolf & Tseng, 2012).

Clinical and Pre-Clinical Applications of NAC

The cysteine pro-drug *N*-acetylcysteine (NAC) is an antioxidant and anti-inflammatory compound that is commonly used to treat acetaminophen poisoning (Yarema et al., 2009). NAC has also been used to treat a multitude of psychiatric disorders and neurological diseases, including autism, Alzheimer's disease, trichotillomania, and many others (Deepmala et al., 2015). NAC is thought to drive

system xc^- and inhibits both drug- and cue-induced reinstatement of drug seeking (Kupchik et al., 2012; Moussawi et al., 2009; Ramirez-Niño et al., 2013; Zhou & Kalivas, 2008). As mentioned previously, reinstatement of drug seeking is associated with glutamate release into the NA and a large body of evidence suggests that glutamatergic stimulation of postsynaptic mGluR5 receptors potentiates drug-seeking behavior (Kumaresan et al., 2009; Moussawi et al., 2009; Smith et al., 2017; Wang, Moussawi, Knackstedt, Shen, & Kalivas, 2013). Particularly, NAC-mediated inhibition of cue-induced cocaine reinstatement depends on restoring homeostatic signaling between mGluR2/3 and mGluR5 receptors (Kupchik et al., 2012). Indeed, stimulation of mGluR5 receptors in the NAcore has been shown to increase cue-induced cocaine reinstatement (Wang et al., 2013) and systemic antagonism with 2-methyl-6-(2-phenylethynyl)pyridine (MPEP) has been shown to attenuate cue-induced nicotine reinstatement (Bespalov et al., 2005). Nevertheless, given NAC's intimate involvement with redox-sensitive signaling mechanisms, particularly regarding antioxidant and anti-inflammatory processes, it is also likely that its therapeutic efficacy depends on these mechanisms (Elbini Dhouib et al., 2016). For instance, chronic cocaine self-administration increases oxidative stress. Specifically, cocaine increases *S*-glutathionylation and decreases the expression of GSH-*S*-transferase pi (GSTpi), which has been shown to regulate cocaine-induced neurobehavioral plasticity (Uys et al., 2011).

As mentioned above, drugs such as NAC and ceftriaxone have been shown to upregulate both GLT-1 and xCT. As well, restoration of these two proteins is associated with decreases in drug- and cue-induced reinstatement of drug seeking (Alhaddad, Das, & Sari, 2014; Knackstedt, Melendez, & Kalivas, 2010; Sari, Smith, Ali, & Rebec, 2009;

Sondheimer & Knackstedt, 2011). Interestingly, a recent study demonstrated that chronic NAC administration successfully inhibits cue-induced cocaine reinstatement when xCT expression is suppressed and that chronic NAC treatment is ineffective if GLT-1 is not restored (Reissner et al., 2015). In addition, this study also found that inhibition of GLT-1 expression along with systemic administration of NAC significantly augmented cue-induced reinstatement, an effect that was blocked by administration of the mGluR5 negative allosteric modulator 3-[(2-methyl-4-thiazolyl)ethynyl]pyridine (MTEP) (Reissner et al., 2015). Thus, while previous studies would suggest that system xc^- activity is necessary for NAC to attenuate reinstatement, recent evidence suggests that the mechanism through which NAC elicits its therapeutic effects may be more complex. Understanding the mechanistic properties of NAC's therapeutic potential may provide useful insights into the molecular underpinnings of cue-induced relapse across drugs of abuse.

Research Aims and Hypotheses

Vivo-morpholino and viral-mediated gene transfer strategies were utilized during extinction training to manipulate GLT-1 and NF-κB signaling, respectively, to examine their role in NAC's ability to inhibit cue-induced nicotine seeking. We hypothesized that NAC would inhibit conditioned nicotine-seeking behavior and that this process is both GLT-1- and NF-κB-dependent. Specifically, we predicted that (1) blocking the restoration of GLT-1 via NAC would prevent NAC from inhibiting reinstatement and (2) that driving NF-κB signaling would also occlude these restorative processes. In addition, we hypothesized that NAC treatment and GLT-1 knockdown would alter neuroimmune signaling. Therefore, we predicted that (3) NAC treatment would inhibit TNFα

expression and that (4) GLT-1 knockdown would elevate CD40 expression (a marker of pro-inflammatory activation of microglia and other immune cells).

CHAPTER 3

METHODS & MATERIALS

Subjects

93 Male Sprague Dawley rats (Charles River, 250-300 g) were individually housed on a 12-hour reverse light-dark cycle and received 20 g of food/day and *ad libitum* water. Experimentation was conducted during the dark phase. All experiments conducted adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and were approved by the Arizona State University Institutional Animal Care and Use Committee.

Drugs and Viral Vectors

Nicotine tartrate (MP Biomedicals, LLC, Solon, OH, USA) was dissolved in 0.9% saline and the pH adjusted to 7.4. Nicotine doses were calculated based on free base weight. NAC (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 27 mg/ml sodium hydroxide in saline to physiological pH immediately before injection. Vivo-morpholinos (Gene Tools, LLC, Philomath, OR, USA) were dissolved in sterile phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl) and stored at room temperature. The GLT-1 antisense (AS) vivo-morpholino sequence is 5'-TGTTGGCACCCTCGGTTGATGCCAT-3'. The reverse sequence was used as a control (CTRL). HSV vector titers were as follows: HSV-IKKdn (1.4x108 TU/mL), HSV-IKKca (2.0x108 TU/mL), or HSV-GFP (1.9x108 TU/mL).

Surgical Procedures

Rats were anesthetized with ketamine HCL (100 mg/kg, i.m.) and xylazine (8 mg/kg, i.m.) and underwent surgical implantation of intravenous catheters and intracerebral guide cannulae. SILASTIC catheters (Dow Corning, Midland, MI, USA) were inserted 3 mm into the right jugular vein, exited subcutaneously through a dermal hole between the shoulder blades, and were attached to a stainless-steel cannula held within a backpack (Instech Laboratories, Plymouth Meeting, PA, USA). Immediately following catheter implantation, rats were fixed onto a stereotaxic frame and intracerebral guide cannulae (26G, 15 mm, PlasticsOne, Roanoke, VA, USA) were implanted bilaterally into the NAcore (+1.5 A/P, +1.8 M/L, -5.5 D/V, from Paxinos & Watson, 2007). Cefazolin (100 mg/kg) and meloxicam (1 mg/kg) were administered at the end of surgery. Meloxicam was administered for three days following surgery to provide analgesia. Cefazolin and heparin (100 usp/kg) were infused through the catheter for seven days following surgery and heparin alone was administered daily throughout self-administration to maintain catheter patency.

Nicotine self-administration and extinction training

Behavioral testing took place in operant chambers containing two-levers (an active right lever and an inactive left lever), a stimulus light above each lever, and a food receptacle (Med Associates, Inc., Fairfax, VT, USA). Prior to surgery and SA procedures, rats received a 15 hour food training session where active lever presses dispensed a food pellet (45 mg, Bio-Serv, Flemington, NJ, USA) into a receptacle. Food delivery was not paired with conditioned cues. Following food training, rats underwent nicotine SA for 2 hours/day on a fixed-ratio 1 (FR-1) schedule of reinforcement where each active lever press resulted in a single infusion of nicotine (0.02 mg/kg/infusion) that was paired with a

compound stimulus light and auditory tone followed by a 20-second time-out period. Inactive lever presses had no consequence, but responses were still recorded. SA criteria were set at a minimum of 10 infusions per session for 10 sessions and a 2:1 active-to-inactive lever press ratio. Upon completion of SA, rats entered extinction training, where active lever presses no longer resulted in nicotine infusions or associated cues. Extinction training persisted for 14-16 days prior to a 2-hour cue-induced reinstatement test. Active lever presses during reinstatement testing resulted in presentation of nicotine-paired cues but not nicotine. Immediately following reinstatement testing, animals were rapidly decapitated and NAcore tissue was collected for glutamate uptake, electrophysiology, or western blot analysis (described in further detail below). Only animals that extinguished to below 65 active lever presses prior to the onset of vivo-morpholino and/or NAC treatment were included in the study as described previously (Reissner et al., 2015).

Microinjection and drug treatment procedures

Experiment 1

Beginning on day 10 of extinction training, rats received 3 days of bilateral microinjections (1 µl, 0.5 µl/min, 30 pmol/injection) of GLT-1 AS or CTRL sequence vivo-morpholino 2-hours following each extinction session. Microinjectors were placed 2 mm below the guide cannula into the NAcore and were left in place for 1 min following the injection to allow for the morpholino to fully diffuse through the tissue. Rats were sham injected one day prior to their first microinjection. On days 12 to 16 of extinction, rats received NAC (100 mg/kg, i.p.) or saline 2-hours prior to each extinction session. This treatment regimen was based on previous studies that demonstrated an increase in cystine-glutamate exchange and GLT-1 expression following NAC treatment, a decrease

in cue-induced cocaine reinstatement, as well as effective down-regulation of GLT-1 following morpholino treatment. (Knackstedt, Melendez, & Kalivas, 2010; Moussawi et al., 2011; Reissner et al., 2012, 2015). Figure 1A depicts the experimental timeline.

Experiment 2

On day 10 of extinction, rats received an acute intra-NAcore microinjection of either HSV-GFP, HSV-IKKdn, or HSV-IKKca (1 µl, 0.5 µl/min). Rats were sham microinjected the day before receiving HSV. NAC (100 mg/kg, i.p.) was administered for 5 days beginning on day 10 of extinction exactly as described above. Figure 4A illustrates a timeline of the treatment protocol. For both experiments, cannula placement was visually inspected prior to tissue collection (as described below) and only animals with cannula placement in the NAcore were included in the study.

Tissue preparation, western blotting, and immunohistochemistry

Experiment 1

To examine GLT-1 expression, animals were rapidly decapitated immediately following a 2-hour reinstatement test and NAcore tissue was collected and homogenized in 200 µl of ice-cold sucrose buffer containing HEPES and sucrose (pH=7.4) and supplemented with 1:100 protease and phosphatase inhibitors. Samples were centrifuged at 1,000 x g for 10 mins at 4°C, resulting in a pellet that was re-suspended in another 200 µl of ice-cold sucrose buffer containing 1:100 protease and phosphatase inhibitors. The pellet was centrifuged again at 1,000 x g for 10 mins. Supernatants were combined and centrifuged at 12,000 x g for 20 mins. The resulting pellet was re-suspended in 30 µl of RIPA lysis buffer containing protease and phosphatase inhibitors and centrifuged at 10,000 x g for 5 mins to remove insoluble matter. To examine CD40 and TNFα

expression, NAcore tissue was collected and homogenized in 200 µl of ice-cold RIPA lysis buffer and supplemented with 1:100 protease and phosphatase inhibitors. Samples were centrifuged at 10,000 x g for 5 mins at 4°C, followed by collection of the supernatant. Protein concentrations were determined by the BCA method (Thermo Scientific). Samples were prepared in 4x lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and incubated for 10 mins at 70°C. Equal microgram quantities of protein were loaded onto a 4-12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane using semi-dry transfer conditions for 6 mins (iBlot, Invitrogen). Membranes were blocked for 2 hours in tris-buffered saline plus 0.1% Tween-20 (TBST) containing 5% non-fat milk or bovine serum albumin (BSA) and incubated overnight at 4°C in primary antibody (see Table 1). Following incubation, membranes were washed 6 x 5 mins in TBST, incubated in secondary antibody (see Table 1) for 1 hour at room temperature, and washed 6 x 5 mins in TBST. Proteins were detected using ECL chemiluminescent substrate (Thermo Scientific) and band density analyzed using NIH ImageJ software. Protein expression was normalized to calnexin or GAPDH.

Experiment 2

For western blot experiments, tissue was homogenized exactly as described above and centrifuged at 10,000 x G for 5 mins at 4°C. Supernatants were collected, processed, and probed for GLT-1 exactly as described above. Phosphorylated IκBα (phospho-IκBα) and total IκBα expression was also analyzed to verify molecular activity of viral constructs. Proteins were detected and normalized to GAPDH as described above and densities were analyzed using NIH ImageJ software. To validate viral expression of the fluorescent reporter (EGFP) and to confirm neuronal transduction of IKK mutants,

immunohistochemistry was performed. Here, rats were sacrificed via ketamine overdose (200 mg/kg, i.p.) for transcardiac perfusion with ice-cold 4% paraformaldehyde (PFA) in phosphate buffer (PB) three days after intra-NAcore HSV injection, when viral transgene expression is at its peak (Lachmann, 2004). Brains were post-fixed overnight in 4% PFA and 30% sucrose for at least 48-hours prior to collection of 40 µm-thick slices of the NAcore at 20°C. Slices were washed 3 x 10 mins in phosphate buffered saline (PBS) and blocked for 2-hours in 5% normal goat serum (NGS) + PBS + 0.1% Triton X-100 (PBST). Slices were then incubated overnight at 4°C in anti-GFP (see Table 1) and then washed for 3 x 10 mins in PBST. Next, slices were incubated in secondary antibody (goat anti-chicken, Alexa Fluor® 488, see Table 1) for 2-hours, washed 3 x 10 mins in PBST, and then washed once for 10 mins in PBS. Finally, slices were mounted and cover-slipped with Vectashield® mounting medium and slides were stored at 4°C until analyzed. Images were captured on a confocal microscope at 20x and 63x magnification at 488 nm.

Statistics

Experiment 1

Active lever pressing during a 2-hour cue-induced reinstatement test (Figure 1A) in morpholino-treated rats that received NAC or vehicle injections was analyzed using a two-way ANOVA, with "treatment" (NAC vs vehicle) and "morpholino" (AS versus CTRL) as fixed factors. GLT-1 expression (Figure 1D) was similarly analyzed using a two-way ANOVA. Lastly, two-way ANOVAs were used to analyze TNFα (Figure 2B) and CD40 (Figure 2C) expression, with "treatment" and "morpholino" as fixed factors. *Post hoc* comparisons were made using a Bonferroni or Tukey HSD multiple

comparisons test. Significance level was set at $\alpha = 0.05$ for all analyses using SPSS software.

Experiment 2

Active lever pressing during a 2-hour cue-induced reinstatement test (Figure 4C) in virus-treated rats that received NAC or vehicle injections was analyzed using a two-way ANOVA, with "treatment" (NAC vs vehicle) and "virus" (HSV-GFP vs HSV-IKKdn vs HSV-IKKca) as fixed factors. GLT-1 expression (Figure 5) was similarly analyzed using a two-way ANOVA. Phospho-IκBα:IκBα expression between HSV-IKKdn and HSV-IKKca was analyzed using a two-tailed unpaired Student's t-test. *Post hoc* comparisons were made using a Bonferroni or Tukey HSD multiple comparisons test. Significance level was set at $\alpha = 0.05$ for all analyses using SPSS software.

CHAPTER 4

RESULTS

Experiment 1

Vivo-morpholino knockdown of GLT-1 impairs NAC-mediated inhibition of cue-induced reinstatement

Here, we hypothesized that GLT-1 AS treatment in the NAcore would impair NAC-mediated suppression of cue-induced reinstatement and GLT-1 expression. Figure 1B depicts lever presses and infusions during nicotine SA and lever presses during extinction training. A two-way ANOVA revealed a significant "treatment" X "morpholino" interaction (but no main effects of treatment or morpholino alone) ($F_{(1,27)} = 5.978$, $p = 0.02$). *Post hoc* comparisons revealed a significant decrease in cue-induced nicotine reinstatement during a 2-hour session due to NAC treatment, which was blocked

by GLT-1 AS (Figure 1C, *p<0.05). Interestingly, rats treated with GLT-1 AS and NAC did not reinstate above CTRL-Vehicle conditions as previously reported with cue-induced cocaine reinstatement (Reissner et al., 2015). A two-way ANOVA examining GLT-1 expression revealed significant main effects of "treatment" ($F_{(1,21)}$ = 10.96, p<0.001), "morpholino" ($F_{(1,21)}$ = 11.08, p<0.05), and a significant "treatment" X "morpholino" interaction ($F_{(1,21)}$ = 4.36, p<0.05). *Post hoc* comparisons showed that CTRL-NAC treatment increased GLT-1 expression in the NAcore relative to all other treatment conditions (Figure 1D, *p<0.05). Taken together, these results indicate that NAC attenuates cue-induced nicotine seeking behavior and that this mechanism is dependent on NAcore GLT-1 expression.

NAC and vivo-morpholino knockdown of GLT-1 alter neuroimmune signaling in the NAcore

We hypothesized that NAC may modulate glutamatergic plasticity and subsequent nicotine-seeking behavior through immunomodulatory mechanisms. TNFα expression in the NAcore was assessed in NAC-treated rats following cue reinstatement, regardless of morpholino treatment. A two-way ANOVA revealed a significant main effect of "treatment" on TNFα expression ($F_{(1,29)}$ = 8.77, p<0.05). *Post hoc* comparisons revealed that NAC treatment, regardless of morpholino, significantly attenuated TNFα expression (Figure 2B, *p<0.05). Furthermore, a two-way ANOVA revealed a significant main effect of "morpholino" on CD40 expression ($F_{(1,27)}$ = 38.40, p<0.001). *Post hoc* comparisons indicated that regardless of NAC treatment, GLT-1 AS alone was sufficient to upregulate CD40 (predominantly expressed by microglia and other immune cells in the CNS (Kawahara et al., 2009)) (Figure 2C, *p<0.05). Together, these results suggest that while

NAC inhibits NAcore TNFα, suppressing GLT-1 may exacerbate microglial activation regardless of NAC treatment.

Experiment 2

Validation of viral constructs

Prior to examining the neurobehavioral effects of NF-κB manipulation on cue-induced nicotine reinstatement, we sought to validate the neurotropism and functional activity of HSV vectors. Rats receiving HSV-IKKca exhibited significantly elevated phosphorylation of IκBα compared to HSV-IKKdn-treated rats (Figure 3D, $t_{(14)} = 2.38$, $p = 0.032$) three days post-injection. In addition, confocal microscopy performed on fixed NAcore tissue revealed successful viral transduction and expression of the GFP reporter within NAcore MSNs (Figures 3A-C).

NF-κB signaling in the NAcore regulates cue-induced nicotine seeking and NAC's attenuating effects on nicotine seeking behavior

In this experiment, we hypothesized that constitutive activation of NF-κB would impair NAC-mediated attenuation of cue-induced nicotine reinstatement as well as inhibit GLT-1 expression in the NAcore. Figure 4B depicts lever presses and infusions during self-administration and lever presses during extinction training. A two-way ANOVA revealed a significant main effects of "treatment" ($F_{(1,38)} = 18.74$, $p < 0.001$) and "virus" ($F_{(2,38)} = 13.341$, $p < 0.001$), as well as a significant "treatment" X "virus" interaction ($F_{(2,38)} = 9.938$, $p < 0.001$) on active lever presses during a 2-hour cue reinstatement test (Figure 4C). *Post hoc* analyses revealed that rats receiving HSV-GFP + Vehicle, HSV-IKKca + NAC, and HSV-IKKca + Vehicle all significantly reinstated relative to extinction and displayed significantly higher reinstatement compared to the other treatment conditions (p

< 0.05). These results indicate that the inhibition of cue-induced nicotine reinstatement by NAC is sensitive to NF-κB signaling, which suggests that the therapeutic efficacy of NAC may depend on its immunomodulatory activity described in Experiment 1.

NF-κB signaling regulates GLT-1 expression in the NAcore following nicotine self-administration and extinction

Considering that constitutive activation of NF-κB occluded the inhibitory effects of NAC on reinstatement, we hypothesized that HSV-IKKca, regardless of NAC treatment, would result in impaired GLT-1 expression in the NAcore. A two-way ANOVA revealed a significant main effect of "virus" ($F_{(2,34)}$ = 12.103, p < 0.001), with no significant main effect of "treatment" or "treatment" X "virus" interaction (Figure 5). *Post hoc* analysis of "virus" revealed significantly higher GLT-1 expression in GFP-treated rats compared to both HSV-IKKdn- and HSV-IKKca-treated rats (p < 0.001). Taken together, these results indicate that NAC itself did not alter total levels of GLT-1 expression, but that constitutive activation or inhibition of NF-κB may suppress GLT-1 expression regardless of NAC treatment.

CHAPTER 5

DISCUSSION

GLT-1 Mediates the Inhibitory Effects of NAC on Cue-Induced Nicotine Seeking

The present findings indicate that GLT-1 expression is necessary for NAC to inhibit cue-induced nicotine seeking. The inhibition of GLT-1 expression using an antisense vivo-morpholino (GLT-1 AS) significantly suppressed GLT-1 protein levels in the NAcore even in the presence of NAC. This suppression of GLT-1 expression was associated with an increase in cue-induced nicotine reinstatement. Unlike what has been

observed with cue-induced cocaine reinstatement (Reissner et al., 2015), the combination of GLT-1 AS and NAC did not augment reinstatement significantly above what was observed in vehicle controls. Downregulation of GLT-1 is a consistent drug-induced neuroadaptation that has been observed across drug classes (Alhaddad, Das, & Sari, 2014; Gipson et al., 2013b; Knackstedt, Melendez, & Kalivas, 2010; Roberts-Wolfe & Kalivas, 2015; Sari, Smith, Ali, & Rebec, 2009; Shen, Scofield, Boger, Hensley, & Kalivas, 2014), and restoration of GLT-1 expression is associated with a decrease in cue-induced cocaine reinstatement (Knackstedt, Melendez, & Kalivas, 2010; Trantham-Davidson, LaLumiere, Reissner, Kalivas, & Knackstedt, 2012). Given these previous findings and the current results, up-regulation of GLT-1 via NAC may inhibit cue-induced nicotine seeking akin to cue-induced cocaine seeking. However, considering NAC did not potentiate reinstatement when GLT-1 levels were suppressed, the mechanism by which NAC inhibits cue-induced nicotine reinstatement may not be entirely analogous to that of cue-triggered cocaine seeking. Provided that inhibiting GLT-1 expression and driving cystine-glutamate exchange increased extracellular glutamate concentrations, it is likely that mGluR2/3 stimulation would have increased, thereby decreasing presynaptic glutamate release. Therefore, the reinstatement of nicotine seeking observed in rats treated with NAC and GLT-1 AS may not be the consequence of solely inhibiting GLT-1 expression. Indeed, GLT-1 knockdown may have neuroimmune consequences that could alter synaptic plasticity, as described below. While nicotine self-administration shares common neurobiological consequences with other drugs of abuse, nicotine differentially alters NAcore proteins associated with glutamatergic signaling as compared to cocaine and heroin (e.g., AMPA and NMDA receptor subunit composition

Conrad et al., 2008; Gipson et al., 2013b; Shen et al., 2011). Taken together, the present findings highlight the need to examine nicotine-specific mechanisms underlying cue-triggered nicotine relapse in order to improve treatment outcomes and guide the development of new and effective pharmacotherapies.

NAC Inhibits TNFα Expression and GLT-1 Knockdown Upregulates CD40

Regardless of morpholino treatment, rats treated with NAC exhibited an attenuation of pro-inflammatory TNFα expression in the NAcore relative to vehicle-treated controls. In addition, rats treated with GLT-1 AS, regardless of NAC treatment, exhibited enhanced CD40 expression, which is a marker of pro-inflammatory (M1) activation of microglia, macrophages, and other immune cells (Walker & Lue, 2015). These findings corroborate previous studies that have demonstrated the anti-inflammatory potential of NAC (Oka et al., 2000; Schneider et al., 2017; Sury et al., 2006) as well as the role of dysregulated glutamatergic signaling in neuroinflammatory disease processes (Kaindl et al., 2012; Noda & Beppu, 2013; Wei et al., 2012). Indeed, the interactions between TNFα, CD40, and NF-κB are complex. Previous work has suggested that transcriptional activation of CD40 is mediated by interferon gamma (IFN-γ), which also induces TNFα production and subsequent activation of NF-κB (a transcriptional regulator of CD40) similar to pathological levels of extracellular glutamate (Benveniste, Nguyen, & Wesemann, 2004). While microglia do not produce significant levels of IFN-γ, peripheral immune cells (i.e., T-cells) that infiltrate into the brain parenchyma due to drug-induced disruptions of the blood brain barrier (Clark et al., 2013; Hawkins et al., 2004) do release IFN-γ. This positive feedback loop results in a robust immune response that may increase extracellular glutamate through TNFα-induced

downregulation of GLT-1 (Olmos & Lladó, 2014; Sitcheran et al., 2005). Overall, this cascade of pro-inflammatory events could result in enhanced postsynaptic excitatory potential in MSNs within the NAcore, which could be a driving force underlying both cue-induced drug-seeking behavior as well as persistent dysregulation of glutamatergic signaling.

Nicotine, as opposed to other drugs of abuse, is capable of directly modulating neuroimmune signaling in the CNS through its full-agonist activity at the α_7 nAChR. Cholinergic activity at α_7 nAChRs on microglia plays a significant role in modulating microglial responses to inflammatory stimuli, where acute nicotinic activation of microglial α_7 exerts an anti-inflammatory effect (Shytle et al., 2004). However, nicotine exposure can also disrupt the blood brain barrier as mentioned above (Hawkins et al., 2004) and induce oxidative stress and NF-κB activation (Barr et al., 2007). Here, we demonstrate that subchronic NAC treatment significantly attenuated pro-inflammatory TNFα expression in the NAcore, which was measured immediately after a 2-hour cue reinstatement test (Figure 2B). TNFα, which is a known modulator of learning, memory, and glutamatergic plasticity (Albensi & Mattson, 2000; Beattie et al., 2002; Stellwagen, Beattie, Seo, & Malenka, 2005; Stellwagen & Malenka, 2006), is primarily derived from microglia under pathological conditions (Gregersen, Lambertsen, & Finsen, 2000; Welser-Alves & Milner, 2013). Indeed, our results corroborate previous findings indicating an inhibitory effect of NAC on brain TNFα expression (Oka et al., 2000; Saleh, 2015; Sury et al., 2006). Moreover, microglia are known to express both ionotropic and metabotropic glutamate receptors that mediate neuroinflammatory processes (Biber et al., 2001; Kaindl et al., 2012; Noda & Beppu, 2013). Interestingly, TNFα both increases surface expression

of GluA2-lacking calcium permeable AMPA receptors and decreases surface expression of $GABA_A$ receptors within hippocampal slices, resulting in significant increases in excitatory synaptic strength (Stellwagen et al., 2005). This effect was shown to occur within 15 minutes of TNFα treatment, which is the same time point at which we have previously observed rapid post-synaptic potentiation of MSNs during cue-induced nicotine reinstatement (Gipson et al., 2013b). Recently, it has been suggested that TNFα might *decrease* excitatory activity at striatal synapses and inhibit cocaine-induced synaptic plasticity (Lewitus et al., 2016). However, this study did not utilize a range of physiologically-relevant concentrations of TNFα (compare to Habbas et al., 2015), and utilized 5 days of experimenter-delivered cocaine as opposed to SA, which are different models of drug delivery that produce profoundly distinct neurobehavioral consequences (Namba, Tomek, Olive, Beckmann, & Gipson, 2018). For example, nicotine SA, but not experimenter-delivered nicotine, decreases GLT-1 in the NAcore (Gipson et al., 2013b; Knackstedt et al., 2009). Taken together, it is possible that nicotine, through its direct actions on microglial nAChRs, could uniquely alter stimulus-induced neuroimmune activity that functions as a modulator of glutamatergic plasticity and subsequent drug-seeking behavior.

As highlighted above, microglial sensitivity to changes in extracellular glutamate may underlie a significant immunomodulatory mechanism that shapes neuron-glia crosstalk and subsequent drug-seeking behavior. Interestingly, exogenous glutamate has been shown to rapidly increase glutamate uptake within 15 minutes of exposure *in vitro* (Duan, Anderson, Stein, & Swanson, 1999). Thus, an activity-dependent compensatory immunomodulatory mechanisms that are sensitive to rapid elevations in extracellular

glutamate may underlie relapse-dependent neurobehavioral plasticity. Considering TNFα downregulates GLT-1 through activation of NF-κB (Sitcheran et al., 2005), it is possible that glutamate overflow-induced activation of microglia and subsequent release of TNFα could produce long-lasting inhibition of GLT-1 expression. We demonstrate here that rats treated with GLT-1 AS morpholino exhibited marked elevations in CD40 expression (Figure 2C), which is a known marker of pro-inflammatory M1 activation of microglia and other immune cells (Walker & Lue, 2015). Assuming GLT-1 knockdown contributed to elevated levels of extracellular glutamate and impaired glial glutamate uptake, it is possible that this could explain the significant increase in CD40 expression observed in antisense-treated rats. Thus, despite treatment with NAC and inhibition of TNFα, pro-inflammatory activation of microglia induced by GLT-1 knockdown is a possible mechanism that might explain why GLT-1 antisense occluded the therapeutic efficacy of NAC as observed in the present study.

NF-κB Signaling Regulates Both GLT-1 Expression and Cue-Induced Nicotine Seeking

As an extension of the aforementioned findings, we sought to examine the specific role of NF-κB in regulating cue-induced nicotine seeking as well as the therapeutic efficacy of NAC. Rats receiving HSV-IKKdn demonstrated an attenuation of cue-induced reinstatement, regardless of NAC treatment. Conversely, rats receiving a HSV-IKKca reinstated nicotine seeking in response to contingent cues. Interestingly, HSV-IKKca also blocked the attenuating effects of NAC on cue-induced reinstatement. Combined with the above findings that NAC inhibits TNFα expression, these results suggest that inhibition of pro-inflammatory activation of NF-κB may be a defining

mechanism underlying NAC's therapeutic efficacy. Surprisingly, while HSV-IKKdn alone was sufficient to inhibit cue-induced reinstatement, it inhibited GLT-1 expression similar to HSV-IKKca-treated conditions, regardless of NAC treatment. Overall, these findings suggest that while NF-κB regulates NAC's effects on cue-induced nicotine seeking and GLT-1 expression, NF-κB may also mediate nicotine-seeking behavior through GLT-1-independent mechanisms. While seemingly inconsistent with the aforementioned findings from Experiment 1, this conclusion is supported by recent findings demonstrating that AAV-mediated overexpression of GLT-1 is insufficient to inhibit cue-induced cocaine reinstatement (Logan, LaCrosse, & Knackstedt, 2018).

TNFα signaling and the NF-κB pathway regulate learning, memory, and synaptic plasticity (Albensi & Mattson, 2000; Kaltschmidt & Kaltschmidt, 2015; Meffert et al., 2003; O'Neill & Kaltschmidt, 1997), including synaptic plasticity associated with drug-seeking behavior (Ang et al., 2008; Clark et al., 2013; Crews, Zou, & Qin, 2011; Cui et al., 2014; Russo et al., 2009). Here, we confirm that inhibiting NF-κB alone is sufficient to inhibit cue-induced nicotine seeking despite concomitant downregulation of GLT-1. As mentioned previously, NF-κB is a bidirectional regulator of GLT-1 (Sitcheran et al., 2005). Therefore, it is reasonable to posit that constitutive activation (by HSV-IKKca) or dominant negative inhibition (by HSV-IKKdn) of NF-κB could result in a net decrease in GLT-1 expression. Indeed, NF-κB signaling has been shown to be necessary for neuron-dependent induction of GLT-1 expression in astrocytes (Ghosh, Yang, Rothstein, & Robinson, 2011). Nevertheless, our results support a previous study that demonstrated that NF-κB signaling in the nucleus accumbens facilitates cocaine-induced changes in dendritic spine morphology as well as the formation of cocaine conditioned place

preference (Russo et al., 2009). Considering the finding that HSV-IKKca blocked the inhibitory effect of NAC on cue-induced nicotine reinstatement and that these HSV vectors produced transgene expression in MSNs (Figure 3A-C), it is possible that NAC may inhibit cue-triggered relapse through inhibition of pro-inflammatory activation of neuronal NF-κB. Figure 6 provides an illustration of a proposed mechanism of action through which NAcore glutamate overflow induced by exposure to drug-paired cues could activate microglia and a pro-inflammatory signaling cascade that ultimately facilitates nicotine-seeking behavior.

Alternative Potential Mechanisms Underlying the Effects of NAC

Previous work has demonstrated that high concentrations of NAC *in vitro* increase excitatory postsynaptic currents (EPSCs) on MSNs via activation of mGluR5, whereas low concentrations of NAC decrease EPSCs on MSNs through an increase in cystine-glutamate exchange and subsequent stimulation of presynaptic mGluR2/3 receptors (Kupchik et al., 2012). Other studies have also indicated that glutamatergic signaling between system xc^- and presynaptic mGluR2/3 receptors mediates the effects of NAC on cocaine reinstatement (Kau et al., 2008; Moran et al., 2005). Additionally, a recent study has shown that inhibiting GLT-1 expression and chronically administering NAC potentiates cue-induced cocaine reinstatement through activation of mGluR5 (Reissner et al., 2015). Considering chronic nicotine exposure is associated with a decrease in system xc^- function and mGluR2/3 expression (Knackstedt et al., 2009; Liechti, Lhuillier, Kaupmann, & Markou, 2007), and cue-induced nicotine reinstatement is associated with an increase in extracellular glutamate (Gipson et al., 2013b), it is likely that extracellular glutamate levels were increased in rats treated with NAC and GLT-1

AS. While not directly quantified in the present study, this potential elevation in extracellular glutamate could have increased postsynaptic excitability via mGluR5, effectively blocking the observed inhibitory effects of NAC on cue-induced nicotine reinstatement. However, the lack of potentiation of reinstatement in rats treated with NAC and GLT-1 AS suggests that alternative, nicotine-specific neurobiological mechanisms may underlie NAC's therapeutic potential, as highlighted above.

Group I metabotropic glutamate receptors have been implicated in nicotine seeking behavior and antagonism of mGluR1/5 receptors has been shown to inhibit nicotine self-administration (Paterson, Semenova, Gasparini, & Markou, 2003; Tronci, Vronskaya, Montgomery, Mura, & Balfour, 2010) as well as cue- and nicotine-induced reinstatement (Bespalov et al., 2005; Dravolina et al., 2007). However, the role of these receptors on conditioned behavior is not uniform. Studies have demonstrated that, unlike cocaine, the reward-enhancing effects and the conditioned-rewarding effects of nicotine are not reduced by antagonism of mGluR5 receptors (McGeehan & Olive, 2003; Palmatier, Liu, Donny, Caggiula, & Sved, 2008). Given the present findings, the potential role of mGluR5 in cue-induced nicotine reinstatement after suppressing GLT-1 expression and driving cystine-glutamate exchange remains somewhat unclear. Therefore, future studies should examine the impact of mGluR5 antagonism on cue-induced nicotine reinstatement in rats treated with NAC and GLT-1 antisense vivo-morpholino.

Concluding Remarks

Clinical studies investigating NAC as a treatment for substance use disorders have shown inconsistent results. One study investigating NAC in cocaine-dependent

individuals found that NAC may suppress cocaine withdrawal and craving (LaRowe et al., 2006), while another more recent study found that NAC did not suppress cocaine use unless individuals had achieved abstinence prior to the onset of treatment (LaRowe et al., 2013). Similarly, another study showed that NAC demonstrated a trend towards reducing withdrawal symptoms in nicotine-dependent individuals but had no effect on nicotine craving (Schmaal et al., 2011). Additionally, an earlier placebo-controlled study showed that NAC had no effect on nicotine craving or withdrawal and only showed a decrease in self-reported cigarette use per day when two outliers were removed due to excessive alcohol consumption (Knackstedt et al., 2009). Considering these findings, it is likely that NAC may be most effective in preventing relapse during a period of abstinence. Indeed, most preclinical studies implicating NAC as a potentially effective pharmacotherapeutic have utilized a model of reinstated drug seeking, where animals are placed into extinction training or forced abstinence following drug self-administration. In addition to NAC's effects on GLT-1 and cystine-glutamate exchange, NAC also exhibits antioxidant and anti-inflammatory properties that may underlie its observed therapeutic effects on cue-induced nicotine reinstatement (Zafarullah, Li, Sylvester, & Ahmad, 2003). For instance, pro-inflammatory TNFα recruits NF-κB to inhibit GLT-expression (Sitcheran et al., 2005). Interestingly, NAC has been demonstrated to inhibit TNFα-induced activation of NF-κB (Oka et al., 2000). Ceftriaxone has also been shown to increase GLT-1 expression through a NF-κB-dependent mechanism (Lee et al., 2008). Finally, as demonstrated in the aforementioned studies, inhibition of pro-inflammatory activation of NF-κB is likely a relevant mechanisms underlying NAC's therapeutic mechanism, which includes its restorative effects on glutamatergic signaling. Intriguingly, a recent report has

demonstrated that enhancing NAC's bioavailability via nanoparticles significantly improves its anti-inflammatory potential (Markoutsa & Xu, 2017). NAC is notorious for its poor oral bioavailability and patients in clinical settings must take large doses to achieve the desired therapeutic outcome, which represents a significant limitation of NAC as a therapeutic (McClure et al., 2014; Minarini et al., 2017). Given the present findings, which highlight the role of immunomodulation in regulating nicotine-seeking behavior, improving NAC's bioavailability may enhance its anti-inflammatory potential and thus its therapeutic efficacy. As such, future studies should further investigate the potential role of neuroimmune function in modulating relapse-dependent synaptic plasticity and subsequent drug-seeking behavior, which may yield valuable insight into the cellular and molecular mechanisms that drive addiction and relapse processes.

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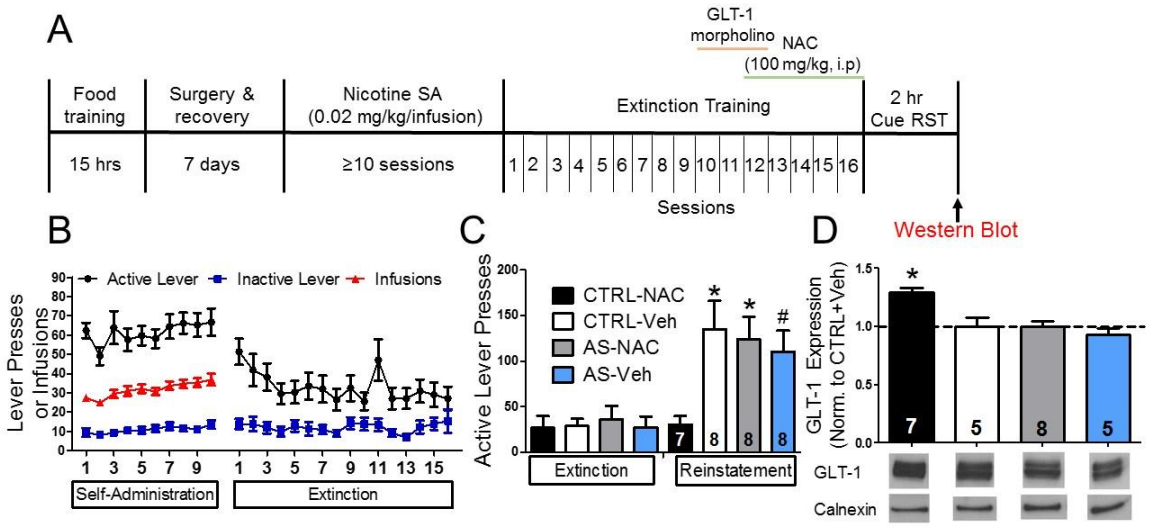

Figure 1. GLT-1 Knockdown Impairs NAC Inhibition of Cue-Induced Reinstatement. (A) Timeline of experimental procedures. (B) Rats self-administered nicotine (0.02 mg/kg/infusion) on an FR-1 schedule until criteria were met. Following self-administration, rats were placed into extinction training, where some received once-daily vivo-morpholino microinjections between days 10 and 12, followed by NAC (100 mg/kg, i.p.) or vehicle injections between days 12 and 16. (C) Average active lever presses during the last two extinction sessions and during a 2-hr cue-induced nicotine reinstatement session (26 hours following last NAC injection). NAC significantly attenuated active lever pressing relative to saline controls. This effect was blocked by administration of GLT-1 antisense vivo-morpholino. *$p < 0.05$ and $^{\#}p < 0.10$. (D) GLT-1 expression across treatment groups, normalized to CTRL-Vehicle (denoted by dashed line). NAC significantly increased GLT-1 protein expression as compared to vehicle controls. Additionally, GLT-1 antisense vivo-morpholino suppressed GLT-1 levels in NAC-treated rats. *$p < 0.05$. Error bars = SEM.

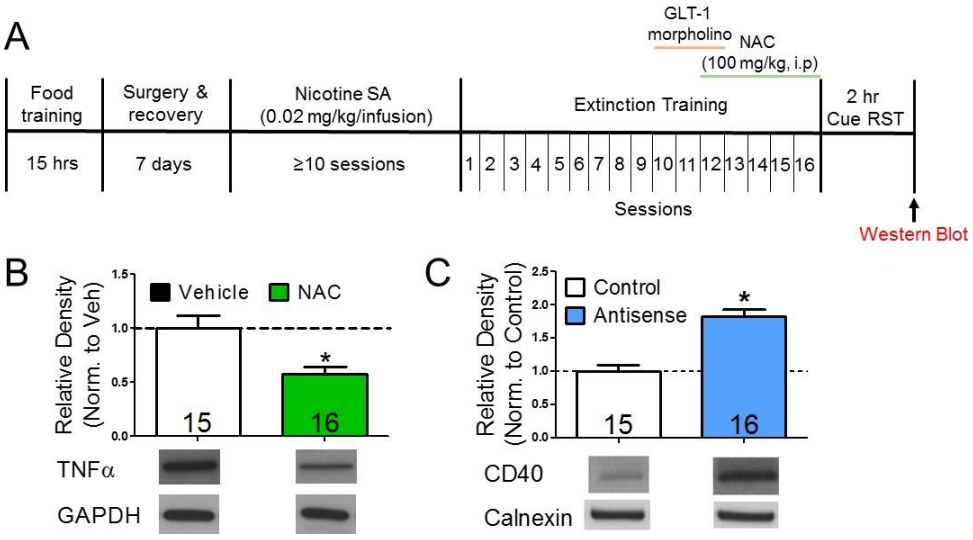

Figure 2. Nicotine Self-Administration and GLT-1 Knockdown Alter Neuroimmune Signaling. (A) Timeline of experimental procedures. (B) TNFα expression following nicotine SA, extinction, and cue-induced reinstatement. NAC significantly inhibited TNFα expression measured after reinstatement. *$p < 0.05$. (C) CD40 expression following nicotine self-administration, extinction, and cue-induced reinstatement. GLT-1 antisense morpholino treatment significantly elevated CD40 expression measured after reinstatement. *$p < 0.05$. Error bars = SEM.

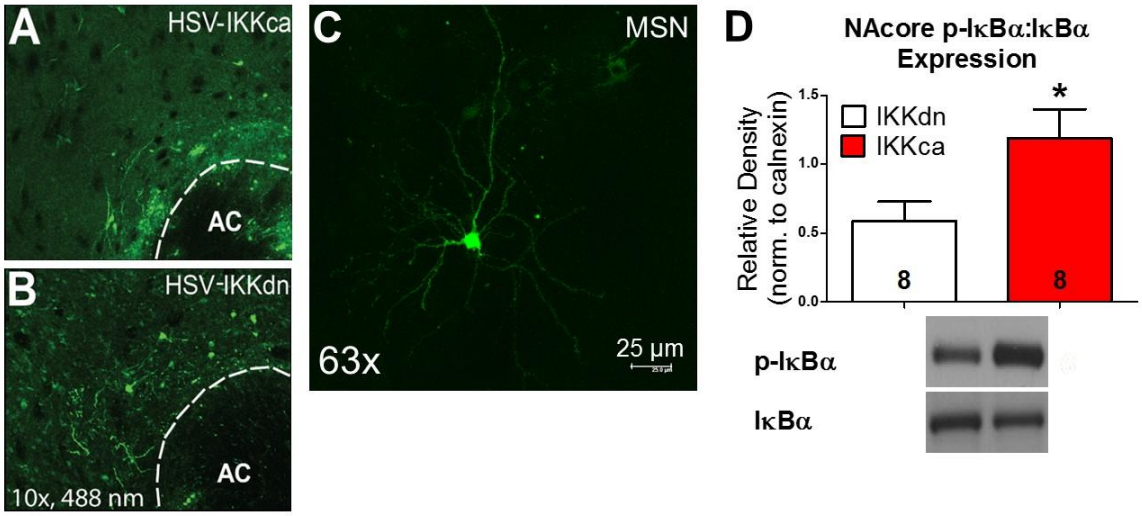

Figure 3. Validation of Viral Constructs. (A) HSV-IKKca and (B) HSV-IKKdn successfully produce GFP expression in the NAcore. (C) Moreover, HSV vectors successfully produce transgene expression in NAcore MSNs. (D) Compared to HSV-IKKdn, HSV-IKKca produces significantly more phosphorylation of IκBα, which is a protein that normally keeps NF-κB in an inhibited state in the cytoplasm until it is phosphorylated. *$p < 0.05$. Error bars = SEM.

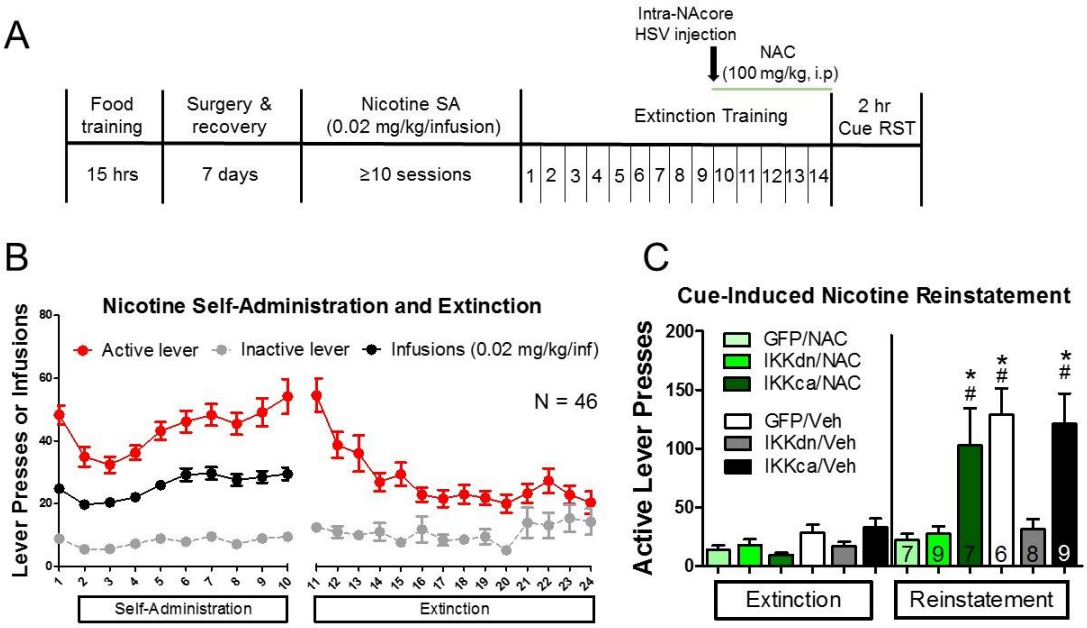

Figure 4. Constitutive Activation of IKK Blocks Attenuating Effect of NAC on Cue-Induced Nicotine Seeking. (A) Timeline of experimental procedures. Rats underwent nicotine self-administration and extinction exactly as described in Experiment 1, except on Day 10 of extinction, rats received an intra-NAcore microinjection of HSV-GFP, HSV-IKKdn, or HSV-IKKca. NAC or vehicle injections also began on Day 10 and persisted through the end of extinction. (B) Lever presses and nicotine infusions across self-administration and lever presses across extinction training. (C) Average active lever presses during the last two extinction sessions and during a 2-hr cue-induced nicotine reinstatement session (26 hours following last NAC injection). Rats receiving HSV-GFP + Vehicle, HSV-IKKca + Vehicle, or HSV-IKKca + NAC significantly reinstated nicotine seeking in response to contingent cues as compared to the other three treatment conditions. #$p < 0.05$ relative to HSV-GFP + NAC, HSV-IKKdn + NAC, and HSV-IKKdn + Vehicle. *$p < 0.05$ relative to respective extinction. Error bars = SEM.

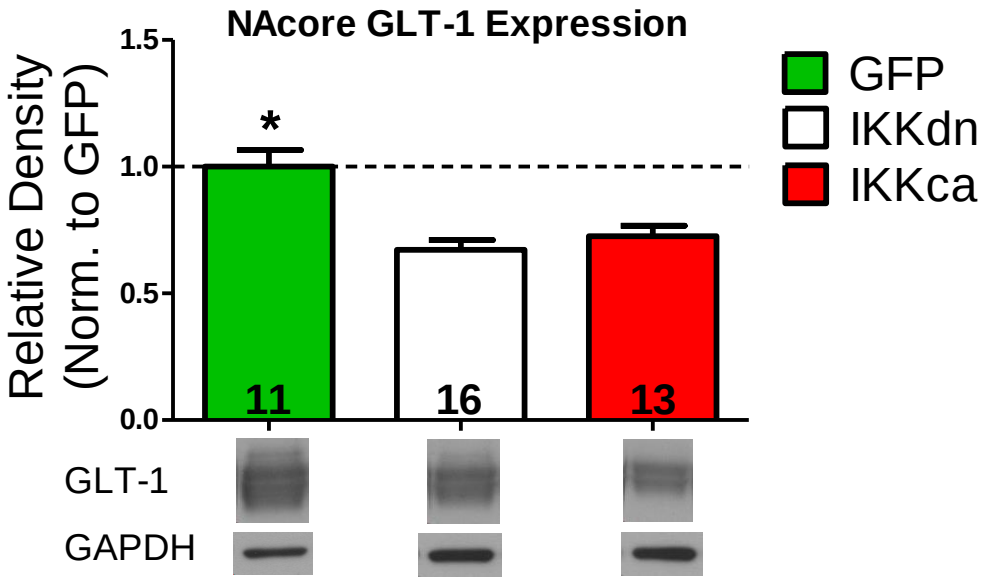

Figure 5. Constitutive Activation or Inhibition of IKK in the NAcore Inhibits GLT-1 Expression. NAC treatment within the HSV-GFP control condition did not increase total GLT-1 protein levels in the NAcore above the vehicle condition. Regardless of NAC or Vehicle treatment, both HSV-IKKca and HSV-IKKdn inhibited GLT-1 expression compared to HSV-GFP controls.*$p < 0.001$. Data normalized to HSV-GFP, denoted by dotted line. Error bars = SEM.

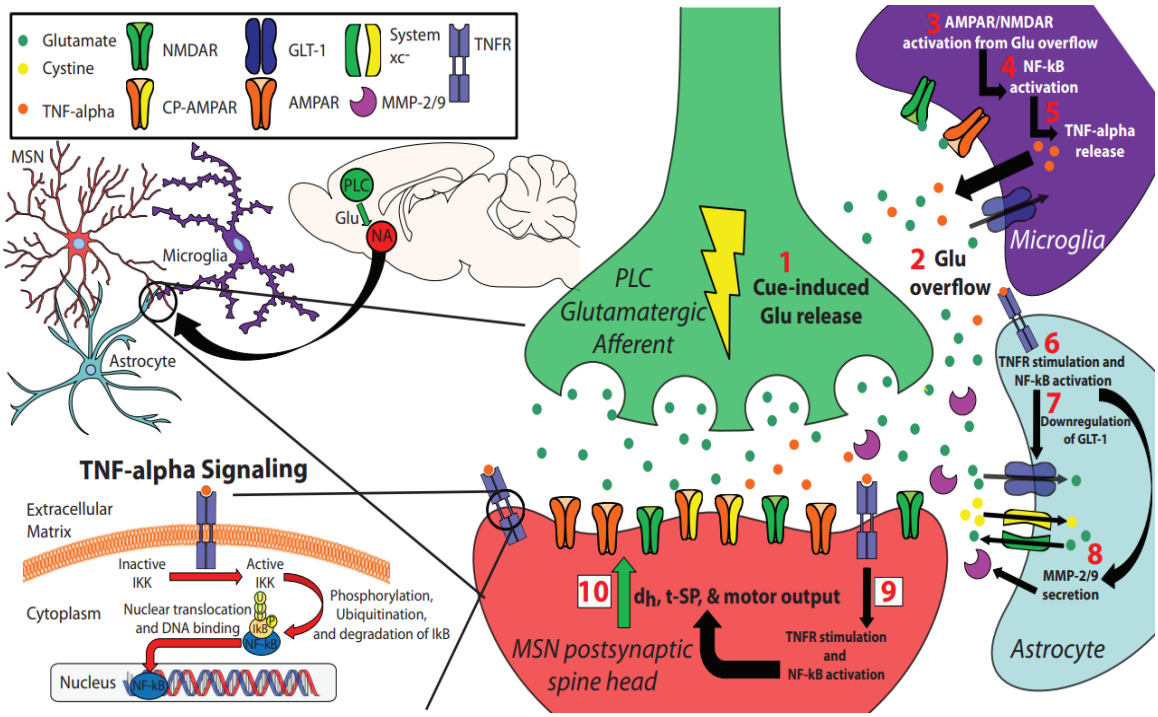

Figure 6. Proposed Mechanism of Action of Immunomodulatory Mechanisms Regulating Relapse-Induced Glutamatergic Plasticity. (1) Cue-induced reinstatement induced glutamate (Glu) release into the NAcore from prelimbic cortex (PLC) afferents, which synapse with NAcore MSNs. (2) This causes glutamate overflow into the extracellular space, which could (3) activate resident microglia through stimulation of ionotropic glutamate receptors, leading to (4) NF-κB activation and (5) TNFα release. Once released, TNFα can bind to TNF receptor 1 (TNFR1) on (6) astrocytes, leading to (7) further downregulation of GLT-1 and (8) MMP activation. As well, TNFα can bind to TNFR1 on neurons, leading to (9) neuronal NF-κB activation and (10) subsequent increases in dendritic spine diameter (d_h) and/or density, transient synaptic potentiation (t-SP), and motor output.

Table 1

Antibody Concentrations for Western Blot Experiments

Target	Catalog #	RRID	1^0 Concentration	2^0 Concentration
GLT-1	Abcam ab41621	AB_941782	1:1,000	1:45,000
CD40	Abcam ab13545	AB_1951619	1:1,000	1:5,000
TNFα	Abcam ab6671	AB_305641	1:1,000	1:2,000
Calnexin	Enzo ADI-SPA-860	AB_10616095	1:1,000	1:25,000
GAPDH	CST D16H11	AB_10622025	1:1,000	1:25,000

Note. All secondaries were Goat pAb to Rb IgG (HRP) (Abcam ab97080; RRID: AB_10679808)