

TREATMENT DURING ABSTINENCE FROM METHAMPHETAMINE IN A RAT MODEL
OF METHAMPHETAMINE USE DISORDER

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DEDICATION

To those I've had the privilege of calling teacher, friend, or family, for whom without I am a blind man touching an elephant.

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TREATMENT DURING ABSTINENCE FROM METHAMPHETAMINE IN A RAT
MODEL OF METHAMPHETAMINE USE DISORDER

Methamphetamine (METH) is a psychostimulant with high abuse potential. Currently there are no pharmacological treatments specific for relapse to METH use disorder. Chronic METH abuse has been associated with changes to the dopamine and glutamate neurotransmitter systems, as well as inflammation. Phosphodiesterase-4 inhibitors are known to affect cAMP involved in dopaminergic and glutamatergic neurotransmission, as well as having anti-inflammatory action. In pre-clinical models, phosphodiesterase inhibitors can reduce behaviors associated with the self-administration of drugs of abuse if given directly before tests of relapse-like behavior. However, they have not been examined in the more clinically relevant context as a treatment for use during abstinence from drugs of abuse. To address this gap, a METH self-administration model in the rat was used in which roflumilast, a phosphodiesterase 4 inhibitor, was administered during the abstinence period before a relapse test. The overarching hypothesis was that roflumilast inhibited inflammation associated with METH self-administration abstinence to reduce subsequent relapse-like behaviors. A detailed behavioral analysis showed that the chronic treatment with roflumilast during 7 days of forced abstinence reduced relapse-like behavior to METH seeking and METH taking. Roflumilast treatment during 7 days of forced abstinence did not affect subsequent sucrose seeking and sucrose taking behaviors. Biochemical analyses of proteins related to dopamine and glutamate neurotransmission did not reveal changes in these neurotransmitter systems, nor was there evidence of overt inflammation. These data suggest that roflumilast may be a treatment for METH use disorder that is effective when taken during abstinence, but further studies related to the mechanism of action of roflumilast are needed.

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LIST OF ABBREVIATIONS

5-HT – 5-hydroxytryptamine
ADHD – Attention Deficit Hyperactivity Disorder
cAMP – Cyclic Adenosine monophosphate
AMPA – alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA – Analysis of variance
B2R – beta-type 2 adrenergic receptor
CD – Cluster of Differentiation
CREB – Cyclic AMP response element binding protein
D1R – Dopamine type-1 receptor
D2R – Dopamine type-2 receptor
DARPP32 – Dopamine- and cAMP-regulated phosphoprotein, MW 32 kDa
DAT – Dopamine transporter
ELISA – enzyme-linked immunosorbent assay
ESPC – Excitatory post-synaptic current
GFAP – Glial fibrillary acidic protein
h – Hour
Iba-1 – Ionized calcium binding adapter molecule 1 / Allograft inflammatory factor 1
IL-1 β – Interleukin-1 beta
IL-6 – Interleukin-6
IV – intravenous
LTD – Long term depression
LTP – Long term potentiation
METH – Methamphetamine
min – Minute
MMP – Matrix metalloproteinase
NMDA – N-methyl-D-aspartate
P- - Phosphorylated
PDE – Phosphodiesterase
PET – Positron emission tomography
SE – Standard Error
SEE – Standard error of the estimate
SEM – Standard error of the mean
TH – Tyrosine hydroxylase
TNF α – Tumor necrosis factor alpha
VMAT – Vesicular monoamine transferase

Chapter 1: Introduction to METH Use Disorder

Methamphetamine (METH) is a psychoactive xenobiotic that exists as a glassy crystal, powder, or pill, and has potent stimulant properties (Chomchai and Chomchai 2015; M. P. Paulus and Stewart 2020). The purpose of the work presented here was to uncover a new potential therapeutic for the treatment for METH use disorder, more colloquially known as METH addiction, and the underlying mechanism of the potential treatment. Though METH has some medical use in the treatment of refractory attention-deficit hyperactivity disorder, narcolepsy, and as an adjunct treatment for obesity, the vast majority of clinical harms associated with METH use stem from the illicit abuse of the drug and its consequences. In this dissertation, METH will be discussed primarily in its context as a drug of abuse.

General Introduction to METH Pharmacology

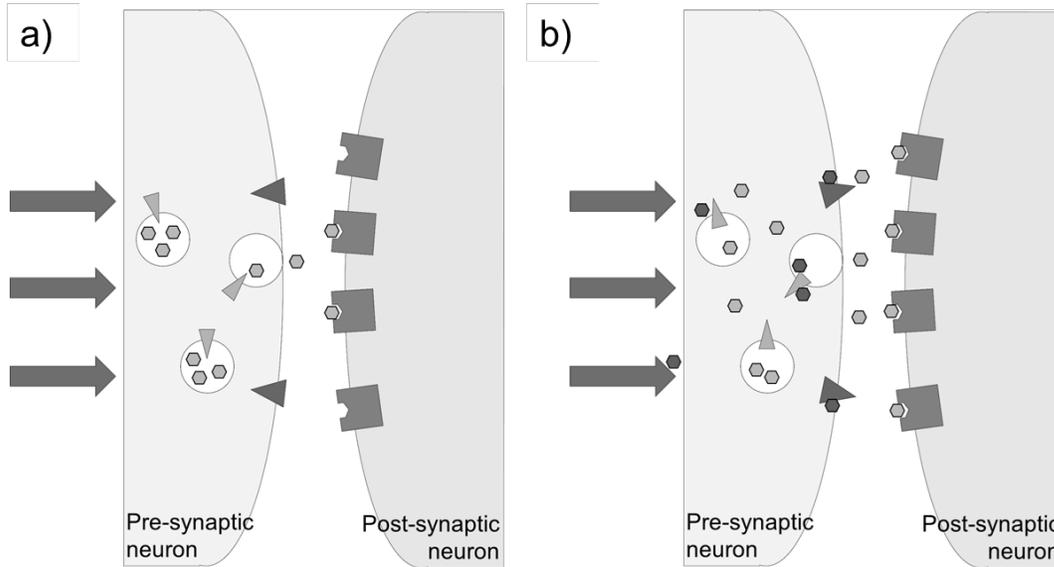
METH is a substituted amphetamine, and substituted amphetamines act largely at the terminals of neurons that release monoamines to increase the synaptic levels of dopamine, serotonin, and norepinephrine (Nichols 1994; Sulzer et al. 2005; Ferrucci et al. 2019). The complete relationship between neurotransmitter release magnitude and the concentration of amphetamines is not fully elucidated, but the established pharmacology of substituted amphetamines was reviewed in exceptional detail by Dr. David Nichols in 1994, and by Sulzer et al., 2005, and the following description draws greatly on these two reviews with updates.

Chemically, METH is a derivative of alpha-methyl-phenethylamine, which is commonly referred to as “amphetamine” (Sulzer et al. 2005). The chemical structure of amphetamine and its chemical derivatives, referred to as substituted amphetamines, resemble catecholamines such as dopamine and norepinephrine. However substituted amphetamines are not considered catecholamines because they lack hydroxyl groups at

the 3 and 4 positions of the phenyl ring (Nichols 1994). This property renders these xenobiotics resistant to catabolism by catechol-O-methyltransferase, an enzyme that degrades catecholamines. Additionally, the presence of an alpha-methyl group confers resistance to degradation by monoamine oxidase and increases their ability to cross the blood-brain barrier. Together, the resistance to degradative metabolism and increased ability to cross the blood-brain barrier help extend the half-life of the molecules as well as allow for their systemic administration, such as by oral or intravenous (IV) administration, to also act in the central nervous system. Previous studies have been primarily on the effects of amphetamine on dopamine. However, many independent but overlapping experiments suggest substituted amphetamines act on a common mechanism with multiple sites of action within neurons to release monoamines further described below, with differences in the degree of individual monoamine release dependent on the chemical substituents on the amphetamine moiety.

Upon administration, substituted amphetamines diffuse across the blood-brain barrier and enter neuron terminals through the dopamine transporter (DAT), or other monoamine transporters, and into the vesicles either through passive diffusion or through vesicular monoamine transferase (VMAT2). As a weak base, substituted amphetamines become protonated in acidic organelles, including the vesicles storing monoamines. When charged, the substituted amphetamines become unable to diffuse out and accumulate within the vesicle. Physiologically relevant concentrations of METH and amphetamine collapse the pH gradient of vesicles from monoamines and allow for the passive diffusion of neurotransmitters out of the vesicle into the cytosol. The increased concentration of neurotransmitters in the cytosol reverses the action of DAT and allows for the passive diffusion of neurotransmitters down their concentration gradient into the extracellular space independent of action-potential mediated neurotransmitter release (Figure 1).

Figure 1



- Dopamine 
- Methamphetamine 
- Tyrosine Hydroxylase 
- Dopamine Receptor 
- Dopamine Transporter 
- Vesicular Monoamine Transporter 

Figure 1: Schematic of the acute effects of METH at the dopaminergic synapse. (a) Dopamine released by pre-synaptic neurons interacts with post-synaptic receptors before it is repackaged into pre-synaptic vesicles by dopamine transporter (DAT) and vesicular monoamine transporters (VMAT). (b) METH reverses the actions of DAT and VMAT and stimulates dopamine synthesis by tyrosine hydroxylase to increase overall synaptic dopamine levels.

Normally, the extracellular concentration of monoamines are largely regulated by their reuptake through their respective plasmalemmal transporters, such as the dopamine through DAT. However, in addition to releasing monoamines into the synapse, substituted amphetamines also inhibit their transporter-mediated reuptake, further

increasing the extracellular concentration of monoamines such as dopamine, norepinephrine, and serotonin. One additional mechanism by which amphetamines inhibit transporter-mediated uptake includes by activating the Trace Amine Associated Receptor 1, TAAR1, which has been demonstrated to activate a cAMP-dependent biochemical cascade that results in the internalization of several transporters, including DAT, NET, and the excitatory amino acid transporter, EAAT3. The internalization of these transporters leads to reduced ability to remove dopamine, norepinephrine, and glutamate from the synapse, contributing to the increased extracellular concentrations of these neurotransmitters (Wheeler et al. 2015; Underhill, Colt, and Amara 2020).

Amphetamines can also increase dopamine production by acting as a competitive inhibitor of monoamine oxidase, as well as by increasing the activity and expression of tyrosine hydroxylase, a critical enzyme in the endogenous synthesis of dopamine. These increased levels of dopamine measured using intracerebral microdialysis correlated to the half-life of the amphetamine (Sharp et al. 1987; Stephans and Yamamoto 1994), suggesting that the acute effects of METH on neurotransmitter levels was dependent on the pharmacokinetics of METH. The half-life of METH smoked or injected intravenously in humans were similar, at 11.2 hours and 12.2 hours respectively (C Edgar Cook et al. 1993), with the half-life for rats estimated at 70 minutes (A. K. Cho et al. 2001).

The mechanism by which METH acts at the monoaminergic synapse continues to be explored. Recent advances include the finding that METH-induced increases in dopamine release may be partially dependent on sigma-receptor signaling leading to amino-acid modifications of VMAT and its kinetics (Hedges et al. 2017). Currently, the understanding of how METH influences other non-monoamine neurotransmitters such as acetylcholine continues to be explored (Ferrucci et al. 2019). However, the general schema of METH inducing monoamine release primarily through its action at

monoamine vesicles and transporters has so far been sufficient to broadly explain most of the acute physiological effects seen in the use of these drugs (Nichols 1994; Sulzer et al. 2005; Ferrucci et al. 2019).

Neurotransmitters affected by METH

The mechanism of action of METH produces large increases in the extracellular monoamines, specifically dopamine, serotonin, norepinephrine (Sulzer et al. 2005). Actions by these monoamines can also induce the release of glutamate in dopamine-dependent polysynaptic pathway (Nash and Yamamoto 1992). In this instance, METH-stimulated dopamine release reduces GABA-dependent inhibition of the thalamus, thereby increasing glutamatergic outflow to the cortex, and cortical glutamatergic outputs to the dorsal striatum (Mark, Soghomonian, and Yamamoto 2004) and the nucleus accumbens (Parsegian and See 2014). Because monoamines have broad roles in regulating neurotransmission in the brain, a brief overview of the main neurotransmitters affected by METH – dopamine, serotonin, norepinephrine, and glutamate – is included.

Dopamine is a neurotransmitter synthesized from the amino acid tyrosine by the rate-limiting enzyme tyrosine hydroxylase (Palmiter 2008). The phosphorylation of tyrosine hydroxylase at the Serine-40 position dramatically increases its rate of enzymatic activity and can be used as an indirect measure of the degree of dopamine synthesis in the brain (Fujisawa and Okuno 2005; Nishi et al. 2008). In the brain, dopamine is primarily synthesized in the nerve terminals of neurons with nuclei contained within midbrain regions, namely the substantia nigra, the ventral tegmental area, and the dorsal median arcuate nucleus (Stagkourakis et al. 2019). The action of dopamine is important to dopaminergic terminals in the striatum, a cluster of nuclei within the basal ganglia, that coordinate and execute planned and motivated behaviors (Haber 2016). The striatum is further subdivided by the dorsal to ventral anatomical axis,

with the dorsal striatum consisting of the caudate and putamen, and the ventral striatum consisting of the nucleus accumbens and the olfactory tubercles (Delgado 2007; McCutcheon, Abi-Dargham, and Howes 2019; Murata 2020).

Dopaminergic pathways can be organized into three major pathways. In the nigrostriatal pathway, nuclei originating from the substantia nigra project to the striatum – both the caudate and the putamen – and serve a role in modulating motor control. In the mesocortical pathway, neurons arising from the ventral tegmental area project to cortical areas including the prefrontal cortex, hippocampus, amygdala, as well as the nucleus accumbens. This pathway serves a role in enacting cognitive and emotional behaviors, as well as modulating working memory and attention (Ogawa and Watabe-Uchida 2018). In the tuberoinfundibular pathway, dopamine produced by cells within the dorsal median arcuate nucleus is released in the median eminence into the hypothalamic circulation, where it acts as a hormone regulating the secretion of the hormone prolactin, and is involved in maternal breast milk production (Stagkourakis et al. 2019). However, prolactin secretion levels have been found to be unchanged after chronic exposure to cocaine (Baumann and Rothman 1993) and dopaminergic neurons of the tuberoinfundibular pathway are largely not thought to be involved in the neuropathology of substance use disorder (Koob and Volkow 2009).

There are five different dopamine receptors, D1, D2, D3, D4, and D5, encoded by five respective genes (Beaulieu, Espinoza, and Gainetdinov 2015). Though they have both G-protein dependent and independent effects, dopamine receptors are generally classified according to their ability to activate or inhibit adenylyl cyclase, which produces the secondary messenger, cyclic adenosine monophosphate (cAMP). D1 and D5 receptors are classified as D1-type receptors (D1R) that activate G_s/olf proteins and ultimately increase concentrations of intracellular cAMP, and D2, D3, and D4 receptors classified as D2-type receptors (D2R) that activate G_i/olf proteins and ultimately

decrease intracellular concentrations of cAMP (Kebabian, Petzold, and Greengard 1972; Girault and Greengard 2004).

Early brain stimulation studies correlated, dopamine release from the ventral tegmental area to areas like the ventral striatum, also known as the nucleus accumbens, to pleasure and learned cues associated with pleasure, whereas low levels of dopamine in the nucleus accumbens are associated with aversion and learned cues associated with aversive stimuli (Wise 1980). However, as the role of dopamine release was explored further, it became clear dopamine is important for learning and reinforcement of behaviors associated with reward, but the relationship between how dopamine responds to environmental stimuli to drive behaviors was found to be much more complex (Steinberg et al. 2014; de Jong et al. 2019; Wise and Robble 2020). After a focus on how dopaminergic neurons fired in response to pleasurable stimuli, it was observed that when specific behaviors produce pleasurable outcomes, dopaminergic neurons fire (Hollerman and Schultz 1998). However, as the behavior is repeated over time and is consistently rewarded, the dopamine response appears to habituate, and dopaminergic fire at a baseline level. Interestingly, when an unexpected outcome occurs, when there is an unexpected negative outcome, such as a behavior not being rewarded in accordance with previous expectations, there is a decrease in the dopaminergic neuron firing rate. Correlatively, if there is an unexpected increased reward, there is an increase in dopaminergic firing rate. This led to the theory that dopamine functioned to encode reward prediction errors (Schultz 2016). More recently, it was found that dopaminergic neuron firing rate can increase in response to aversive stimuli as well. Taken together, one current theories about the function of dopaminergic neuron firing rate and the release of dopamine to the nucleus accumbens is that it is thought to signal the perceived salience for specific stimuli, associating outcomes with

stimuli perceived to be meaningful about future conditions; however, this is still an active area of study (Kutlu et al. 2021).

The specific relationship between D1-type or D2-type dopamine receptor signaling and drug self-administration is complex. However, multiple experiments utilizing self-administration paradigms suggest that D1R-mediated dopaminergic signaling in the nucleus accumbens is the dominant signaling relevant to the self-administration of psychostimulants (Pascoli et al. 2015). Within the context of drugs of abuse, the rate of psychostimulant self-administration maintains stable D1R-mediated dopaminergic signaling the nucleus accumbens (Wise et al., 1995; Weiss et al., 2000). Infusions of dopamine D1-type receptor antagonist, SCH-23390 directly into the nucleus accumbens (Yokel and Wise 1976; Phillips, Robbins, and Everitt 1994), or systemic administration of the D1-type receptor antagonist leads to increases in the rate of drug stimulant self-administration (Koob, Le, and Creese 1987). Experiments targeting D2-type receptors appear to be mixed as the administration of both agonists (Rowlett et al. 2007) and antagonists of D2-type receptors can increase rates of stimulant self-administration (Koob, Le, and Creese 1987; Phillips, Robbins, and Everitt 1994; Caine et al. 2002). Similar to drugs of abuse, optogenetic stimulation of dopaminergic neurons in the ventral tegmental area in mice induces the release of dopamine, and this optogenetic stimulation is sufficient to train self-stimulation behaviors in mice, with seeking of self-stimulation behaviors persistent after 30 days away from the operant chamber where they had previously self-stimulated (Pascoli et al. 2015). Within these mice, only D1R-expressing neurons of the nucleus accumbens demonstrated an increase in the AMPA/NMDA receptor current ratio and not D2R expressing neurons, suggesting that dopamine selectively potentiates glutamatergic signaling in D1R-expressing neurons of the nucleus accumbens to drive drug-seeking behaviors (Nestler and Lüscher 2019; Wright and Dong 2020).

Dopamine action in other brain regions has been shown to mediate a variety of other cognitive functions, including attention and executive function in the prefrontal cortex (Schultz 2007), movement in the caudate and putamen (Palmiter 2008), and the formation of long-term memory in the hippocampus (Berke and Hyman 2000; Rossato et al. 2009). Dysfunction in dopamine signaling has been known to be associated with several disease states outside of substance use disorder. One of the most well characterized is in Parkinson's Disease, where its hallmark rigidity and bradykinesia are associated with deficits in dopamine signaling from the substantia nigra to the striatum, canonically the putamen (Palmiter 2008; Chung et al. 2018). Increasing dopamine and norepinephrine signaling in the prefrontal cortex and hippocampus is thought to underly the efficacy of amphetamines in the treatment of attention-deficit hyperactivity disorder in improving attention and memory deficits (ADHD) (Arnsten 2006; Carmack et al. 2014; Kempadoo et al. 2016). In schizophrenia, the dopamine hypothesis postulates that hypoactivity of dopamine in the prefrontal cortex is thought to mediate the negative symptoms associated with schizophrenia, namely the flattened affect and anhedonia, whereas hyperdopaminergic stimulation to the striatum is thought to be associated with positive symptoms like auditory hallucinations (Der-Avakian and Markou 2012; McCutcheon, Abi-Dargham, and Howes 2019).

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter synthesized from the amino acid tryptophan by the rate-limiting enzyme tryptophan hydroxylase (Gershon and Tack 2007). The majority of central serotonin is produced by the dorsal raphe nuclei of the brain stem, but innervates virtually all basal ganglia components as well as throughout the cerebral cortex (Lidov, Grzanna, and Molliver 1980; Parent et al. 2011; Ogawa and Watabe-Uchida 2018). The receptors for serotonin are organized into 7 different receptor families, with at least 16 different receptor subtypes, nearly all of which are G-protein coupled receptors (GPCR), with the

exception of the 5HT-3 receptors, which are non-selective ligand-gated cation channels (Bacqué-cazenave et al. 2020). Canonically, the 5-HT1 receptor family signals through G-proteins that act to inhibit neuronal firing by activating G-protein sensitive potassium channels (Jeong et al. 2001), while 5-HT2 receptors are Gq-mediated to act using the secondary messengers inositol 1,4,5-triphosphate and diacyl-glycerol. 5-HT4 receptors signal through Gs-coupled GPCRs act primarily in the gastrointestinal system to promote gastric motility. 5-HT5, 5-HT6 and 5-HT7 receptors are still being explored, particularly 5-HT6 and 5-HT7 as they have been found to be antagonized by a wide range of antipsychotic and antidepressant drugs. However, there currently no drugs that can selectively target these receptors (Scotton et al. 2019).

Though serotonin modulates a wide variety of physiological processes, including cardiovascular function, gastrointestinal motility, sleep-wake cycles, and body temperature, in the context of amphetamines, serotonin is important in its mediation of mood and hyperthermia. As early as the 1960's it was postulated that decreased levels of monoamines, principally serotonin, was associated with depression and anxiety, leading to what was termed the serotonin hypothesis of depression (Freis 1954; Coppen 1967; Albert, Benkelfat, and Descarries 2012). Subsequent refinements to the serotonin hypothesis now suggest that antidepressant action at post-synaptic 5-HT1A receptors in the limbic system, or blockade and desensitization of pre-synaptic 5HT1A auto-receptors on in raphe mediate their anti-depressive effects. Stimulation of pre-synaptic 5HT-1A auto-receptors may mediate anxiolytic effects (Żmudzka et al. 2018).

Peripherally, significant serotonin stores exist within platelets, packaged into dense granules by the serotonin transporter, and is secreted during platelet activation. Serotonin in circulation produces vasoconstriction or vasodilation depending on the receptor present in the vascular bed, with significant roles in platelet aggregation and vasoconstriction to produce hemostasis, or in 5-HT1B-mediated cerebrovascular dilation

such as in the pathogenesis of migraine (Berger, Gray, and Roth 2009). Serotonergic output from medullary raphe spinal neurons has been shown to modulate sympathetic nerve vascular tone in a 5HT-2A receptor dependent manner (Ootsuka and Blessing 2005), and mice deficient in centrally-derived serotonin have been observed to have impaired shivering response in hypothermic conditions in a 5-HT1A and 5-HT7 dependent manner (Hedlund et al. 2003; Hodges et al. 2008).

Hyperstimulation of 5-HT receptors is thought to mediate serotonin syndrome, a toxidrome most often associated with selective serotonin reuptake inhibitor overdose, which is characterized by anxiety, agitation, clonus and/or tremor with hyperreflexia and rigidity, diarrhea, and hyperthermia. This clinical syndrome is managed primarily by stopping the suspected serotonergic agents, benzodiazepine administration to produce sedation and reduce agitation and muscular activity, and supportive care with continuous cardiac and temperature monitoring for signs of autonomic instability (Boyer and Shannon 2007; Scotton et al. 2019). There is an increased risk of precipitating this syndrome with the co-administration of drugs that increase extracellular serotonin levels, such as two different substituted amphetamines like methylene-dioxy-methamphetamine and METH, or with a prescribed monoamine oxidase inhibitor, such as phenelzine (Silins, Copeland, and Dillon 2007).

Norepinephrine and epinephrine are catecholamines released by the sympathetic nervous system and account for the canonical “fight-or-flight” response, producing the well characterized changes such as increased cardiac output, blood pressure, and metabolism (Stanford 2020). METH activates the sympathetic nervous system by inducing the release of epinephrine and norepinephrine from sympathetic nerve terminals (Matsumoto et al. 2014), as well as by activating in the brainstem to drive the downstream physiology (Ferrucci et al. 2019). These catecholamines classically act on α_1 , α_2 , β -type receptors and are differentiated by their respective G-protein coupled

receptor signaling: α_1 receptors are coupled to $G\alpha_q$ proteins and are generally postsynaptic and are most known to drive vasoconstriction on activation; α_2 receptors are coupled to $G\alpha_i$ receptors and are classically presynaptic where activation leads to attenuation of sympathetic response of the autonomic nervous system; β type adrenergic receptors are classically coupled to G_s proteins and activates adenylyl cyclase. Canonically, the activation of the β_1 subtype increasing heart rate and cardiac contractility, the activation of β_2 receptors is associated with vasodilation, bronchodilation, and relaxation of smooth muscle groups, and β_3 receptors are associated with metabolic effects, particularly lipolysis in adipocytes (William Tank and Lee Wong 2014). Of note, β_1 and β_2 receptors are expressed throughout the brain, including in the medial prefrontal cortex by both interneurons (Liu et al. 2014), and astrocytes and microglia, and their action continues to be explored (Ryan et al. 2020).

Norepinephrine is synthesized from dopamine by dopamine β -hydroxylase at the nerve terminals of catecholaminergic neurons (Coyle and Axelrod 1972), principally at the locus coeruleus and the nucleus of the solitary tract (Bahtiyar et al. 2020).

Norepinephrine has been shown to be released throughout the brain, but is most well studied for its role in mediating vigilance and sleep-wake cycles in the reticular activating system (Sara 2009), physiological responses to stress and emotional stimuli, as well as allowing for memory consolidation in the basal lateral amygdala, hippocampus, or medial prefrontal cortex (Bahtiyar et al. 2020). Stimuli that release norepinephrine also activate the hypothalamic-pituitary-adrenocortical axis to release glucocorticoids (Ulrich-Lai and Herman 2009), and norepinephrine has also been seen to synergize with glucocorticoids released in response to stressors to enhance memory formation and retrieval in a dose-responsive fashion (De Quervain, Schwabe, and Roozendaal 2016). Dysregulation of this system may be involved in the pathogenesis of post-traumatic stress disorder (Hendrickson et al. 2018), and anxiety (Goddard et al. 2010).

Clinically, α_2 agonists and β -blockers have been used with some effect to treat both post-traumatic stress disorder and anxiety (Strawn and Geraciotti 2008; Goddard et al. 2010). Adrenergic receptor modulators are also routinely used to modulate cardiovascular function, with α_1 receptor agonists to raise blood pressure in instances of shock, α_1 receptor antagonists are used as an oral medication to manage refractory hypertension, and β -blockers are routinely used for cardiac rate control while also treating mild hypertension. (Strawn and Geraciotti 2008; William Tank and Lee Wong 2014). Interestingly, recent evidence suggests that exposure to inflammatory signaling induced by lipopolysaccharide and interferon γ may downregulate β_2 receptors on glial cells (Ryan et al. 2020). Additionally, in a rat model of Parkinson's Disease, where lipopolysaccharide is injected into the substantia nigra to reduce dopaminergic cells and induce Parkinsonism, administration of the β_2 receptor agonist, formoterol, attenuated the lipopolysaccharide-induced toxicity (O'Neill et al. 2020). Formoterol administration in this model was also associated with an attenuation of lipopolysaccharide-induced microglial activation. Taken together, this would suggest neuroinflammation may predispose microglia to become insensitive to anti-inflammatory action mediated by β_2 receptor agonism and may exacerbate toxicity mediated by further inflammatory insults.

The role of epinephrine in the central nervous system is not as well established. Epinephrine is converted from norepinephrine released back into the cytoplasm in neurons expressing phenylethanolamine-N-methyltransferase but repackaged into vesicles by VMAT for co-release with other monoamines (Stanford 2020), and has been shown to be released in response to acute physiological stressors, where it contributes to modulating aspects of learning and memory, especially in response to aversive stimuli (Liang and Chen 2021).

METH can also lead to the increase of glutamate in cortical and striatal areas (Mark, Soghomonian, and Yamamoto 2004; Parsegian and See 2014). Glutamate is the

major excitatory neurotransmitter in the brain (Zhou and Danbolt 2014). It is an amino acid synthesized by excitatory neurons, and its signaling in the synapse is terminated by its reuptake by glutamate transporters, most significantly on astrocytes that interface with the synapse (Scofield and Kalivas 2014). Glutamate signals through both ionotropic and metabotropic receptors. Ionotropic receptors include α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, N-methyl-D-aspartate receptors (NMDA), and kainite receptors, with its post-synaptic effect determined primarily by the configuration of glutamate receptors on the recipient cell (Crupi, Impellizzeri, and Cuzzocrea 2019).

Within the context of amphetamines, METH induces the disinhibition of glutamatergic in cortical and striatal regions through a dopamine-dependent polysynaptic pathway (Mark, Soghomonian, and Yamamoto 2004), which can be excitotoxic to dopaminergic terminals (Halpin, Northrop, and Yamamoto 2014). In rats trained to self-administer psychostimulants, glutamate levels in the nucleus accumbens correlated to the act of drug seeking, and blocking glutamate transmission in the nucleus accumbens reduced the reinstatement of cocaine seeking behavior after abstinence (McFarland, Lapish, and Kalivas 2003; Conrad et al. 2008), tying drug seeking behaviors after abstinence to glutamate neurotransmission in the nucleus accumbens (Koob and Volkow 2009).

Clinical Manifestations of Acute METH Use

The effect of METH on these neurotransmitters converge to produce the clinical manifestations of METH use. METH can be introduced into the body by ingestion, snorting, smoking, or intravenous injection (Chomchai and Chomchai 2015). Low doses of METH, about 5-10 mg, produce primarily subjective effects, enhancing mood and liking for the drug, increasing alertness, attention, endurance, and sexuality, and decreasing fatigue and appetite. At moderate doses, about 30 mg, increased release of

norepinephrine from sympathetic nerve terminals become more apparent as METH induces hypertension, tachycardia, hyperthermia, restlessness and agitation, as well as increased anxiety. At higher doses, near 50 mg, dysphoria is more apparent as anxiety, paranoia, aggression, and auditory, visual, and or tactile hallucinations closely resembling symptoms of acute paranoid schizophrenia may develop. For reference, METH can be prescribed in pill form in controlled settings to treat ADHD in young children, or treat obesity in adults, with doses beginning at 5 mg per day, up to 25 mg per day. (Cruickshank and Dyer 2009; Mayo et al. 2019).

METH used illicitly is typically found in the glassy crystal or powder form, smoked, or dissolved to be injected intravenously, though usage patterns vary widely. The estimated use amount varies based on purity of the METH and individual user experience (McKetin, Kelly, and McLaren 2006). The effects of METH are dose-dependent, and subjective feelings of euphoria may be near instantaneous when taken by smoking or intravenous injection. The elimination half-life of METH in humans is approximately 11 hours, and this extended half-life distinguishes METH clinically from intoxication with other stimulants (C E Cook et al. 1992; 1993). However, the acute positive subjective effects such as euphoria reportedly diminish and give way to dysphoria after 4 hours, whereas other effects such as restlessness, agitation, tachycardia, and hypertension remain for much longer with a stronger correlation to the elimination half-life. In those that abuse METH illicitly, this may drive a pattern of binge dosing to maintain the acute subjective effects. Two general abuse patterns have been reported and are not mutually exclusive. The first is a pattern where subjects smoke small doses of METH between intervals as short as 30 minutes and stop in the late afternoon so as to “come down” to be able to sleep at night, estimating 200 mg to 1 g of METH used in the day. The second is a pattern where subjects will self-administer drug in increasing doses on 2 - 3 day binges, consuming 2 – 4 g (~30-60 mg/kg) of METH

without sleep, after which they rest over several days (A. K. Cho and Melega 2002; Cruickshank and Dyer 2009).

Acute tolerance cardiovascular effects of METH, but not the positive subjective effects, has been shown to develop over two weeks of daily administration of 10 mg METH in controlled settings (Perez-Reyes et al. 1991; Harris et al. 2003). Though several studies investigated the mechanisms of tolerance to the neurotoxicity of METH (Halpin, Collins, and Yamamoto 2014), there are fewer studies on the mechanism of tolerance to the acute effects of METH that may explain patterns of METH use. Of those, in a preclinical model of chronic METH use, rats were pretreated with escalating doses of METH subcutaneously over 14 days up to 12 mg/kg on the 14th day, then challenged with a METH “binge” with 24 mg/kg of METH on the 15th day (O’Neil et al. 2006). The study used microdialysis to find that the escalating METH pre-treatment did not affect the concentration of METH in the caudate-putamen measured during the binge; however, the pre-treatment attenuated METH binge-induced release of dopamine in the same region, as well as METH-induced hyperthermia. These effects correlated with decreases in DAT, and this decrease in DAT is believed to contribute to the decreased METH-induced dopamine release (O’Neil et al. 2006). Together, these studies would suggest that the pattern of METH use in humans may be related to pharmacodynamics, not pharmacokinetics of METH (A. K. Cho and Melega 2002; O’Neil et al. 2006); but given that epidemiological evidence suggests that intravenous administration appears to be associated with higher rates of drug dependence compared to other routes of drug administration, this idea remains to be further explored (Novak and Kral 2011).

Clinical management of acute amphetamine intoxication revolves primarily around reducing harms associated with agitation and sympathetic overactivation. METH overdose is very similar to serotonin syndrome, and medical complications related to amphetamine abuse largely result from sympathetic overactivation driving a range of

cardiovascular insults related to vasoconstriction and hypertension, such as intracerebral hemorrhage, pulmonary congestion or edema, distributed coagulopathies, or cerebral or myocardial infarction due to cardiac dysrhythmia or vasospasm (Cruickshank and Dyer 2009; Matsumoto et al. 2014; M. P. Paulus and Stewart 2020). Seizures are an additional potential complication due to METH (Wodarz et al. 2017). Due to the spectrum of risks posed by METH, patients presenting in emergency settings with acute amphetamine intoxication or at risk for a fatal overdose from psychostimulants are often treated with benzodiazepines, in conjunction with anti-hypertensives and external cooling as necessary (Chiang 2011; Boyer, Seifert, and Hernon 2019). In addition to some anti-hypertensive action by benzodiazepines independent of its action at GABA receptors (Colussi et al. 2011; Yilmaz et al. 2011), benzodiazepines are used due to their sedative action in the management of stimulant-induced agitation. The prevalence of METH related psychoses is near 40% and is thought to be one of the defining features of METH intoxication; benzodiazepines act as a sedative to manage the extreme agitation and potential for violence accompanying amphetamine intoxication, as well as reduce hypertension and the risk of hyperthermia or rhabdomyolysis induced by agitation and increased musculoskeletal activity (Lecomte et al. 2018).

Withdrawal from METH peaks approximately 24 hours after last use and is characterized by a state of dysphoria with increases in sleeping and appetite, craving, bradycardia and symptoms related to depression, including fatigue, anhedonia, and anxiety. These symptoms are alleviated within a week of abstinence, though changes in sleep, appetite, depression, and METH craving can persist for 2 - 3 weeks. Symptom severity is generally correlated to age and lifetime duration of amphetamine use (C. McGregor et al. 2005; Zorick et al. 2010). Several treatments for alleviating symptoms associated with amphetamine withdrawal have been tested, including antidepressants like mirtazapine (Cruickshank et al. 2008); the 5HT-3 antagonist, ondansetron (Johnson

et al. 2008); pexacerfont , an antagonist for corticotropin releasing factor 1 (Morabbi et al. 2018); and stimulants such as dexamphetamine and modafinil (Longo et al. 2010; Galloway et al. 2011; N. Lee et al. 2013). Currently, only the stimulants dexamphetamine and modafinil have been shown to be effective in reducing symptoms of withdrawal from amphetamines. It is noted however, reducing withdrawal symptoms has not consistently reduced relapse to METH (Shoptaw et al. 2009; Chan et al. 2019; Siefried et al. 2020).

Clinical Manifestations of Chronic METH Use and METH Use Disorder

Though significant mortality exists from the acute intoxication of METH, the recurrent use of substances of abuse despite periods of abstinence is what defines this group of compounds and exacerbates the morbidity and mortality associated with drugs of abuse (Koob and Volkow 2009). A descriptive retrospective study of over 300 individuals that examined the transition of METH use behaviors reported that the median number of METH uses before craving was 6.5 uses, with transition from casual to compulsive use within 3 months of first exposure (Z. Wang, Li, and Zhi-min 2017). Longitudinal studies have observed that approximately 61% of individuals seeking treatment relapse within the first year (Brecht and Herbeck 2014; M. P. Paulus and Stewart 2020).

Epidemiological evidence showed that approximately half of adult METH users in the United States report more than 100 days of METH use out of each year, and it is estimated that approximately half of these individuals fit the criterion for METH Use Disorder (C. M. Jones, Compton, and Mustaquim 2020). METH use disorder falls under the broader category of stimulant use disorder in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and is characterized by significant impairment or distress due to an inability to reduce drug use despite physical or social consequences, tolerance to

the drug of abuse, and cravings for the drug of abuse (*Diagnostic and Statistical Manual of Mental Disorders : DSM-5*. 2013; M. P. Paulus and Stewart 2020).

The neurocircuitry of substance use disorder is still being elucidated. However, it has become clear that a few interconnected regions are highly implicated in the disease process (Koob and Volkow 2016). Currently, the prevailing framework by which substance use disorder is understood is as a disease that occurs in three stages that differentiate along the motivations behind relapse to drugs of abuse: intoxication, withdrawal/negative affect, and preoccupation/craving. Within this framework, the first stage driving relapse to drugs of abuse is the intoxication stage, where drugs of abuse are used primarily for subjective effects. Imaging studies have suggested that drug intoxication produces a fast and steep release of dopamine or activation of dopaminergic pathways within the basal ganglia which are associated with a subjective “high” (N. D. Volkow et al. 2004). Dopamine release to the nucleus accumbens, a nucleus important for the execution of planned behaviors (Lüscher and Malenka 2011), and the dorsal striatum, a brain region implicated in the execution of habitual behaviors (Belin and Everitt 2008), is thought to be important for creating incentive salience, formation of an association of between stimuli and motivational importance and ultimately the long-term neuroadaptations of the reinforcing effects of drugs of abuse (Koob and Volkow 2016).

The second motivational stage driving relapse is withdrawal. Withdrawal from drugs of abuse is associated with negative affect, including subjective feelings of increased depression and anxiety. Because these symptoms can be alleviated by relapse to drugs of abuse, it is thought that withdrawal is a motivator of relapse (C. McGregor et al. 2005; Wodarz et al. 2017). In pre-clinical models, the withdrawal period has been associated with a hypodopaminergic state, with reduced levels of extracellular dopamine in the nucleus accumbens during abstinence (Parsons, Smith, and Justice 1991; Acquas and Di Chiara 1992; Weiss et al. 1992) and was suggested that relapse to

drugs of abuse occurred in order to escape this negative affect state (Koob and Le Moal 2005). Interestingly, withdrawal-induced negative affective states have also been correlated to increases in the release of corticotropin releasing factor and norepinephrine to the extended amygdala (Koob and Volkow 2016). However, human trials utilizing pexacerfont, a corticotropin releasing factor 1 receptor antagonist (Kwako et al. 2015), or prazosin, an α 1 receptor antagonist (Wilcox et al. 2018), have both failed to produce reductions in relapse to alcohol use disorder in randomized control trials, suggesting further investigations targeting these factors with respect to withdrawal and the extended amygdala may require further research.

The third stage driving relapse to drugs of abuse is the preoccupation/craving stage, a stage characterized by the persistence of drug-seeking despite the absence of symptoms related to intoxication or withdrawal, and can occur in response to cues despite extended periods of abstinence from the drug (Koob and Volkow 2016). Interestingly, this phenomenon appears to be dependent on glutamatergic transmission in the nucleus accumbens and not dopamine (McFarland, Lapish, and Kalivas 2003). Interestingly, pre-clinical models have demonstrated increases in drug-seeking behavior after extended periods of abstinence, a phenomenon referred to as the incubation of craving (Grimm et al. 2001). This behavioral phenotype, in face value, is similar to the state of craving in humans, (Venniro, Caprioli, and Shaham 2016). In these pre-clinical models, the incubation of craving is associated with increased GluA2-lacking AMPA receptors accumulating on the surface of the nucleus accumbens in preclinical models that self-administered cocaine (Conrad et al. 2008), suggesting that drug-seeking after extended periods of abstinence is driven by glutamatergic neurotransmission in the nucleus accumbens (Koob and Volkow 2016). A similar phenomenon has been reported to occur with METH self-administration in rats as well (Scheyer et al. 2016).

This framework was created to conceptualize the changes that occur within the brain with regards to how they may drive relapse to substance use disorders (Koob and Volkow 2009). With that in mind the recurrent use of METH is associated with a range of neurological differences that are distinct from the state of acute METH intoxication. Metanalyses of chronic METH abusers show the largest deficits in neurocognitive tests related to learning, executive functions (i.e. poor scores on response inhibition measured by Stroop tests, problem solving measured by Trail Making tests), memory, and attention (Schwendt et al. 2009). There are relatively few prospective studies on the effects of long-term METH abuse in humans with evidence beyond self-report data. Therefore, it is challenging to discern whether neurological differences in chronic METH users are due to the chronic use of the drug, or if they were pre-existing factors that may have precipitated the drug use (Salo et al. 2011; Dean et al. 2013).

Studies utilizing Positron Emission Tomography (PET) revealed that chronic METH abusers relative to healthy controls have reduced stored dopamine in the caudate and putamen that normalize on re-imaging after 1 week abstinence (Boileau et al. 2016). Studies also showed reduced levels of DAT in the prefrontal cortex, caudate, putamen, and nucleus accumbens even after varying durations of abstinence from METH, but with some potential for recovery with abstinence; (McCann et al. 1998; N. D. Volkow, Chang, Wang, Fowler, Leonido-Yee, et al. 2001; Sekine et al. 2001), decreased D2/3 receptor binding in the caudate (N. D. Volkow, Chang, Wang, Fowler, Ding, et al. 2001; Boileau et al. 2012; G. J. Wang et al. 2012); and increased D3 receptor binding in the substantia nigra (Boileau et al. 2012). These findings are in line with cadaveric studies on individuals with known METH abuse histories that have died either due to METH intoxication or with detectable amounts of METH in their bodies. These METH users had an dopamine reduced by 50-60% and reduced DAT protein by 30-40%, and reduced tyrosine hydroxylase by ~20% in the caudate, putamen, and nucleus accumbens

compared to control cohorts matched for age, sex, and postmortem death-to-autopsy interval (Wilson et al. 1996; Moszczynska et al. 2004). Additionally, there was a 25-30% decrease in the maximal dopamine-induced cAMP production in cadaveric brain homogenates taken from the nucleus accumbens, caudate, putamen, and frontal cortex (Tong et al. 2003). This effect is thought to be mediated by a desensitization of the D1-type dopamine receptor, as D1-specific agonists used to stimulate the dorsal striatum in rats pre-treated with amphetamine appears to also have reduced cAMP relative to control (Roseboom and Gnegy 1989). Interestingly, chronic METH use was also associated with nearly a two-fold increase in Parkinsonian symptoms when compared to general population and individuals with chronic cocaine use (Callaghan et al. 2012).

Animal models of METH use that experimentally recapitulate the depletion of dopamine, DAT, and tyrosine hydroxylase, demonstrated that these changes are due to toxicity of dopaminergic nerve terminals (Halpin, Collins, and Yamamoto 2014), leading to an overall hypodopaminergic state (Callaghan et al. 2012; Shin et al. 2017). It has been theorized that the drive to alleviate this hypodopaminergic state contributes to relapse to drugs of abuse (Koob and Mason 2016). One mechanism by which METH induces dopaminergic neurotoxicity is through inflammation and the immune reaction to METH (Halpin, Collins, and Yamamoto 2014). METH has a number of immune consequences including activation of microglia and astrocytes (Thomas et al. 2004; Narita et al. 2006). Microglia and astrocytes are both immune cells in the brain that respond to external insults and can be measured in laboratory settings (Mander, Jekabsone, and Brown 2006). Microglia upregulate the expression of ionized calcium binding adaptor molecule 1 (Iba-1) or Cluster of Differentiation 68 (CD68), in inflammatory states, and these proteins can be detected by immunohistochemistry or by Western blot (He and Crews 2008; Hendrickx et al. 2017). Astrocytic response to cellular injury, including inflammation, can be detected by their increased expression of glial

fibrillary acidic protein (GFAP), a cytoplasmic protein upregulated in states of inflammation, using immunohistochemistry or by Western blot (Eng, Ghirnikar, and Lee 2000; Krasnova et al. 2010). METH has been shown to stimulate both astrocytic and microglial activation within 3 days of exposure in preclinical models (Thomas et al. 2004; Narita et al. 2006), as well as the expression of pro-inflammatory cytokines in the striatum (Sriram, Miller, and O'Callaghan 2006; Gonçalves et al. 2017), and blocking this activation is correlated to changes in METH-induced behaviors and neuropathology (Asanuma et al. 2003).

One of the first human studies on METH and inflammation used PET and found increased levels of activated microglia in the midbrain, striatum, thalamus, orbitofrontal cortex, and insular cortex of METH users that had been abstinent from METH an average 2 years relative to an age, gender, and education matched cohort (Sekine et al. 2008). A subsequent histopathological study for microglia and astrocytes revealed that chronic METH users who died from complications related to METH intoxication had increased numbers of microglia in their caudate, putamen, and nucleus accumbens relative to controls (Kitamura et al. 2010). Taken together, while it is understood that METH activates the innate brain immune system, how this activation contributes to METH use disorder, including relapse after abstinence, is still being explored (Coller and Hutchinson 2012).

Interestingly, as METH has been known to increase striatal levels of pro-inflammatory cytokines (Sriram, Miller, and O'Callaghan 2006; Gonçalves et al. 2017), pro-inflammatory cytokines have also been shown to be able to modulate glutamate receptor expression on neurons. For instance, TNF α has been shown to be able to induce the insertion of GluA1-type AMPA receptors on hippocampal pyramidal cells and medium spiny neurons of the dorsal striatum and thereby increase recorded miniature excitatory post-synaptic currents (Beattie et al. 2002; Stellwagen et al. 2005; Lewitus et

al. 2014). Furthermore, it is known that glutamate levels in the nucleus accumbens correlated to the act of drug seeking in rats trained to self-administer psychostimulants, and glutamate transmission in the nucleus accumbens was necessary for the reinstatement of cocaine seeking behavior after abstinence (McFarland, Lapish, and Kalivas 2003; Conrad et al. 2008). This would suggest that neurochemical changes driving relapse may also be tied inflammatory processes by way of GluA1-type AMPA receptors in the nucleus accumbens, and changes in both glutamate receptors and inflammatory mediators may contribute to the neuropathology of METH use disorder.

Gap in the Literature and Hypothesis

In 2018, an estimated 1.6 million adults in the United States used methamphetamine (METH) during the past year and approximately 50% of those individuals met the criteria for METH use disorder (C. M. Jones, Compton, and Mustaquim 2020). Overdoses related to the acute abuse of METH and other substituted amphetamines are a clear area of societal concern in the United States, as the mortality associated with METH use has been increasing. However, it is the chronic relapsing to METH abuse that is of clear clinical need. Psychosocial interventions remain the first-line treatment, these studies and programs suffer from poor compliance, high rates of discontinuation, and a poor understanding of how they address the underlying neurobiology associated with METH use disorder (M. P. Paulus and Stewart 2020; Ronsley et al. 2020).

The primary gap in the literature revolves around effective treatments for METH use disorder. Relapse to drug taking remains a significant challenge to patient care. So far, mood stabilizers such as selective serotonin reuptake inhibitors or mixed serotonin/norepinephrine reuptake inhibitors have been tried with the rationale of reducing dysphoria associated with METH withdrawal but have not shown efficacy in

reducing relapse (Torrens et al. 2005; Chan et al. 2019; Ronsley et al. 2020) Given that chronic METH use is associated with depletions in dopamine in the basal ganglia in both non-human primates and rats (Ricaurte et al. 2005; Krasnova et al. 2010), L-DOPA, a precursor to dopamine and used to treat Parkinson's Disease, was initially considered as a potential treatment, but pre-clinical models showed that L-DOPA potentiates dopamine terminal toxicity related to subsequent amphetamine use (Weihmuller, O'Dell, and Marshall 1993; Thomas, Francescutti-Verbeem, and Kuhn 2008). Partial dopamine receptor agonists have been tried with the rationale of restoring dopamine signaling, and low dose amphetamines have been tried in a strategy to mitigate dysphoria and craving associated with withdrawal, and some limited success in reducing METH use was found when substituting METH for another stimulant, methylphenidate (Chan et al. 2019). However, though this strategy mitigates withdrawal symptoms after METH intoxication, substitution may not be an effective strategy to reduce relapse, as relapse often occurs in the absence of physical signs of withdrawal from the drug (Morley et al. 2017). Guiding patients through extensive periods of drug abstinence is not always sufficient to prevent drug cravings that can drive drug seeking despite the absence of withdrawal symptoms (Shoptaw et al. 2009; Pérez-Mañá et al. 2013; Chan et al. 2019).

In searching for alternative strategies to treat METH use disorder, pre-clinical evidence has suggested that inhibitors of phosphodiesterase (PDE), an enzyme involved in the degradation of the intracellular cyclic nucleotide signaling molecules, may be a novel treatment for METH use disorder. The PDE family is known to control spatially and functionally distinct pools of cAMP, with their action determined by their binding partners (Houslay and Adams 2003). Since the creation of PDE-specific inhibitors, PDE4 inhibition has received attention as PDE4 inhibitors have been shown to enhance learning and memory in models of Alzheimer's disease (Gong et al. 2004), neural recovery after traumatic brain injury (Vogel et al. 2017), as well as reduce relapse-like

behaviors in pre-clinical models of substance use disorder (Olsen and Liu 2016). For instance, broad spectrum PDE inhibitors propentofylline and ibudilast reduced METH-induced conditioned place preference in mice (Narita et al. 2006), and stress-induced or METH-primed reinstatement of METH seeking behavior in rats (Beardsley et al. 2010).

In humans, a randomized, double-blind clinical trial testing ibudilast against placebo did not detect a difference in METH abstinence rates (Heinzerling et al. 2020). However, ibudilast is a non-specific inhibitor of a variety of PDE isoforms and there is a body of pre-clinical evidence suggesting that the PDE4 enzyme, which hydrolyzes cAMP may be a more selective and effective target (Figure 2). Given that ibudilast is a non-selective PDE inhibitor with an IC_{50} for PDE4 of 5 μ M (Allcock et al. 2011), and roflumilast is a PDE4-specific inhibitor with an IC_{50} for PDE4 of 0.7nM (Rabe 2011), the greater potency of roflumilast may be a meaningful clinical difference.

The PDE4 inhibitor used in the majority of published pre-clinical neurological studies, rolipram, is not FDA-approved due to its adverse side-effect profile and the production of dose-limiting nausea (Blokland et al. 2019). Given its promise as a target, PDE4 inhibitors with more favorable side-effect profiles have been synthesized, including roflumilast, which is already approved by the United States Food and Drug Administration (FDA) (Spina 2008; *Orange Book : Approved Drug Products with Therapeutic Equivalence Evaluations*. 2020). Currently, there are very few studies using these approved PDE4 inhibitors to treat substance use disorder (Blokland et al. 2019). As these drugs have reduced regulatory hurdles before entering clinical trials, there are clear advantages in utilizing an approved PDE4 inhibitor like roflumilast in investigating new treatments for METH use disorder (Morley et al. 2017).

Figure 2

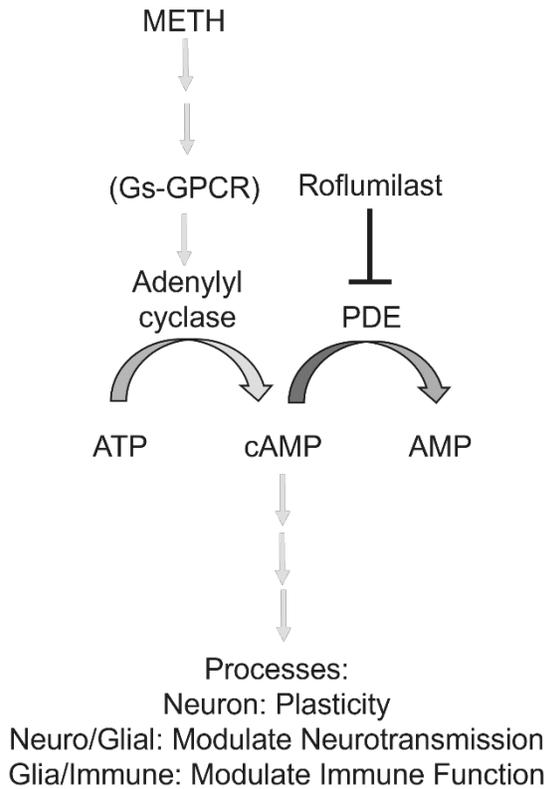


Figure 2: General schematic of cAMP

signaling. METH induces cAMP signaling through mediators activating Gs-coupled GPCRs, such as dopamine. This drives cAMP-mediated cellular processes, including neural adaptations in neurons, modulation of neurotransmission in neurons and glia and immune modulation in immune cells, including glia. METH also induces dopaminergic toxicity, reducing cAMP-mediated signaling. Roflumilast can replete cAMP deficits by inhibiting PDE and increasing intracellular cAMP.

Interestingly, PDE4 inhibitors also demonstrate anti-inflammatory effects (Spina 2008), and METH has been shown to activate both microglia and astrocytes in the brain as well as increase levels of cytokines, including TNF α (Narita et al. 2006; Krasnova et al. 2010; Gonçalves et al. 2017). Separately, TNF α has been shown to be able to induce the insertion of GluA1-type AMPA receptors on hippocampal pyramidal cells and medium spiny neurons of the dorsal striatum and thereby increase recorded miniature excitatory post-synaptic currents (Beattie et al. 2002; Stellwagen et al. 2005; Lewitus et al. 2014). Other pro-inflammatory cytokines including interleukin 1 beta (IL-1 β) and interleukin 6 (IL-6), can also influence the firing properties of neurons (Katsuki et al. 1990; Bellinger, Madamba, and Siggins 1993; Schneider et al. 1998; Tancredi et al. 2002; Pribiag and Stellwagen 2014). Though glutamatergic neurotransmission in the nucleus accumbens is a key mediator in relapse-like behaviors to METH (Rocha and

Kalivas 2010), the inflammatory contribution to glutamate neurotransmission in the nucleus accumbens with regards to relapse-like behaviors is an active area of investigation (Lacagnina, Rivera, and Bilbo 2016).

Given that there is clinical evidence suggesting that there is overall decreased dopamine-induced cAMP signaling, putatively by decreased dopaminergic terminals due to METH-induced toxicity (Tong et al. 2003; Schwendt et al. 2009); as well as increased inflammation in the nucleus accumbens of chronic METH users (Sekine et al. 2008; Kitamura et al. 2010), PDE4 inhibition may be well positioned to act on dopaminergic, glutamatergic, or inflammatory mechanisms to reduce relapse METH use disorder. Therefore, the first hypothesis tested is that administration of roflumilast, an FDA-approved PDE4 inhibitor, during abstinence would reduce relapse-like behaviors to METH in a rat model of METH use disorder. Additionally, we will test whether these behavioral effects are specific for METH using a sucrose self-administration model as a control. Rats can be trained to self-administer sucrose under similar experimental conditions as drug self-administration (Figlewicz et al. 2011), and this model has previously been used to measure how different potential treatments for substance use disorder would affect motivated behaviors not associated with the drug (Shaham et al. 2003). As similar dopaminergic, glutamatergic, or inflammatory dysregulations have not been reported in abstinence from sucrose self-administration, roflumilast treatment will leave resumption of sucrose self-administration behaviors intact (Figure 3).

Furthermore, this dissertation seeks to understand the potential mechanism by which roflumilast may act. Given any changes in relapse-like behavior to METH, we will explore dopaminergic, glutamatergic, or inflammatory changes within the nucleus accumbens that are associated with a behavioral phenotype. The nucleus accumbens specifically will be explored in this preliminary investigation as it is most consistently

identified as a mediator of relapse-like behaviors (Olsen and Liu 2016; Nestler and Lüscher 2019; Wright and Dong 2020).

Figure 3

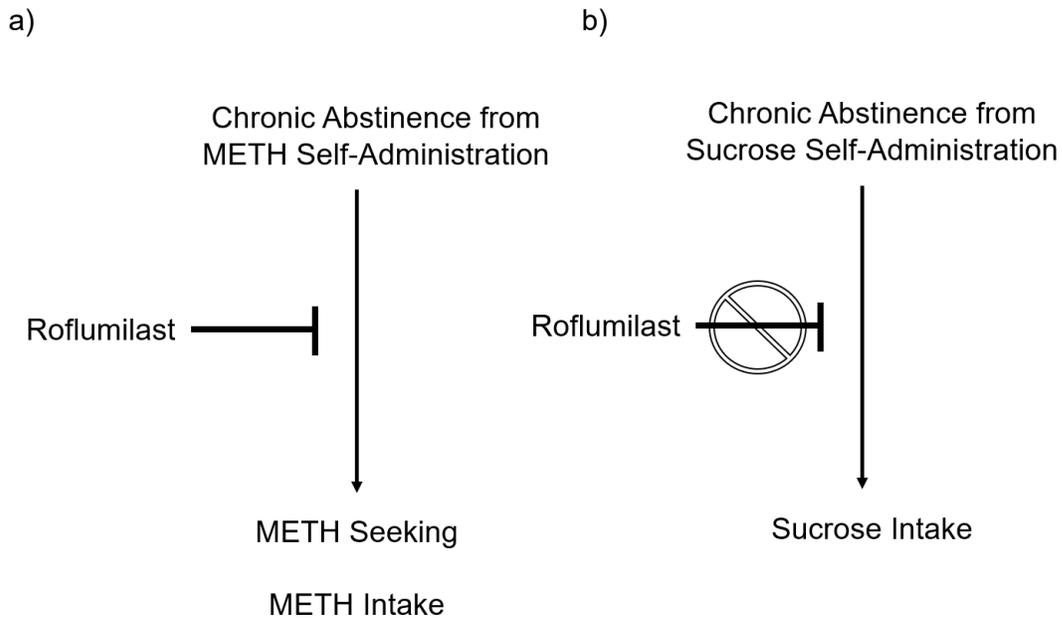


Figure 3: Diagram representing the hypothesized effect of roflumilast on relapse. (a) Abstinence from METH self-administration would lead to increases in METH seeking and taking behavior. Roflumilast, a PDE4 inhibitor, given during the abstinence from METH, would decrease the subsequent relapse to METH seeking and METH taking behaviors. (b) Roflumilast administered during abstinence would not affect the resumption of behaviors related to sucrose self-administration.

Other brain regions have been implicated, including the prefrontal cortex (McFarland, Lapish, and Kalivas 2003; Parsegian and See 2014), extended amygdala (Pelloux et al. 2018), and the dorsal striatum (Fuchs, Branham, and See 2006; Harada et al. 2021). However, given that reduced D1R-mediated dopaminergic neurotransmission in the nucleus accumbens has been observed to increase the rate of

drug self-administration (Phillips, Robbins, and Everitt 1994) and that chronic METH is associated with dopaminergic toxicity including in the nucleus accumbens (Tong et al. 2003), we will investigate potential dopaminergic mechanisms that may influence the rate of METH self-administration. Although multiple brain regions that can also mediate relapse and the rate of drug self-administration converge on the nucleus accumbens including the prefrontal cortex (A. McGregor and Roberts 1995; McFarland, Lapish, and Kalivas 2003) and extended amygdala, specifically the basolateral nucleus of the amygdala, (Koob and Volkow 2016; Pelloux et al. 2018), the bed nucleus of the stria terminalis (Epping-Jordan, Markou, and Koob 1998) and central nucleus of the amygdala (Alleweireldt et al. 2006; Cain, Denehy, and Bardo 2007; X. Li, Zeric, et al. 2015), these factors suggest that the nucleus accumbens is an important effector in relapse-like behaviors. Though certainly other brain regions should be explored, this dissertation seeks to explore how the nucleus accumbens specifically changes with regards to METH self-administration and abstinence, as well as with roflumilast treatment.

Conclusion

This dissertation seeks to fill the gap in the treatment of METH use disorder by exploring the efficacy of a specific phosphodiesterase inhibitor, roflumilast, in the treatment of relapse to METH, as well as potential mechanisms of action. Chapter 2 describes the effects of roflumilast on relapse to METH in rats. Chapter 3 investigates potential mechanisms by which METH and phosphodiesterase action on glutamate and dopamine neurotransmission. Chapter 4 describes potential mechanisms by which the anti-inflammatory action of roflumilast mediate its effects on relapse to METH. Overall the work of this dissertation sought to test a new intervention for METH use disorder and uncover the mechanism underlying the intervention.

Chapter 2: Roflumilast Treatment during Forced Abstinence from METH Reduces Relapse to METH Seeking and METH Taking

Introduction

METH use disorder is categorized under the broader term stimulant use disorder in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and is characterized as a pattern of significant impairment and distress consequent to drug use (M. Paulus 2015). The general pattern of METH intake in humans is an escalation of intake drug quantity when transitioning from casual and experimental use to an established use disorder, to such a point where additional dosing does not have increased subjective effect. Of note, accurate self-report of this escalation of intake in amphetamines was possible due to a period after World War 2 where injectable amphetamines were able to be prescribed for the treatment of heroin use disorder in the United States, and unregulated prescription capabilities resulted in the flagrantly unethical distribution of injectable amphetamines (Kramer, Fischman, and Littlefield 1967; A. K. Cho and Melega 2002). Current criteria for METH use disorder include cravings for METH, tolerance to METH, continued METH use regardless of known physical or social consequences, and unsuccessful efforts to reduce METH use. METH overdoses have been steadily increasing in the United States between 2011 and 2018 (Han et al. 2021). Specific pharmacotherapies for METH addiction are lacking, and relapse to drug taking remains a significant challenge to patient care (Shoptaw et al. 2009; Pérez-Mañá et al. 2013; Chan et al. 2019).

Pre-clinical models of METH use disorder have long used the rat self-administration model due to its face validity in modeling of behaviors associated with METH use disorder, and for the testing potential treatments (Grimm et al. 2001; Kitamura et al. 2006; Panlilio and Goldberg 2007). Drug seeking and taking behavior has

long been known to be stimulated by environmental cues, and similarly, relapse-like behaviors in rats can be stimulated in response to cues associated with prior drug availability such as environmental context, or cue-lights previously activated in conjunction with drug-administration (cue-induced); or by interoceptive cues produced by exposure to the drugs themselves (drug-induced) (Robinson et al. 2014; Venniro, Caprioli, and Shaham 2016).

Pre-clinical evidence has suggested that inhibitors of phosphodiesterase (PDE), an enzyme involved in the degradation of the intracellular cyclic nucleotide signaling molecules, may be a novel treatment for METH use disorder. Of the 11 different isoenzymes within the PDE class, the PDE4 enzyme, which hydrolyzes cAMP, has the largest body of evidence in its use to treat mood disorders (Houslay and Adams 2003; Keravis and Lugnier 2012), and may mediate the reduction of drug seeking behaviors (Self and Nestler 1995; Olsen and Liu 2016). The PDE4 specific inhibitor, rolipram, has been shown to reduce the self-administration behaviors related to cocaine (Knapp et al. 1999; Thompson et al. 2004), alcohol (Wen et al. 2012), and heroin (Lai et al. 2014). However, the design of these pre-clinical studies has limited clinical utility because the PDE4 antagonist was administered during the acquisition of the drug seeking behavior, or as a pre-treatment immediately before a relapse test. There are few if any studies evaluating relapse to drug following treatment with PDE inhibitors during abstinence from established drug self-administration.

To address these gaps in the literature, the current study was designed to test whether a pharmacological intervention administered during abstinence from established METH self-administration behaviors would reduce relapse behaviors. The decisions to treat during abstinence, then measure relapse to METH after a forced abstinence were made to increase face validity in modeling human relapse and treatment paradigms (Katz and Higgins 2003; Pelloux et al. 2018; Chan et al. 2019). Two distinct relapse tests

were used to determine the effect of roflumilast: the METH seeking test, a relapse test where operant behavior was not rewarded to assess resumption of METH seeking behavior after abstinence; and a METH taking test, a relapse test where operant behavior was rewarded with METH to assess resumption of METH intake after abstinence. The selective and FDA-approved PDE4 inhibitor, roflumilast, was administered during the forced abstinence from METH self-administration (*Orange Book : Approved Drug Products with Therapeutic Equivalence Evaluations*. 2020). We hypothesized that in rats with established METH self-administration behaviors, treatment with roflumilast during abstinence would reduce relapse-like behavior with regards to METH self-administration.

Materials and Methods:

Subjects

Male Sprague-Dawley rats (225-250 g, Envigo, Indianapolis, IN) were used in all experiments. Rats were housed 2 per cage in a temperature and humidity controlled facility under a 12 hour light/dark cycle (7:00 AM – 7:00 PM) with a minimum 4 day acclimation period before any experimentation. Food and water were available ad libitum throughout the experiment. Experiments and surgical procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and approved by Indiana University Institutional Animal Care and Use Committee.

Jugular Vein Catheterization

Rats were anesthetized with a mixture of ketamine (75 mg/kg, Henry Schein, NDC 11695-0702-1) and xylazine (5 mg/kg, Akorn, Cat. NDC 59399-110-20) given as an intraperitoneal injection. The anterior cervical region and mid-scapular regions were

depilated and sanitized, then slow-release buprenorphine was administered as a subcutaneous injection for analgesia (1 mg/kg, Buprenorphine SR-LAB, ZooPharm). After accessing the jugular vein, a 3.5 Fr polyurethane catheter (Access Technologies, Cat. AT-RJVC-0612A) pre-filled with antibiotic flushing solution (0.01% cefazolin, w/v (Fisher, Cat AC455210010) and 10 U/mL heparin (Meitheal, NDC 71288-402-02) in 0.9% sterile saline (UltiGiene NDC 50989-885-17)) was inserted towards the heart and secured with silk suture. The catheter was tunneled through the subcutaneous plane to the midline scapular region and secured to a 22-gauge cannula port (Plastics One, Cat. 313-000BM-15-5UP/SPC) pre-filled with antibiotic flushing solution. The port was capped from air with removable heat-sealed tygon tubing (Fisher/Cole-Parmer, Cat. 06419-01). The incisions were sutured closed (Henry-Schein, Cat. 1006830), and topical 2% lidocaine and triple antibiotic cream were applied to the wounds. Rats singly housed in clean home cages recovered a minimum of 3 days before any additional experimental manipulation. Catheter patency was maintained by daily 0.2 mL infusions of antibiotic flushing solution for 3 days following surgery. After 3 days and throughout the rest of the METH self-administration paradigm, catheter patency was maintained with flushing solution (10 U/mL heparin in 0.9% sterile saline).

Self-Administration

Rat behaviors were measured in a sound-attenuating self-administration chamber (MedAssociates, Cat. ENV0008CTT). Chambers were equipped with an active reward-paired lever and inactive lever with cue-lights above each lever. When the active lever was pressed, its cue-light was active for 5 seconds while 1 reward was delivered. During this 5 second period, further presses on the active lever were recorded but no additional rewards were delivered until this period ended and the cue-light turned off. Inactive lever presses were recorded but had no programmed consequences.

Figure 4 diagrams the experimental paradigms used in the study. Rats were naïve to self-administration chambers before the 14 days of self-administration. Immediately before and after self-administration sessions, catheters were flushed with 0.2 mL of flushing solution (10 U/mL heparin in 0.9% saline). Rats requiring increased flushing pressure suggesting catheter patency compromise were removed from the experiment and excluded from analysis. For 14 days, rats were taken from their home cage, placed in the chamber with unrestricted access to food and water where behaviors were recorded over 6 hours between 10 AM and 4 PM, then returned to home cages.

Forced abstinence and treatment began at the end of 14th day of self-administration. By the end of the 14th session, rats were balanced into treatment groups receiving vehicle or roflumilast. Within METH groups, animals were sorted into two groups based on weight, total METH self-administered, as well as active lever pressing, inactive lever pressing, and METH self-administered in the last three sessions. Saline animals were sorted into two groups based on weight, total saline administered, and as well as active lever pressing, inactive lever pressing, and saline administered in the last three sessions. T-tests were run to ensure that each of these measures did not differ between the matched groups, and then each group was arbitrarily assigned to be treated with either vehicle or roflumilast to form 4 groups: Saline x Vehicle, Saline x Roflumilast, METH x Vehicle, and METH x Roflumilast. The same At the end of the 14th session, rats returned to their home cages and then treated according to their treatment group. Treatment consisted of either roflumilast (1.5 mg/kg, p.o. via syringe gavage) (Sigma, Cat. SML 1099) or vehicle (1:4 DMSO, Phosphate Buffered Saline, p.o. via syringe gavage) every other day, with the last day of treatment was the day before the relapse test. The dose of roflumilast was determined in pilot studies where roflumilast was suggested to reduce relapse-like behavior without affecting rat body weights. Rats were reintroduced to the self-administration chamber for the relapse test only. Each rat

experienced only a single relapse test that lasted 6 hours between 10 AM and 4 PM.

Relapse tests are described in further detail separately below.

For rats that self-administered METH, rewards during METH self-administration sessions were on a fixed ratio schedule where 1 press was required for 1 reward unit, an FR1 schedule. Presses at the active lever activated the cue-light and an infusion pump that delivered 1 reward unit, which was 0.1 mL of 0.1 mg/kg (+) Methamphetamine (METH) (Sigma-Aldrich, Cat. M8750) dissolved in heparinized saline (1 U/mL Heparin in 0.9% saline) delivered intravenously over the course of 1 second. METH concentrations in syringes were tailored to each rat's weight \pm 5 mg. A 5 second timeout period followed each reward during which active lever presses were recorded but did not deliver rewards. As each reward was delivered to a METH self-administering rat, a yoked control rat would receive a non-contingent infusion of 0.1 mL of heparinized saline. For yoked saline control rats, presses at both the active lever and the inactive lever were recorded but no rewards were delivered. Levers automatically retracted at the end of the session. The concentration of METH infusions was adjusted for each session to accommodate changes in rat body weight.

Sucrose self-administration was used to serve as a control to measure how roflumilast would affect more naturalistic reward behaviors. Rats that self-administered sucrose also underwent jugular vein catheterization to control for stressors and effects related to the surgery and chronic implantation of the catheter. Rewards during sucrose pellet self-administration were also on an FR1 schedule. Presses at the active lever activated a cue-light and a pellet dispenser that delivered 1 reward unit, which was 1 sucrose pellet (Fisher, Cat. 14-726-596). A 5 second timeout period followed each reward during which active lever presses were recorded but did not deliver rewards. Rats then underwent 7 days of abstinence and treatment, followed by a relapse test.

During the relapse test, lever presses resulted in the delivery of sucrose pellets. Each rat experienced only a single relapse test that lasted 6 hours between 10 AM and 4 PM.

Relapse Tests:

METH seeking test: Rats were reintroduced to the self-administration chamber after forced abstinence and pressing of the active lever activated the cue-light above the lever, but no drug was delivered.

METH taking test: Rats were reintroduced to the self-administration chamber after forced abstinence and pressing of the active lever activated the cue-light above the lever and the infusion pump, which delivered 0.1 mL of 0.1 mg/kg of METH intravenously (FR1 schedule).

Sucrose taking test: Rats were reintroduced to the self-administration chamber after forced abstinence and pressing of the active lever activated the cue-light above and the pellet dispenser, which delivered 1 sucrose pellet (FR1 schedule).

Statistical Analyses:

Statistical analyses were performed using Sigma Plot 13. Single day data of number of active lever presses was analyzed using two-way repeated measures analysis of variance (ANOVA). METH intake per 6 hour session in mg/kg, and sucrose intake per 6 hour session in number of pellets were analyzed using one-way repeated measures analysis of variance (ANOVA). Data in figures 5 (a, b, c), 6 (a, b), 7 (a, b), 8 (a, b, c) and Table 1 are reported as means (M) \pm standard error of the mean (SEM). The time courses of relapse test reported data in 15 minute bins. Data were analyzed using two-way repeated measures ANOVA, and data in figures 5 (c), 6 (c), 7 (b) and 8 (c) are reported as M \pm SEM. Significant differences in all ANOVA were followed by Holm-Sidak's method for post-hoc analysis. Data plots of METH intake over time were

analyzed using nonlinear regressions using an inverse second order polynomial function by the equation $f = y_0 + (a / x) + (b / x^2)$. Regression equations were reported with coefficients of determination (R^2) and standard error of the estimates (SEE). Calculated regression coefficients were presented with standard errors (SE) in figures 5 (d) and 7 (c). Data in figures were reported with regression lines and their 95% confidence intervals. For all experiments, statistical significance was set at $p < 0.05$.

Figure 4

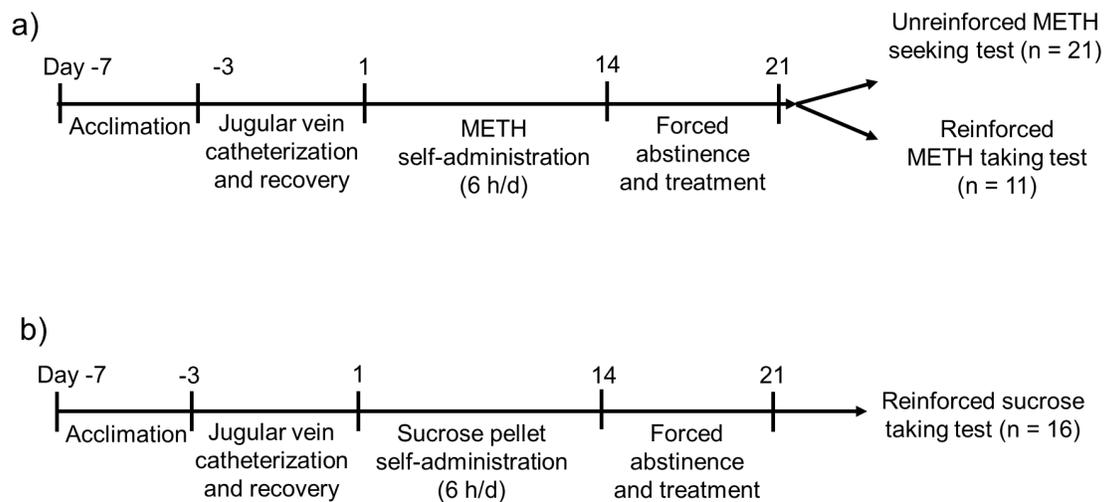


Figure 4: Timeline of the experiment design. (a) METH self-administration paradigm. Rats underwent METH self-administration or yoked saline administration for 14 days before being sorted into treatment groups and undergoing forced abstinence and treatment, followed by a relapse test. (b) Sucrose self-administration paradigm. A separate cohort of rats underwent the same paradigm but self-administered sucrose pellets instead of METH.

Results:

Rats escalated METH self-administration from Day 1

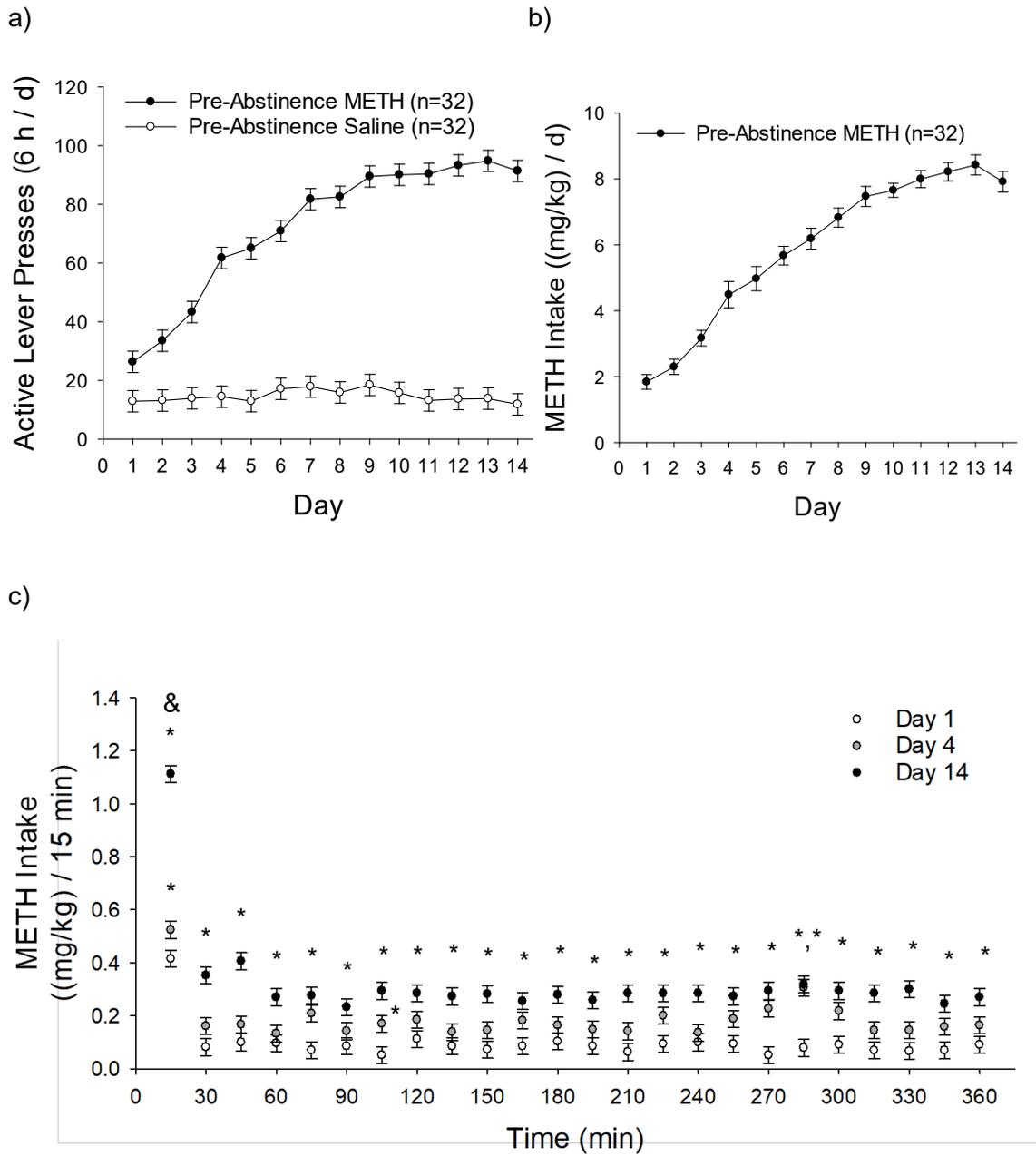
After jugular vein catheterization, rats underwent METH self-administration or yoked saline administration (Figure 4). Rats increased their active lever pressing from Day 1 values (Figure 5a) as analyzed by a two-way repeated measures ANOVA. The number of active lever presses depended on days within the self-administration paradigm, and whether the animals were self-administering METH or a yoked saline control, as revealed by a significant interaction ($F_{(13,806)} = 35.37$, $p < 0.001$). METH self-administering rats pressed the active lever more than yoked saline controls (METH, $M = 72.47 \pm 2.54$; Saline, $M = 14.64 \pm 2.54$; $F_{(1,806)} = 260.21$, $p < 0.001$), and increased their active lever pressing across the 14 days of self-administration ($F_{(13,806)} = 39.12$, $p < 0.001$). Post-hoc analyses using the Holm-Sidak method confirmed that METH self-administering rats pressed the active lever more than yoked saline control rats from Day 1 (Saline Day 1, $M = 12.91 \pm 3.64$; $t = 2.60$, $p < 0.05$). Within the METH self-administering rats, these rats increased their active lever pressing from Day 1 values by Day 3 (Day 1, $M = 26.31 \pm 3.64$; Day 3 = 43.31 ± 3.64 ; $t = 4.43$, $p < 0.001$) and plateaued in their active lever pressing by Day 7, the earliest day where differences from Day 14 active lever presses are not statistically significant. Yoked saline control rats did not differ in their active lever pressing between any day in the self-administering paradigm.

The increase in active lever pressing corresponded with an increase in METH intake as revealed by a one way repeated measures ANOVA (Figure 5b). Rats escalated their METH intake across days ($F_{(13,403)} = 125.642$, $p < 0.001$), such that the self-administered METH increased from Day 1 values by Day 3 onward (Day 1, $M = 1.84 \pm 0.22$; Day 3, $M = 3.17 \pm 0.24$; $t = 4.65$, $p < 0.001$). METH intake by rats plateaued by Day 9, the earliest day where differences from Day 14 METH intake are not statistically significant (Day 9, $M = 7.47 \pm 0.32$; Day 14, $M = 7.92 \pm 0.32$; $t = 1.55$, $p = 1$).

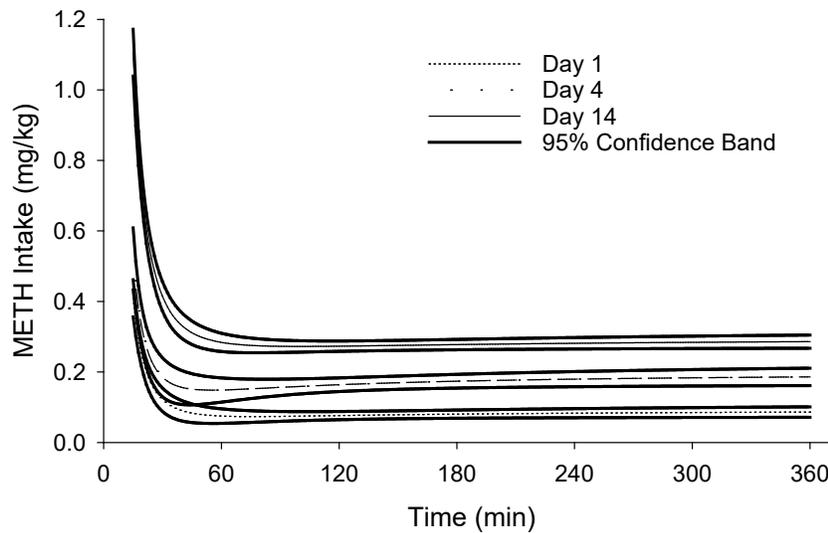
To understand the pattern of self-administration within the 6 hour self-administration session, the time courses of METH intake on Day 1, Day 4, and Day 14 were analyzed using a two-way repeated measures ANOVA (Figure 5c). METH intake over the 6 hour session increased from Day 1 by Day 4 and Day 14 (Day 1, $M = 0.096 \pm 0.018$; Day 4, $M = 0.19 \pm 0.018$; Day 14, $M = 0.32 \pm 0.018$; $F_{(2,1472)} = 40.69$, $p < 0.001$). The METH intake per 15 minutes also changed within each session ($F_{(23,1472)} = 29.59$, $p < 0.001$), and the change depended on the number of days of METH self-administration as indicated by a significant interaction between the METH self-administered and the number of days of METH self-administration ($F_{(46,1472)} = 4.11$, $p < 0.001$). Post-hoc analysis using the Holm-Sidak method analyses comparing rats revealed that rats increased their METH intake overall from Day 1 values (Day 4 vs Day 1, $t = 3.64$, $p < 0.001$; Day 14 vs Day 1, $t = 8.97$, $p < 0.001$; Day 14 vs Day 4, $t = 5.33$, $p < 0.001$). Rats increased their peak dose of METH from Day 1 values, as measured by the METH intake in the first 15 minutes of the session in Figure 3c (Day 1 peak dose, $M = 0.42 \pm 0.03$; Day 4 peak dose, $M = 0.52 \pm 0.03$; Day 14 peak dose, $M = 1.11 \pm 0.03$; Day 1 vs Day 4, $t = 2.16$, $p < 0.05$; Day 1 vs Day 14, $t = 13.79$, $p < 0.001$; Day 4 vs Day 14, $t = 11.63$, $p < 0.001$). After self-administering of a peak dose, rats maintained a constant rate of METH intake, as METH intake per 15 minutes did not differ significantly between subsequent time points after 30 minutes.

To better analyze differences in the basal rate of METH intake, nonlinear regression on the cumulative METH intake against time was performed for the METH Intake on Days 1, 4, and 14 (Figure 5c) (Day 1: $R^2 = 0.152$, $SEE = 0.156$; Day 4: $R^2 = 0.069$, $SEE = 0.259$; Day 14: $R^2 = 0.42$, $SEE = 0.196$). The maintenance dose of METH per 15 min, here defined as y_0 , increased with the number of days of METH self-administration from Day 1 (0.0940 (mg/kg)/15min, $SE = 0.0103$) to Day 4 (0.201 (mg/kg)/15min, $SE = 0.0172$) to Day 14 (0.299 (mg/kg)/15min, $SE = 0.0130$).

Figure 5



d)



Coefficients of the Regression Line $f = y_0 + (a / x) + (b / x^2)$			
Coefficient (SE)	Coefficient, y_0 mg/kg	Coefficient, a mg/kg * min	Coefficient, b mg/kg * min
Day 1 $R^2 = 0.152$ SEE = 0.156	0.0940 (0.0103) ($p < 0.0001$)	-3.107 (1.379) ($p = 0.0246$)	117.465 (21.0551) ($p < 0.0001$)
Day 4 $R^2 = 0.0690$ SEE = 0.259	0.201 (0.0172) ($p < 0.001$)	-5.773 (2.299) ($p = 0.0122$)	158.551 (35.0932) ($p < 0.0001$)
Day 14 $R^2 = 0.419$ SEE = 0.196	0.299 (0.0130) ($p < 0.0001$)	-5.201 (1.737) ($p = 0.0028$)	259.620 (26.518) ($p < 0.0001$)

Figure 5: Rats escalate METH self-administration behaviors. (a) Active lever pressing over 14 days. (b) METH intake over 14 days. (c) Time course of METH intake on Days 1, 4 and 14 analyzed by two-way repeated measures ANOVA. (*, $p < 0.05$ compared to Day 1; &, last time point where $p < 0.05$ compared to the reduced constant levels, the time point at which METH intake did not differ significantly between subsequent time points). (d) Nonlinear regression with fitment according to a second order inverse polynomial function, and corresponding coefficients. $n=32$ /group.

Roflumilast prevented weight gain during abstinence in rats that self-administered METH

Rats were sorted into balanced groups for treatment with either roflumilast or vehicle on Day 14 according to METH intake (Figure 5b) and Day 14 active lever pressing (Figure 5a). Mean mass after treatment is reported in Table 1. A three-way ANOVA was performed to analyze the effects of METH (vs Saline), Roflumilast (vs Vehicle), and time (Pre- vs Post-abstinence) on mass. The three-way ANOVA revealed that there was a significant interaction between status and time ($F_{(1,122)} = 11.688$, $p < 0.001$), as well as treatment and time ($F_{(1,122)} = 5.063$, $p = 0.026$), but not between status and treatment ($F_{(1,122)} = 0.0291$, $p = 0.865$), nor a three-way interaction between status vs treatment vs time ($F_{(1,122)} = 0.510$, $p = 0.477$). Main effect analyses demonstrated the effect of status (Saline vs METH) was significant ($F_{(1,122)} = 32.836$, $p < 0.001$); the effect of time (Pre vs Post) was significant ($F_{(1,122)} = 10.015$, $p = 0.002$), but the effect of treatment (Vehicle vs Roflumilast) was not statistically significant ($F_{(1,122)} = 2.244$, $p = 0.137$).

Post-hoc analysis using the Holm-Sidak method of pairwise multiple comparisons are reported as follows. In an analysis of time within Saline rats, mass did not significantly change between Pre- and Post-abstinence ($t = 0.178$, $p = 0.859$). In an analysis of time within METH rats, mass significantly increased Post-abstinence compared to Pre-abstinence ($t = 4.690$, $p < 0.001$). In an analysis of status within Pre-abstinence rats, mass was significantly higher in Saline rats compared to METH rats ($t = 6.469$, $p < 0.001$). In an analysis of status within Post-abstinence rats, mass was not significantly different between METH and Saline rats ($t = 1.634$, $p = 0.105$). In an analysis of time within rats treated with Vehicle, mass significantly increased Post-abstinence compared to Pre-abstinence ($t = 3.801$, $p < 0.001$). In an analysis of time within rats treated with Roflumilast, mass was not significantly different in rats treated with Roflumilast Pre- or Post-abstinence ($t = 0.652$, $p = 0.516$). In an analysis of

treatment Pre-abstinence, mass did not significantly differ between Vehicle and Roflumilast rats ($t = 0.532$, $p = 0.596$). In an analysis of treatment Post-abstinence, mass was significantly higher in rats treated with Vehicle compared to rats treated with Roflumilast ($t = 2.650$, $p = 0.009$).

Within rats that self-administered sucrose, a two-way repeated measures ANOVA was performed measuring the effect of treatment (Roflumilast vs Vehicle) and abstinence (Pre- vs Post-abstinence) on mass. The two-way repeated measures ANOVA revealed there was not a statistically significant interaction between treatment and abstinence ($F_{(1,14)} = 1.730$, $p = 0.209$). Simple main effects analysis showed that treatment did not have a statistically significant effect on mass ($F_{(1,14)} = 0.0192$, $p = 0.892$), and that time did not have a statistically significant effect on mass ($F_{(1,14)} = 0.0651$, $p = 0.802$).

Table 1

Average Weights of Rats by Treatment Group`				
	Pre-treatment Vehicle	Post-treatment Vehicle	Pre-treatment Roflumilast	Post-treatment Roflumilast
METH self-administration (n = 32, 16-17/group)	275.31 ± 2.98	302.31 ± 2.88	279.94 ± 3.63	290.18 ± 5.45
Yoked-saline (n= 32, 16/group)	303.69 ± 3.57	307.31 ± 4.24	303.31 ± 3.85	298.25 ± 4.57
Sucrose pellet self-administration (n = 16, 8/group)	316.50 ± 4.56	321.50 ± 6.96	319.63 ± 4.53	316.25 ± 6.95

Table 1) Average weights of rats in the experimental groups before and after abstinence and treatment. Data are presented as means ± SEM.

Roflumilast treatment during abstinence reduced relapse to METH seeking

Rats that self-administered METH and their yoked saline controls were sorted into treatment groups on Day 14 balanced according to their weight, total METH self-administered, active lever pressing, inactive lever pressing, and METH self-administered in the last three sessions. Saline animals were sorted into treatment groups based on weight, total saline administered, and active, inactive, and saline administered in the last three sessions (Figure 6a). A two-way ANOVA was performed to analyze the groups before abstinence and treatment using status (Saline vs METH) and treatment (Vehicle vs Roflumilast) as main factors. During 14 days of self-administration, METH self-administering rats pressed the active lever more than yoked saline control rats (METH, $M = 95.25 \pm 4.02$; Saline, $M = 10.41 \pm 4.02$; $F_{(1,38)} = 222.64$, $p < 0.001$). There were no differences in active lever presses in METH self-administering rats before abstinence and treatment with roflumilast or vehicle, or in yoked saline control rats before treatment with roflumilast or vehicle (Pre-treatment: Saline x Vehicle ($M = 10.82 \pm 5.55$) vs Saline x Roflumilast ($M = 10.00 \pm 5.82$), $t = 0.102$, $p = 0.919$; METH x Vehicle ($M = 93.40 \pm 5.82$) vs METH x Roflumilast ($M = 97.09 \pm 5.55$), $t = 0.459$, $p = 0.649$).

A two-way ANOVA was performed to assess active lever pressing after forced abstinence and treatment using the same factors, status (Saline vs METH), and treatment (Vehicle vs Roflumilast). The number of active lever presses depended on the treatment received during abstinence, as indicated by a significant two-way interaction between status and treatment ($F_{(1,38)} = 44.41$, $p < 0.001$), (Figure 6b). Main effect analysis of status (METH vs Saline) showed that rats that previously self-administered METH pressed the active lever more (METH, $M = 50.83 \pm 2.07$; Saline, $M = 12.53 \pm 2.07$; $F_{(1,38)} = 170.56$, $p < 0.001$). Main effect analysis of treatment showed roflumilast treatment during abstinence reduced active lever pressing (Vehicle, $M = 42.28 \pm 2.07$; Roflumilast, $M = 21.08 \pm 2.07$; $F_{(1,38)} = 52.29$, $p < 0.001$). Post-hoc analyses using the

Holm-Sidak method revealed that amongst rats that previously self-administered METH, roflumilast treatment significantly reduced active lever pressing compared to vehicle (METH x Vehicle, $M = 71.2 \pm 3.00$; METH x Roflumilast, $M = 30.46 \pm 2.86$; $t = 7.23$, $p < 0.001$). Within the yoked-saline control rats, active lever pressing did not differ between vehicle or roflumilast (Saline x Vehicle, $M = 13.36 \pm 2.86$; Saline x Roflumilast, $M = 11.70 \pm 3.00$; $t = 0.401$, $p = 0.691$). Within rats treated with vehicle, post-hoc analysis revealed that rats that previously self-administered METH pressed the active lever more than saline control rats ($t = 13.947$, $p < 0.001$). Within rats treated with roflumilast, post-hoc analysis revealed that rats that previously self-administered METH pressed more than saline control rats ($t = 4.52$, $p < 0.001$).

To study the pattern of active lever pressing during the METH seeking test, the time course of active lever pressing during the relapse test was analyzed using a two-way repeated measures ANOVA, with treatment (Vehicle vs Roflumilast) and time (each 15 min time point) as factors (Figure 6c). Analysis was restricted to rats that previously self-administered METH. There was a significant interaction between treatment and time ($F_{(23,437)} = 8.64$, $p < 0.001$), with a main effects analysis demonstrating that lever pressing decreased over time ($F_{(23,437)} = 48.43$, $p < 0.001$), and roflumilast treatment reduced active lever pressing ($F_{(1,437)} = 58.84$, $p < 0.001$). Rats treated with roflumilast pressed the active lever less compared to vehicle (Vehicle, $M = 2.967 \pm 0.16$; Roflumilast, $M = 1.27 \pm 0.15$; $t = 7.67$, $p < 0.001$). Post-hoc analysis using the Holm-Sidak method comparing roflumilast and vehicle treatment revealed that most statistically significant differences between groups occurred within the first 150 minutes (Vehicle vs Roflumilast at 150 min, $t = 3.37$, $p < 0.001$, $p > 0.05$ for all later time points). Within vehicle rats, the rate of active lever pressing declined to a constant rate not significantly different in subsequent time points by 90 minutes. The roflumilast group declined to a constant rate by 45 minutes.

Figure 6

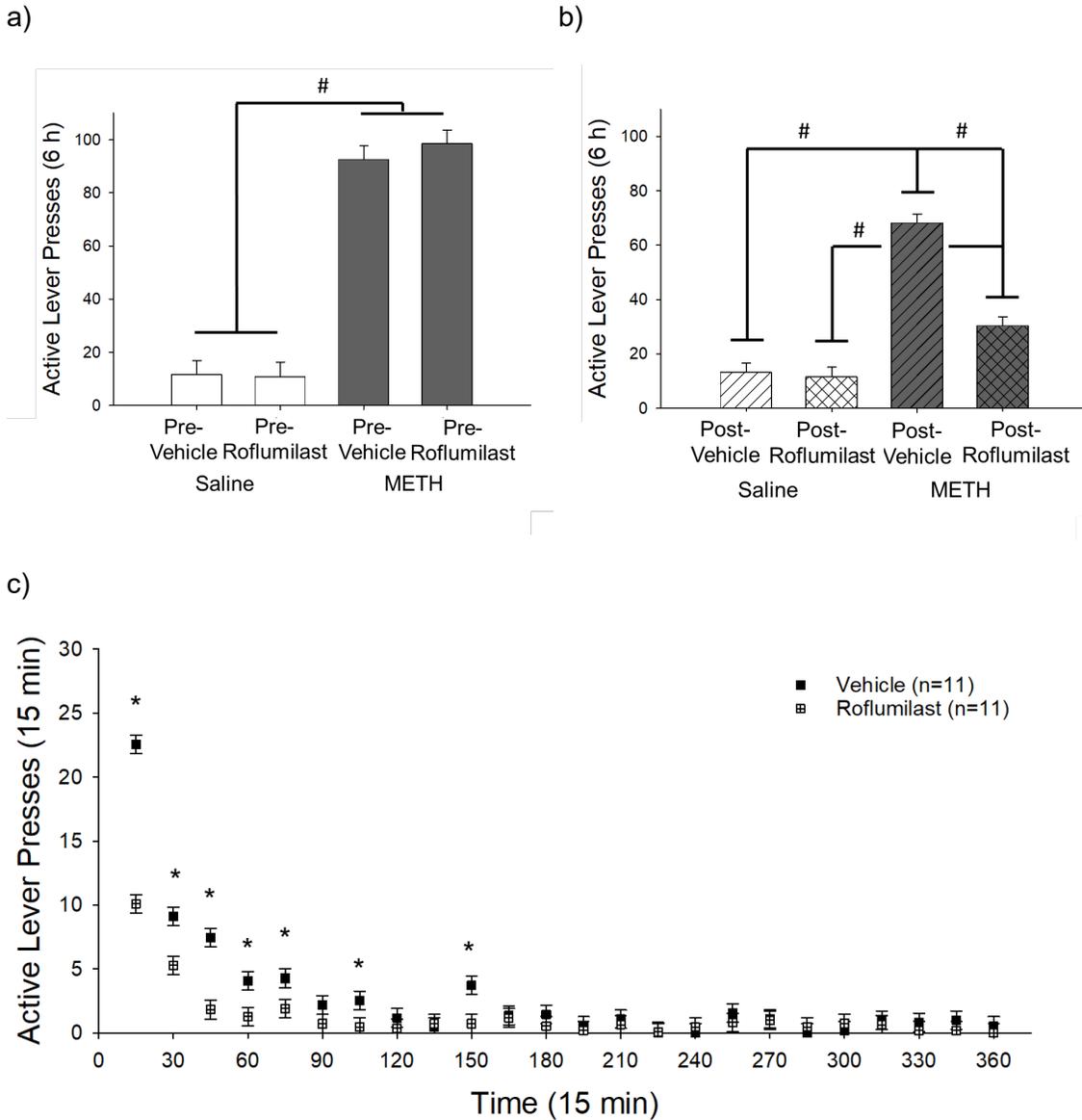


Figure 6: Roflumilast reduces relapse to METH seeking behavior. (a) Average active lever pressing on Day 14 before abstinence and treatment analyzed by two-way ANOVA (#, $p < 0.001$). (b) Active lever pressing during the METH seeking test when active lever presses were not rewarded with METH analyzed by two-way ANOVA (#, $p < 0.001$). (c) Time course of active lever pressing in the METH seeking relapse test using a two-way repeated measures ANOVA. (*, $p < 0.05$ compared to vehicle). $n=10-11$ /group.

Roflumilast treatment during abstinence reduced relapse to METH taking

Rats that had self-administered METH and yoked saline controls were sorted into two groups that were balanced according to their average METH self-administration on Day 14, further described in the Methods (Figure 7a). A two-way repeated measures ANOVA was performed to analyze the effect of roflumilast treatment and abstinence on METH self-administration using treatment (Vehicle vs Roflumilast) and abstinence (Pre- vs Post-abstinence) as main factors. There was a significant interaction between treatment and abstinence on METH Intake ($F_{(1,9)} = 12.503$, $p = 0.006$). A main effects analysis demonstrated that abstinence had a significant effect on METH intake ($F_{(1,9)} = 136.383$, $p < 0.001$), and that treatment itself did not have a significant effect on METH intake ($F_{(1,9)} = 1.695$, $p = 0.225$). Post-hoc analyses using the Holm-Sidak method demonstrated there were no significant differences in METH self-administration between groups before treatment with roflumilast or vehicle (Pre-Vehicle, $M = 7.77 \pm 0.62$; Pre-Roflumilast, $M = 7.77 \pm 0.56$, $t = 0.007$, $p = 0.995$). After 7 days of forced abstinence and treatment roflumilast decreased relapse-like behaviors to METH self-administration compared to vehicle (Post-Vehicle, $M = 5.40 \pm 0.62$; Post-Roflumilast, $M = 3.35 \pm 0.56$; $t = 2.449$, $p = 0.032$). Abstinence itself was associated with a statistically significant decrease in METH intake between Pre- and Post-abstinence values in both vehicle ($t = 5.512$, $p < 0.001$) and roflumilast groups ($t = 11.283$, $p < 0.001$).

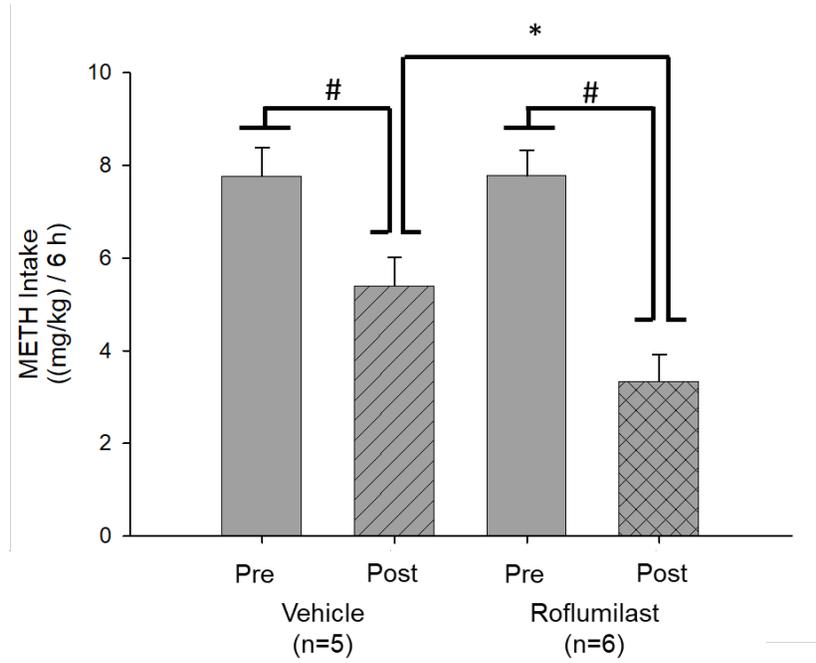
The within session time course of METH intake during the METH relapse test after 7 days of forced abstinence was analyzed using a two-way repeated measures ANOVA with treatment (Vehicle vs Roflumilast) and time (15 min time points to 360 min) as main factors (Figure 7b). METH intake was determined by a significant interaction between treatment and time ($F_{(23,207)} = 1.793$, $p = 0.018$). A simple main effects analysis revealed a significant effect due to both treatment (Vehicle vs Roflumilast, $F_{(1,207)} = 5.836$, $p = 0.039$) and time ($F_{(23,207)} = 9.470$, $p < 0.001$). Post-hoc analyses using the

Holm-Sidak method revealed that that roflumilast reduced METH intake relative to vehicle at the 15 minute time point (Vehicle at 15 min, $M = 1.020 \pm 0.0675$; Roflumilast at 15 min, $M = 0.533 \pm 0.616$, $t = 5.329$, $p < 0.001$) and the 105 min time point (Vehicle at 105, $M = 0.360 \pm 0.675$; Roflumilast at 105 min, $M = 0.083 \pm 0.0616$, $t = 3.029$, $p = 0.003$). Within all groups, rats declined to constant rates of METH self-administration by 30 minutes, the time point after which METH intake did not differ significantly between subsequent time points (15 vs 105 min, $t = 7.346$, $p < 0.001$)

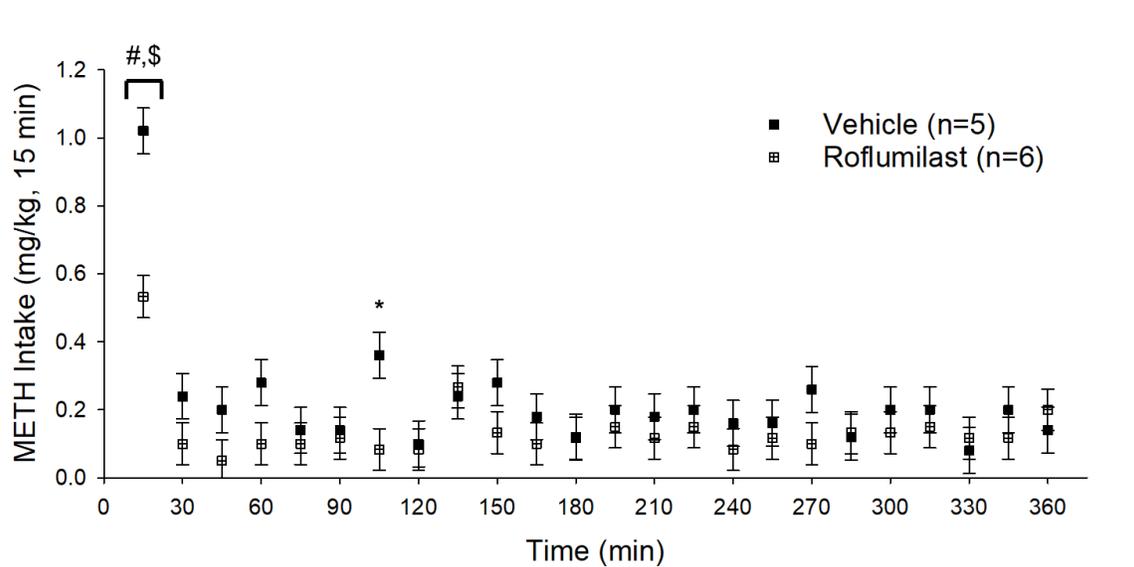
To better analyze differences in the basal rate of METH intake, nonlinear regression on the cumulative METH intake against time was performed for the METH Intake on for rats treated with vehicle or roflumilast that underwent the METH taking test (Figure 7c) (Vehicle: $R^2 = 0.559$, $SEE = 0.148$; Roflumilast: $R^2 = 0.246$, $SEE = 0.147$). The maintenance dose of METH per 15 min, here defined as y_0 , was similar between the rats treated with vehicle and roflumilast (Vehicle: $y_0 = 0.201$ (mg/kg)/15min, $SE = 0.025$) (Roflumilast: $y_0 = 0.167$ (mg/kg)/15min, $SE = 0.023$).

Figure 7

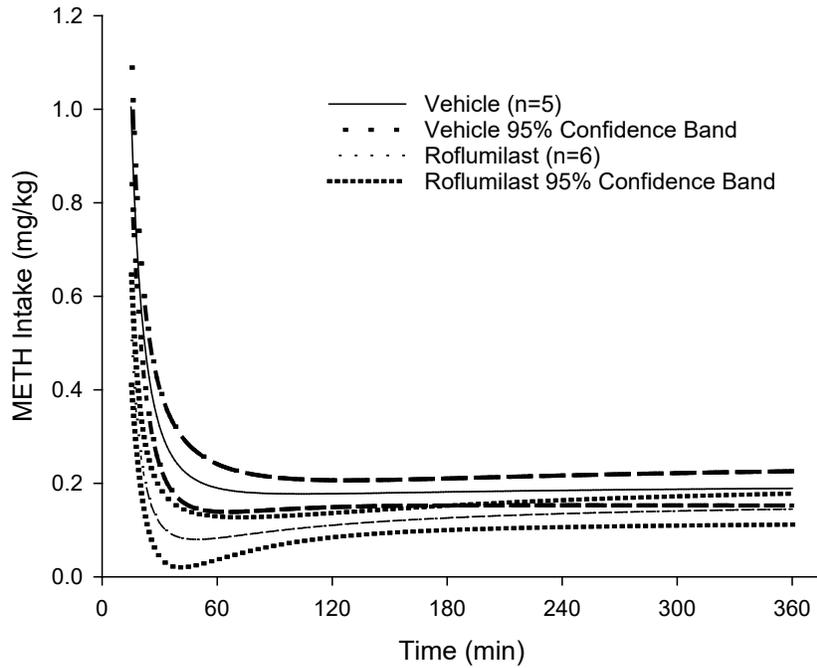
a)



b)



c)



Coefficients of the Regression Line $f = y_0 + (a / x) + (b / x^2)$			
Coefficient (SE)	Coefficient, y_0 (mg/kg)	Coefficient, a (mg/kg * min)	Coefficient, b (mg/kg * min)
Vehicle $R^2 = 0.559$ SEE = 0.148	0.201 (0.025) ($p < 0.0001$)	-4.876 (3.383) ($p = 0.152$)	254.004 (51.639) ($p < 0.0001$)
Roflumilast $R^2 = 0.246$ SEE = 0.147	0.167 (0.023) ($p < 0.001$)	-8.537 (3.065) ($p = 0.0061$)	209.473 (36.761) ($p < 0.0001$)

Figure 7: Roflumilast reduces relapse to METH taking behavior. (a) Average METH intake compared before and after abstinence, and roflumilast or vehicle treatment (*, $p < 0.05$; #, $p < 0.001$). (b) METH intake during the METH taking relapse test, where lever pressing was rewarded with METH (#, $p < 0.001$, compared to all other timepoints; \$, $p < 0.001$, comparing vehicle and roflumilast treatment; *, $p < 0.05$, comparing vehicle and roflumilast treatment). (c) Non-linear regression of METH intake during the METH taking relapse test with fitment according to a second order inverse polynomial function, and corresponding coefficients. $n=5-6$ /group.

Roflumilast during abstinence did not affect resumption of sucrose self-administration

To investigate whether the reduction in self-administrative behaviors was specific to METH, a sucrose self-administration paradigm was utilized (Figure 4b). Like the METH self-administration paradigm, a one way repeated measures ANOVA revealed that rats increased sucrose pellet self-administration during the 14 days of access to sucrose pellets (Figure 8a). Sucrose pellet intake increased across days ($F_{(13, 195)} = 26.613$, $p < 0.001$) such that rats escalated their intake of sucrose pellets from Day 1 values by Day 6 (Day 1, $M = 16.81 \pm 2.62$; Day 6, $M = 52.75 \pm 11.10$; $t = 3.51$, $p = 0.026$). Sucrose pellet intake plateaued by Day 6, the earliest day where differences from Day 14 are not statistically significant.

Rats that self-administered sucrose pellets were sorted into two groups balanced according to their sucrose pellet self-administration on Day 14 (Figure 8b). A two way repeated measures ANOVA was performed to analyze the effect of roflumilast treatment and abstinence on sucrose self-administration using treatment (Vehicle vs Roflumilast) and abstinence (Pre- vs Post- Abstinence) as main factors. There was no significant interaction on sucrose pellet intake due to treatment or abstinence ($F_{(1, 14)} = 1.123$, $p = 0.307$). Main effects analysis did not observe a difference due to abstinence ($F_{(1, 14)} = 0.0213$, $p = 0.886$), or due to treatment ($F_{(1, 14)} = 0.496$, $p = 0.493$)

An analysis of the within session time course of sucrose self-administration after 7 days of forced abstinence and treatment was performed (Figure 8c). Rates of sucrose pellet self-administration declined within the session ($F_{(23, 322)} = 7.71$, $p < 0.001$), with no significant effect of roflumilast treatment ($F_{(1, 322)} = 0.996$, $p = 0.335$), and no significant interaction between time and treatment ($F_{(23, 322)} = 0.987$, $p = 0.482$). A post-hoc analysis using the Holm-Sidak method between time points revealed that by 60 min, sucrose intake did not differ significantly between subsequent time points (Time 60 vs 75 min, $t = 0.940$, $p = 1.00$).

Figure 8

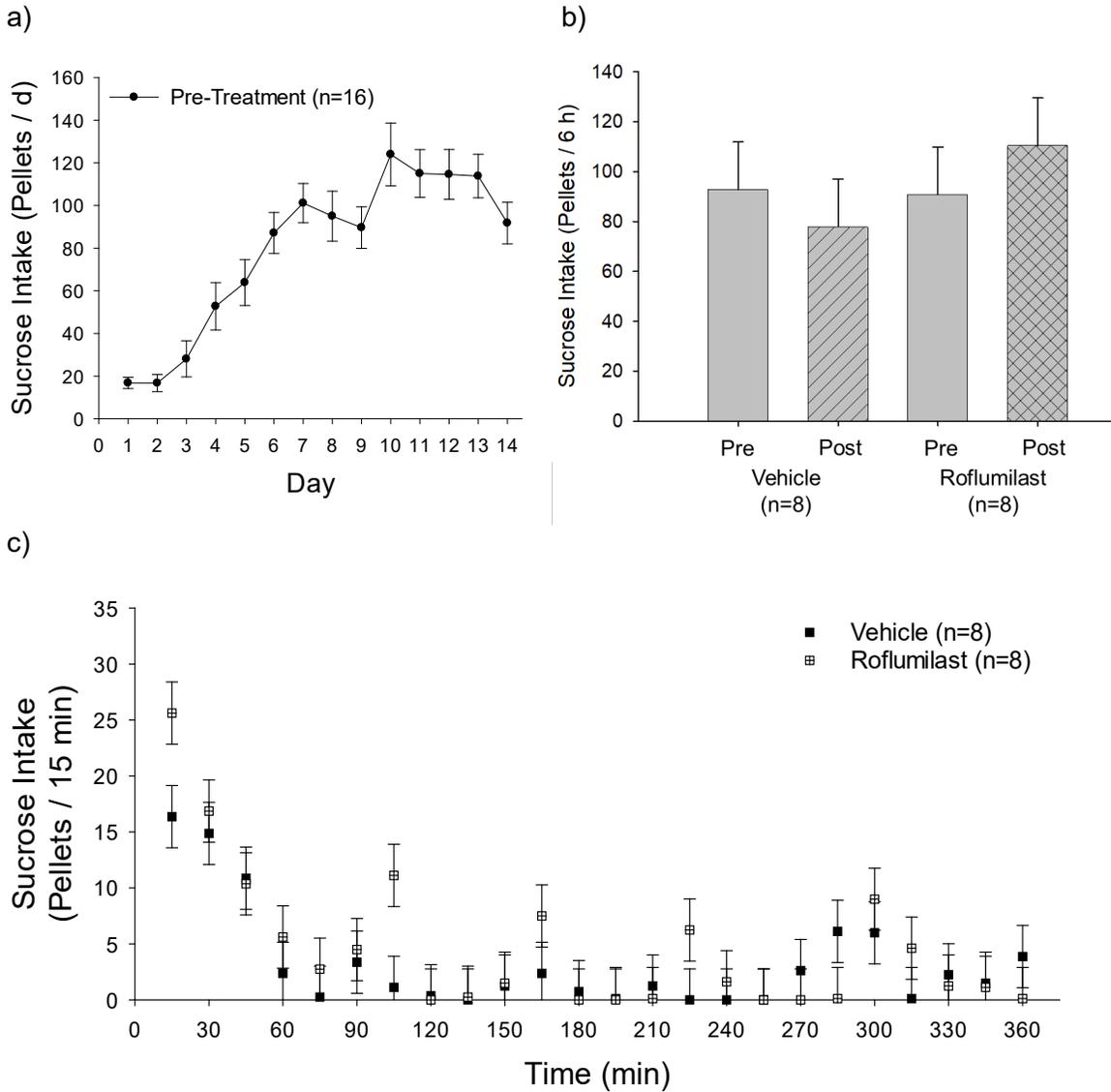


Figure 8: Roflumilast did not affect resumption of sucrose self-administration. (a) Sucrose pellet self-administration on Days 1-14. (b) Sucrose pellet self-administration on Day 14 and after abstinence and treatment. (c) Time course of sucrose pellet self-administration after forced abstinence and treatment. n=8/group.

Discussion:

The PDE4 inhibitor, roflumilast, was administered during forced abstinence from METH self-administration to determine its effect on relapse to METH. Treatment with roflumilast during the abstinence from METH reduced both METH seeking and METH taking behaviors. In rats allowed to self-administer sucrose pellets, roflumilast treatment during abstinence did not affect the resumption of sucrose pellet self-administration.

Limitations of this study include not using yoked-METH rats, which may have revealed lever pressing that occurs due to the motor stimulating effects of METH, and whether this would affect non-contingent active lever pressing, revealing differences between the active self-administration of the drug or the effects of passive drug experience (E. H. Jacobs et al. 2003). Given that previous pre-clinical METH self-administration studies have uncovered differences in the neural changes associated with active or passive METH self-administration (Stefanski et al. 1999), further studies investigating the neurobiology of roflumilast's effect on METH self-administration utilizing this control may be required. However, as roflumilast did not affect the active lever pressing in yoked saline-administered control animals, nor the sucrose self-administered in sucrose self-administering animals, this would suggest that the effect of roflumilast is not due to the depression of motor behavior. Another limitation of the study is that only a single dose of roflumilast was evaluated. This dose was chosen based on pilot studies demonstrating that this dose of roflumilast did not reduce weights in rats, a concern due to the potential for roflumilast to induce the side-effect of gastrointestinal distress and nausea (Spina 2008). However, a dose-response of roflumilast on relapse-like behaviors may have revealed additional information such as whether there is a minimum effective dose or a ceiling effect.

Additionally, it should be noted that all the studies contained within this work describe the effects of a single dose of METH as well. For rats, the number of self-

administered METH infusions within a set-time against the concentration of METH is a linear dose-effect function, with lower doses of METH (0.05 mg/kg/infusion) producing higher infusion numbers than higher doses of METH (0.2 mg/kg/infusion) (Kitamura et al. 2006). However, all groups self-administered approximately 9 mg/kg METH per session, suggesting that alternative dosing would have produced similar effects.

To demonstrate that the reduction in relapse-like behaviors was not due to adverse effects of roflumilast, mass and the resumption of sucrose pellet self-administration were measured after treatment. With regards to mass, the primary changes were that rats that self-administered METH had less mass than yoked-saline control rats, but quickly gained mass to match yoked-saline rats during abstinence. However, rats treated with roflumilast did not gain as much mass as vehicle controls, and generally rats treated with vehicle had more mass than those treated with roflumilast. Within rats that self-administered sucrose, there was not a significant difference in mass between rats that were treated with either vehicle or roflumilast. Given that rats treated with roflumilast during forced abstinence did not reduce their resumption of sucrose pellet self-administration (Figure 8b, c), this suggests that the effects of roflumilast on METH seeking are not due to nausea and gastrointestinal distress that has been reported to accompany PDE4 inhibition (Spina 2008; Beghe, Rabe, and Fabbri 2013). These findings also illustrate that roflumilast did not adversely affect motivation nor the consumption of natural rewards (Papp, Willner, and Muscat 1991; Der-Avakian and Markou 2012; Donahue et al. 2014) and suggests that roflumilast acted on mechanisms specific for METH self-administration.

Consistent with previous studies using rat models of METH self-administration with extended access (6 hour), rats increased their METH intake from their initial exposure on day 1 (Shepard et al. 2004; Kitamura et al. 2006). As an escalation of

intake is one of the criterion features of METH use disorder, this behavioral pattern would suggest that this experimental paradigm may be a good preclinical model for METH use disorder (*Diagnostic and Statistical Manual of Mental Disorders : DSM-5*. 2013). The analysis of the time course of METH self-administration (Figure 5c) revealed that the escalation in METH self-administration was attributed to changes in two phases within each session: an increased initial loading dose phase within the first 15 minutes (Yokel and Pickens 1973; Yokel and Piekens 1974; Lynch and Carroll 2001), and an increased maintenance dose in the remainder of the session (Tsibulsky and Norman 1999; Ahmed and Koob 2005) (Figure 5d). These observations are consistent with previous studies and support the face validity of the approach in modeling of behaviors associated with METH use disorder, and for the testing potential treatments (Grimm et al. 2001; Kitamura et al. 2006; Panlilio and Goldberg 2007).

A treatment for METH use disorder that would be most clinically relevant would be used to treat patients with established METH self-administration behaviors, but are in a period of abstinence from METH. Thus, we implemented our model with these considerations (Shoptaw et al. 2009; Chan et al. 2019). We utilized forced abstinence to best model the behavioral effects of roflumilast on the relapse to METH and avoid potential confounds due to training of extinction itself, as well as for its face value in modeling human relapse (Katz and Higgins 2003; Fuchs, Branham, and See 2006; Pelloux et al. 2018). Using this relapse model, we found that rats with established METH self-administration behaviors could be treated with roflumilast during the forced abstinence from METH to reduce relapse behaviors (Figure 6b, 7a). In the METH seeking test, when active lever presses were not reinforced with METH, roflumilast treatment greatly reduced active lever pressing under extinction conditions (Figure 6c, 6d) (Millan, Marchant, and McNally 2011). Unfortunately, though stressors have been known to precipitate relapse in humans (Koob and Mason 2016) and in pre-clinical

models (Shepard et al. 2004; Mahler et al. 2012). Interestingly, anxiolytics commonly used to treat stress-related disorders such as clonidine or selective-serotonin reuptake inhibitors have not been shown to be effective in reducing relapse (Torrens et al. 2005). Given that stress-induced relapse was not tested in this context, and it remains to be explored whether roflumilast acts to reduce relapse-like behaviors if triggered by stress rather than only environmental cues.

In the METH taking test, where active lever presses were rewarded with METH (Figure 7a), it was shown that 7 days of abstinence reduced METH intake (Figure 7a). Previous literature showed that the steady rate of METH self-administered by rats correlated to maintaining concentrations of METH found in the brain (Yokel and Pickens 1973; Yokel and Pickens 1974). Furthermore, the interruption of dopamine signaling, not norepinephrine or serotonin, affected the rate of METH self-administration (Yokel and Wise 1976; Roberts, Corcoran, and Fibiger 1977). Results from these past experiments suggest roflumilast may also act to reduce METH intake behaviors by altering the pharmacodynamics or pharmacokinetics of METH (Lynch and Carroll 2001; Ahmed and Koob 2005; Siciliano and Jones 2017), potentially by influencing neurotransmission related to dopamine. Therefore, in the next chapter, the effect of roflumilast on proteins related to dopamine neurotransmission will be explored.

In the METH seeking test, where active lever presses were not rewarded with METH (Figure 6), roflumilast reduced unreinforced METH seeking behaviors after 7 days of abstinence. In a METH self-administration study where relapse-like behavior was assessed under similar extinction conditions after 7 days of forced abstinence, relapse-like behaviors was mediated by the accumulation of calcium-permeable AMPA receptors in the nucleus accumbens (Scheyer et al. 2016). Additionally, previous studies showed that glutamate neurotransmission in the nucleus accumbens is necessary for the reinstatement of METH seeking behaviors (Rocha and Kalivas 2010; Parsegian and See

2014). Therefore, in the next chapter, the effect of roflumilast on calcium-permeable GluA1-type AMPA receptors will be explored.

Additionally, previous studies found that ibudilast, a non-specific phosphodiesterase inhibitor with anti-inflammatory action reduced METH-induced locomotor activity and sensitization in mice (Snider et al. 2012) and METH self-administration behavior in rats (Snider, Hendrick, and Beardsley 2013). Additionally, it was shown that AV1103, a pharmacological analogue of ibudilast that possesses anti-inflammatory activity but negligible PDE inhibitory properties had the same effects as ibudilast (Y. Cho et al. 2010). This suggests inflammation itself may mediate the expression of some METH-induced behaviors. Evidence supporting this includes the findings that human METH users in extended abstinence appear to have immune systems that are persistently dysregulated as measured by increased activated brain microglia (Sekine et al. 2008), and increased levels of central and serum pro-inflammatory cytokines (Loftis et al. 2011; Gonçalves et al. 2017; Blaker, Rodriguez, and Yamamoto 2019; M. J. Li et al. 2020). Roflumilast reduces the stimulated release of cytokines in a variety of immune cells (Bundschuh et al. 2001; Kwak et al. 2005; Grootendorst et al. 2007), and this is the basis for its use to treat chronic obstructive pulmonary disorder (Martorana et al. 2005; Gross, Gienbycz, and Rennard 2010; Martinez et al. 2015). As pro-inflammatory cytokines including $TNF\alpha$, $IL-1\beta$, and $IL-6$ can also influence the firing properties of neurons (Bellinger, Madamba, and Siggins 1993; Beattie et al. 2002; Pribrig and Stellwagen 2014), the action of METH and roflumilast on immune dysregulation will be explored as well.

Chapter 3: Preliminary studies suggest abstinence from METH self-administration or treatment with roflumilast during abstinence are not associated with changes in the expression of Tyrosine Hydroxylase, Synaptosomal Dopamine Transporter or Surface GluA1 AMPA Receptors in the Nucleus Accumbens

Introduction

In the last chapter, we found that roflumilast reduced relapse-like behaviors to METH seeking and METH taking. Previous studies suggested that glutamate and its receptors in the nucleus accumbens may be a neural correlate for stimulant seeking behavior after abstinence (Rocha and Kalivas 2010; Scheyer et al. 2016). In a rat model of cocaine self-administration, the duration of forced abstinence correlated with increases in calcium-permeable GluA1 AMPA receptor subunits in the nucleus accumbens and inhibition of these accumulated AMPA receptors reduced cocaine seeking behaviors (Conrad et al. 2008). Moreover, whole-cell patch clamp studies suggested that increases in calcium-permeable AMPA receptors after 7 days of forced abstinence from METH in neurons of the nucleus accumbens mediate METH seeking behavior after 7 days of abstinence (Scheyer et al. 2016). We tested the hypothesis that METH seeking behavior was mediated by the accumulation of AMPA receptors at the plasma membrane of neurons in the nucleus accumbens, and that this increase in GluA1 was blocked by roflumilast.

As described previously, roflumilast reduced METH intake, suggesting roflumilast affected the pharmacodynamics of METH-induced dopamine. Systemic administration of PDE4 inhibitors has also shown to inhibit the self-administration of various drugs of abuse, including cocaine (Thompson et al., 2004; Knapp et al., 1999), alcohol (Wen et al., 2012), and heroin (Lai et al. 2014). It was observed that the rate of psychostimulant self-administration maintains stable dopamine levels the nucleus accumbens (Wise et

al., 1995; Weiss et al., 2000). Interestingly, animals will also respond for infusions of stimulants into the nucleus accumbens, and will increase their rate of drug self-administration with adulteration of the infusate with the dopamine D1-type receptor antagonist, SCH-23390 (Yokel and Wise 1976; Phillips, Robbins, and Everitt 1994), or systemic administration of the D1-type receptor antagonist (Koob, Le, and Creese 1987). Experiments targeting D2-type receptors appear to be mixed as the administration of both agonists (Rowlett et al. 2007) and antagonists of D2-type receptors can increase rates of stimulant self-administration (Koob, Le, and Creese 1987; Phillips, Robbins, and Everitt 1994; Caine et al. 2002). More recently, optogenetic-based activation of the ventral tegmental area inducing dopamine release has been shown to be sufficient to train self-stimulation behaviors in mice (Pascoli et al. 2015). Within these mice, D1R-expressing neurons in the nucleus accumbens had increased AMPA/NMDA receptor current ratio. A similar change was not observed in D2R-expressing nucleus accumbens neurons. Given that this optogenetic model recapitulates changes associated with drugs of abuse, together this would suggest that psychostimulants selectively potentiate glutamatergic signaling in D1R-expressing neurons of the nucleus accumbens (Nestler and Lüscher 2019; Wright and Dong 2020).

It is known that dopamine D1-type receptor signaling is mediated through effects on adenylyl cyclase and the synthesis of cAMP (Beaulieu, Espinoza, and Gainetdinov 2015). Additionally, it was observed that dopamine-induced cAMP production is impaired in the nucleus accumbens of chronic METH users (Tong et al. 2003). Interestingly, modulation of cAMP or its breakdown by PDE4 in the nucleus accumbens can bidirectionally modulate cocaine self-administration (Self et al. 1998; Knapp et al. 1999). These effects are correlated with changes in cAMP-mediated protein kinase A – cyclic AMP response element binding protein (CREB) signaling, which is known to mediate long-term adaptations to dopaminergic signaling (Carlezon et al. 1998; Hollander et al.

2010). The phosphorylation of CREB in the nucleus accumbens has been shown to be decreased in rats trained to self-administer heroin after abstinence, and PDE4 inhibition has been associated with both reducing relapse-like behaviors to heroin seeking and rescuing decreases in phosphorylated CREB (Sun et al. 2015). Together, as cAMP levels affect the rate of METH self-administration, and that modulating cAMP levels using PDE inhibitors could alter drug self-administration, CREB may function as a molecular readout of cAMP signaling and also drive long-term adaptations in response to cAMP signaling.

Chronic METH use is associated with a hypodopaminergic state associated with a loss of dopamine transporter (DAT), tyrosine hydroxylase (TH), and the stimulated induction of cAMP in the striatum, putatively through the loss of dopaminergic terminals due to METH toxicity (Tong et al. 2003; Broom and Yamamoto 2005; O'Neil et al. 2006; Krasnova et al. 2010; Mcfadden et al. 2012). Treatment strategies for another hypodopaminergic disorder, Parkinson's Disease, revolves around dopamine replacement using dopamine precursors to replete dopamine lost from the nigrostriatal pathway using L-DOPA, a dopamine precursor (Callaghan et al. 2012). Interestingly, inhibition of PDE4 in striatal neurons activates tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of dopamine, activates cAMP response element binding protein (CREB) to increase the synthesis and turnover of dopamine (Nishi et al. 2008; Heckman et al. 2018). Accordingly, PDE4 inhibition is currently being investigated as a potential treatment strategy for Parkinson's Disorder as well (Nthenge-Ngumbau and Mohanakumar 2017).

Given that inflammation is a key mediator of METH-induced toxicity (Halpin, Collins, and Yamamoto 2014), and that roflumilast is an anti-inflammatory (Kwak et al. 2005; Spina 2008), we hypothesized that DAT, TH and CREB are downregulated by METH self-administration and abstinence and that these deficits induced by METH self-

administration are rescued by roflumilast. Additionally, we hypothesized that PDE4 inhibition would increase phosphorylated CREB and phosphorylated TH, which may contribute to the reduction of drug-taking behaviors (Figure 9).

Figure 9

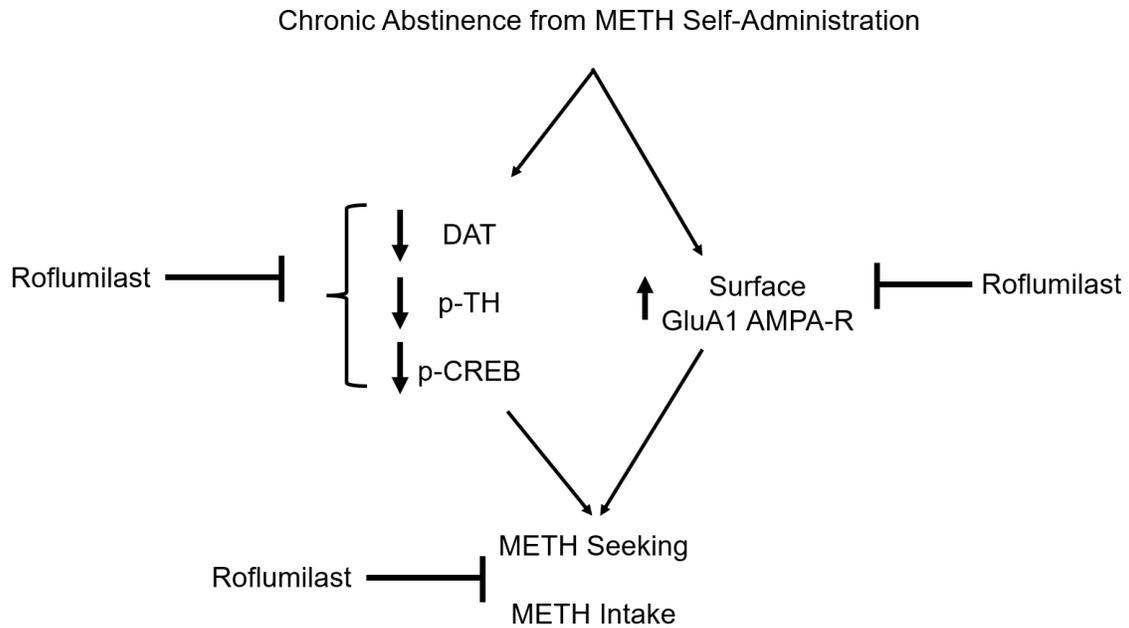


Figure 9: Diagram of hypotheses on how METH and roflumilast affect dopaminergic and glutamatergic proteins in the nucleus accumbens. As chronic METH use is associated with a hypodopaminergic state drives METH taking behaviors, we hypothesized that there would be decreases in DAT, TH, and phosphorylated CREB in the nucleus accumbens. Given that roflumilast reduces relapse to METH taking behaviors, we hypothesize that roflumilast would prevent these changes. Furthermore, as PDE4 inhibition is associated with increased cAMP and has been shown to increase phosphorylation of TH to increase dopamine synthesis, we hypothesized that phosphorylated TH and phosphorylated CREB would be increased in the nucleus accumbens. Additionally, as abstinence from METH self-administration has been

associated with an increase in GluA1-type AMPA receptors that mediate relapse-like behaviors to METH seeking, and roflumilast reduced METH seeking behaviors, we hypothesized that METH would increase surface expression of GluA1 AMPA receptors at the nucleus accumbens, and that roflumilast would block this increase.

Materials and Methods:

Subjects

Male Sprague-Dawley rats (225-250 g, Envigo, Indianapolis, IN) were used in all experiments. Rats were housed 2 per cage in a temperature and humidity controlled facility under a 12 hour light/dark cycle (7:00 AM – 7:00 PM) with a minimum 4 day acclimation period before any experimentation. Food and water were available ad libitum throughout the experiment. Experiments and surgical procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and approved by Indiana University Institutional Animal Care and Use Committee.

Rats underwent 14 days of METH self-administration followed by 7 days of forced abstinence, treatment with roflumilast or vehicle, and relapse test as described in the previous chapter. Following the relapse test, rats were decapitated and their brains were extracted. An ice-cold brain matrix (ASI Instruments, Cat. RBM—4000S) was used to obtain coronal slices. Thin razor blades were inserted at the level of the optic chiasm, with two more blades inserted every 2 mm increments in the caudal direction to yield 2 contiguous coronal slices that were 2 mm thick. Nucleus accumbens was dissected from the face corresponding to Bregma +0.5 mm using the anterior commissure as the landmark (Paxinos et al. 1997).

Biotinylation to measure levels of GluA1 at the plasma membrane surface

Plasma membrane surface proteins of the nucleus accumbens were isolated using a biotin-based protocol as performed in (Conrad et al. 2008; Murray et al. 2019). Freshly dissected nucleus accumbens was incubated in 1 mL of artificial cerebral spinal fluid spiked with 1 mM Sulfo-NHS-S-S-biotin (Pierce, Cat. 21331) for 30 min at 4°C with agitation to covalently bind proteins with an exposed extracellular amine group, and the reaction was quenched with 100 µl of 1M glycine. Samples were then pelleted by spinning at 20,000 RCF at 4°C for 2 min and the supernatant was discarded. The pellet was reconstituted in lysis buffer (25mM HEPES pH 7.4, 500mM NaCl, 2mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 20mM NaF, 0.1% IGEPAL (v/v), and 1X protease and phosphatase inhibitor cocktail (ThermoFisher, CAT. 78442) and homogenized by sonication twice for 5 s each on ice. The tissue homogenate was again pelleted at 20,000 RCF at 4°C for 2 min, and the supernatant was used for analysis. Total protein content was quantified using the Bradford protein assay (BioRad, Cat. 500-0006).

High Capacity NeutrAvidin Agarose Resin (Thermo Sci, Cat. 29204) was prepared using 3 cycles of PBS washes, where 5 volumes of PBS were added to the resin, vortexed into suspension, then pelleted by centrifugation at 3,000 RPM for 1 min, and the supernatant completely decanted and discarded. To separate intracellular and plasma membrane surface protein, an aliquot of the biotinylated sample was incubated in prepared NeutrAvidin Agarose resin overnight at 4°C. The sample-resin suspension was then pelleted at 3,000 RPM for 1 min, and the supernatant was carefully decanted and incubated again with fresh resin under the same conditions. After a second round of incubation and pelleting, the supernatant was carefully decanted and was combined with an equal volume of 2x Laemmli Sample buffer (Bio-Rad, Cat. 161-0737) containing 200mM DTT. This supernatant composed of unbiotinylated protein, represented the intracellular fraction. The biotin-tagged surface proteins bound to the agarose-resin were

released by an incubation with 2X Laemmli Sample Buffer containing 200mM DTT in a volume equal to the input tissue volume. Finally, a separate aliquot of the biotinylated sample was combined with an equal volume of 2X Laemmli Sample Buffer containing 200mM DTT to release biotinylated protein without separation, and this represented the total fraction. All fractions were heated at 97°C for 3 min. After heating, surface proteins were isolated from the NeutrAvidin resin using a centrifugal filter unit 0.45 um (Millipore Cat. UFC30HV00) spun at 10,000 RPM, for 5 min. This supernatant, which represented the surface fraction, was decanted then combined for each subject.

For the analysis of the surface expression of GluA1 AMPA receptors only, equal volumes of the intracellular, surface, and total fractions were analyzed with Western Blot. Equal volumes were used as they correlated with fraction representing the proteins present in each of the plasma membrane, intracellular compartments, with the total tissue fraction used as a comparison for the total protein in both fractions. Each gel was loaded with 10 ug of protein from the total fraction, and with all represented experimental groups to allow for comparison across conditions. Relative optical density units for intracellular or surface fractions were normalized to the subject's total fraction. Total fractions were corrected using the Ponceau method for total protein visualization to account for differences in loading as described in (Conrad et al. 2008).

Ponceau Stain for normalization of biotinylated proteins

Immediately after Western blot transfer, blots were incubated in 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid for 45 minutes with agitation. All incubations and washes were performed at room temperature, and consistent timing between blots was essential for proper comparison. Blots were then washed 2 times with ddH₂O for 2 minutes before imaging using the LAS-4000 Image Analyzer System (FujiFilm) to digitize and quantify the optical density of the protein within each well. To de-stain the blot, blots

were washed 2 times for 1 min in ddH₂O, followed by 2 washes in 0.5% TBS-T for 3 minutes. Blots then proceeded to blocking. Of note, Ponceau stain for normalization of protein loading was used only with blots measuring the surface expression of GluA1 AMPA receptors, and the stain was applied immediately after Western blot transfer in the manner described in Conrad et al., 2008 (Conrad et al. 2008).

Synaptosomal preparation to measure DAT

Synaptosomal fractions were prepared from the dissected brain region via differential centrifugation separation as described previously (Riddle et al. 2002). Tissues were homogenized in ice-cold 0.32 M sucrose in a glass vessel with a smooth Teflon pestle and pelleted at 800 RCF for 12 min at 4°C. The supernatant (S1) was removed and centrifuged at 22,000 RCF for 17 min then 4°C. The resulting pellet (P2) yielded the synaptosomal fraction, which was resuspended in ice cold diH₂O. Protein concentration in the fraction was then quantified using the Bradford protein assay before Western blot.

Tissue homogenization to measure TH and CREB

Brain tissue for protein analyses was dissected from brains on the day of relapse. Brain regions were homogenized by sonication in lysis buffer (25mM HEPES pH 7.4, 500mM NaCl, 2mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 20mM NaF, 0.1% IGEPAL (v/v), and 1X protease and phosphatase inhibitor cocktail (ThermoFisher, CAT. 78442) and twice for 5 s each on ice. The tissue homogenate was spun down at 20,000 RCF at 4 C for 2 min and the supernatant was used for analysis. Total protein content was quantified using the Bradford assay before Western blot.

Western Blot

Samples were loaded onto a 4-12% Bis-Tris NuPage Gel (ThermoFisher, Cat. NP0336) and were run in a MOPS running buffer at 150 V for 90 min. Proteins were transferred onto a PVDF membrane at 27 V for 120 min. Membranes were blocked in 5% non-fat milk in 0.5% TBS-T at room temperature for 1 hour before being incubated in primary antibody overnight at 4 °C. The next day, membranes were washed 4 × 5 min in 0.5% Tris-buffered saline with Tween 20 (0.5% TBS-T), incubated at room temperature for 1 hour with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Membranes were again washed 4 × 5 min in 0.5% TBST and incubated for 2 min in chemiluminescence solution to allow visualization of antigen-antibody complexes (ThermoFisher, Cat. 34580). An LAS-4000 Image Analyzer System (FujiFilm) was used to image and quantify the optical density of proteins of interest. Membranes were then incubated in stripping buffer for 15 min (Fisher, Cat. 2502MI) before blocking and re-probing. Membranes were probed for a maximum of 4 proteins. Of note, Ponceau stain for normalization of protein loading was used only to measure surface expression of GluA1 AMPA receptors. These blots were de-stained as detailed in Ponceau the section above, then blocked and processed like all other blots. For all other protein quantitation, protein loading normalization was performed using Actin as was published by our lab previously (Blaker, Rodriguez, and Yamamoto 2019).

Antibodies used were as follows. Anti-GluR1-NT (NT) Antibody, clone RH95, 1:500 (Millipore Sigma, MAB 2263); Anti-Dopamine Transporter Antibody, 1:1000 (Millipore Sigma, AB2231); Anti-Tyrosine Hydroxylase Antibody, 1:2000 (Millipore, AB152); Anti-Tyrosine Hydroxylase Antibody, PhosphoSer 40 1:500 (Millipore Sigma, AB5935); Anti-CREB (48H2), 1:500 (Cell Signaling Technology, mAb #9197); Anti-Phospho-CREB (Ser 133) (87G3), 1:1000 (Cell Signaling Technology, mAb #9198); Anti-Actin, Clone C4, 1:2000 (Fisher, MAB1501); m-IgGk BP-HRP Mouse Secondary

Antibody, 1:2000 (Santa Cruz, sc-516102); Goat anti-rabbit IgG Antibody, (H+L) HRP conjugate, 1:2000 (Millipore, AP307P).

Statistical Analysis

Protein data were quantitated as relative optical density units. Statistical analyses were performed using Sigma Plot 13. Two-way analyses of variance were performed on all proteins according to the factors of status (METH or yoked-saline control) and treatment (vehicle or roflumilast). Significant differences were followed by Holm-Sidak's method for post-hoc analysis. Data are reported in figures as means \pm standard error of the mean (SEM). For all experiments, statistical significance was set at $p < 0.05$. If a calculated p-value was under 0.05 but not significant, a power analysis was performed with alpha set to 0.05.

Results:

METH abstinence or roflumilast did not affect GluA1 AMPA

Biotinylation was used to fractionate the surface and intracellular compartments to determine differences in glutamate receptor levels in these compartments in the nucleus accumbens after 14 days of METH self-administration followed by 7 days of abstinence and treatment with either vehicle or roflumilast. Figure 10 illustrates the quantification of GluA1 AMPA receptors present in each fractions as a percent of Saline x Vehicle. Surface fractions (10a), intracellular fraction (10b), and total fraction (10c). Figure 10d is a representative Western blot image of GluA1 AMPA receptor, as well as the total protein Ponceau stain. A one-way ANOVA revealed no significant differences between treatment groups in any fraction analyzed. Surface fraction: $F_{(2,9)} = 0.402$, $p = 0.681$; Intracellular fraction: $F_{(2,9)} = 0.277$, $p = 0.764$; Total: $F_{(2,9)} = 0.961$, $p = 0.419$.

Figure 10

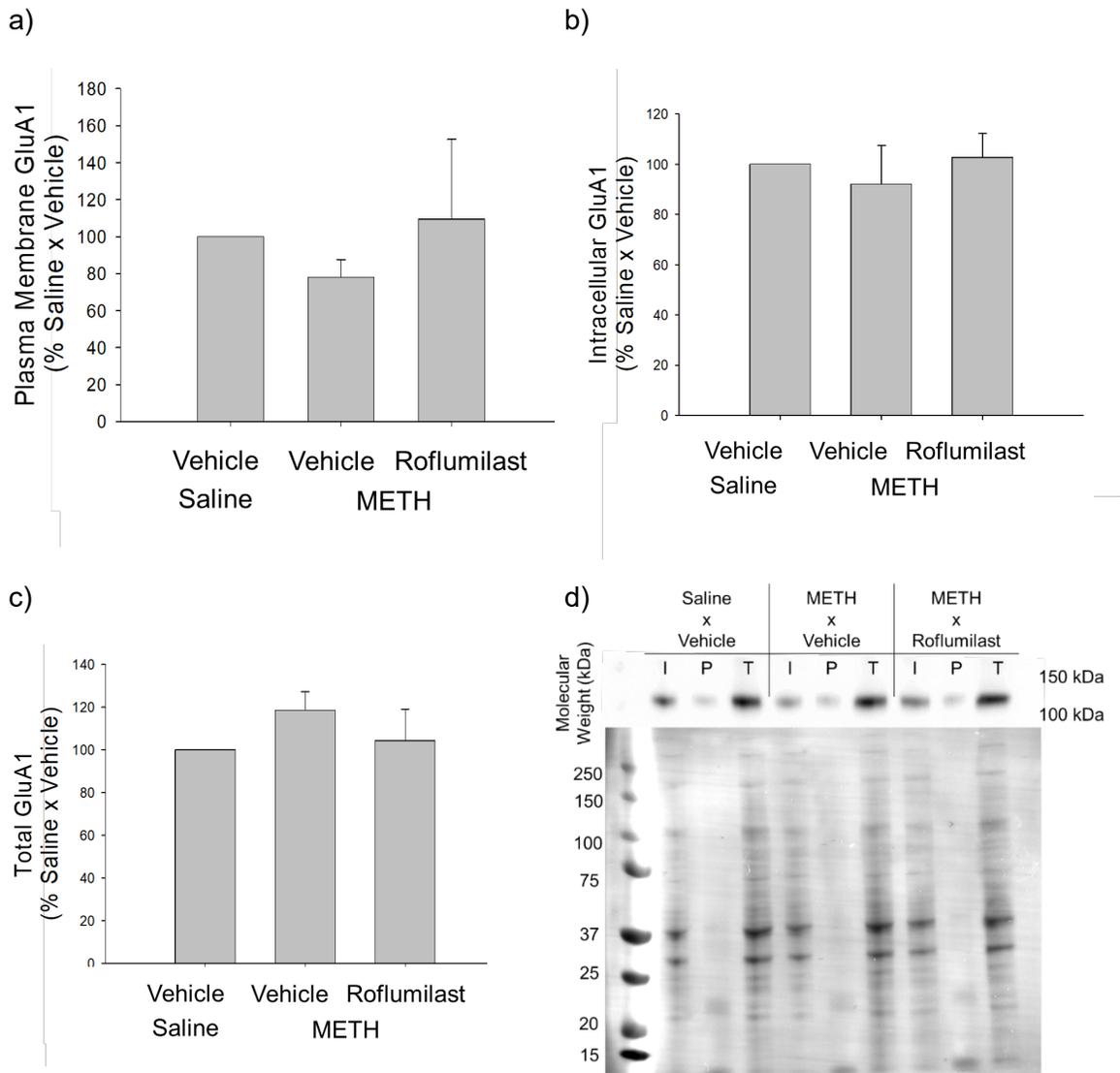


Figure 10: METH abstinence or roflumilast is not associated with changes in GluA1 AMPA Receptors. GluA1 AMPA receptor subunit expression (a) at the plasma membrane, (b) in the intracellular compartment, and (c) in the total tissue homogenate. (d) A representative Western blot of GluA1 AMPA receptors and the total protein Ponceau stain (I = intracellular, P = plasma membrane, T = Total) n=4/group for all fractions.

METH abstinence or roflumilast did not affect Dopamine Transporter or Tyrosine Hydroxylase

Synaptosomes were prepared to determine differences in DAT in the nucleus accumbens after 14 days of METH self-administration followed by 7 days of abstinence and treatment with either vehicle or roflumilast. Figure 11a illustrates the quantification of DAT, and 11b is a representative Western blot of DAT and the actin loading control. Two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. The two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,17)} = 0.0485$, $p = 0.828$). Main factor analysis did not suggest an effect due to status ($F_{(1,17)} = 2.425$, $p = 0.138$), nor treatment ($F_{(1,17)} = 0.227$, $p = 0.640$). Power for performed test with alpha = 0.05 for status was calculated at 0.203.

Western blot of the whole nucleus accumbens tissue was used to determine whether there were differences tyrosine hydroxylase and its phosphorylation in the nucleus accumbens following 14 days of METH self-administration and 7 days of abstinence and treatment. Figure 12 illustrates the quantifications of phospho-TH (Figure 12a), total TH (Figure 12b), and the relative expression of phospho-TH compared to total TH (12c). Figure 12d shows the representative Western blots of phospho-TH, total TH, and the actin loading control.

Two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. For phosphorylated TH normalized to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,15)} = 0.834$, $p = 0.375$). Main factor analysis did not suggest an effect due to status ($F_{(1,15)} = 0.0930$, $p = 0.765$), nor treatment ($F_{(1,15)} = 3.043$, $p = 0.102$). Power for performed test with alpha = 0.05 for treatment was calculated at 0.268.

For total TH normalized to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,15)} = 1.170$, $p = 0.297$). Main factor analysis did not suggest an effect due to status ($F_{(1,15)} = 0.103$, $p = 0.752$), nor treatment ($F_{(1,15)} = 1.438$, $p = 0.249$). Power for performed test with alpha = 0.05 for interaction was calculated at 0.0672; power for treatment was calculated at 0.0951.

For phosphorylated TH normalized to total TH and Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,15)} = 0.125$, $p = 0.728$). Main factor analysis did not suggest an effect due to status ($F_{(1,15)} = 2.091$, $p = 0.169$), nor treatment ($F_{(1,15)} = 1.274$, $p = 0.259$). Power for performed test with alpha = 0.05 for status was calculated at 0.165.

Figure 11

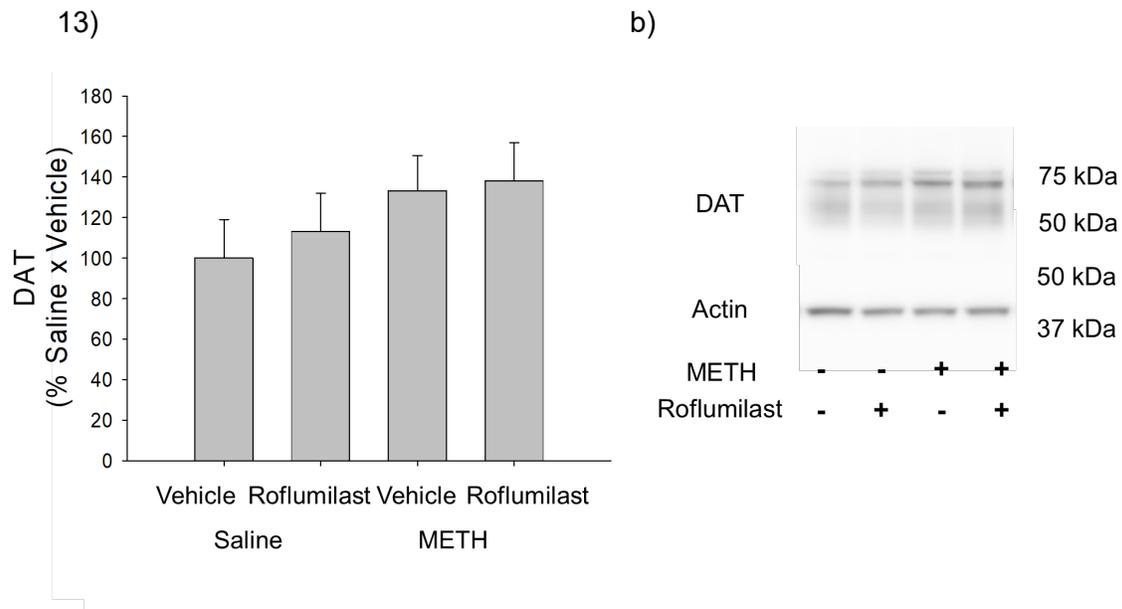
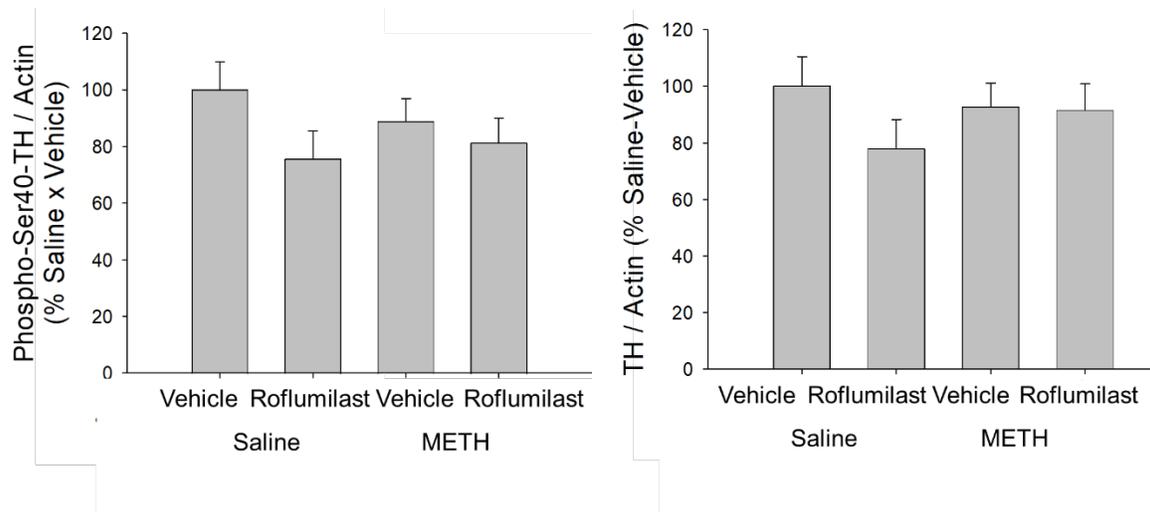


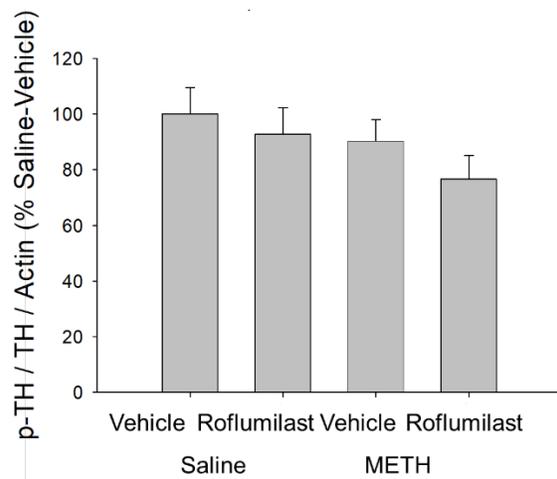
Figure 11: METH abstinence or roflumilast is not associated with changes in synaptosomal DAT. (a) Synaptosomal expression of DAT, (b) and a representative Western blot of DAT and Actin. N=4-5/group.

Figure 12

a)



c)



d)

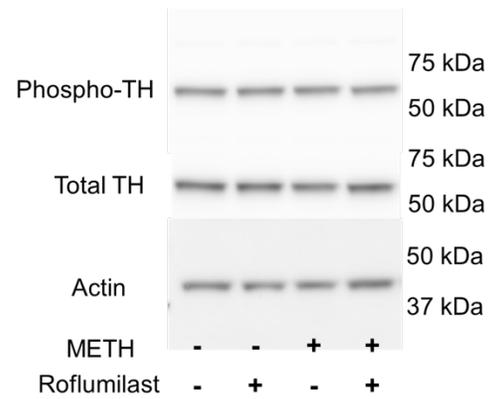


Figure 12: METH abstinence or roflumilast is not associated with changes in TH expression or phosphorylation. (a) Phosphorylated TH, (b) total TH, and (c) phosphorylated-TH expression relative to total TH in tissue homogenate. (d) Representative Western blot of phosphorylated TH, total TH, and actin. N=4-5/group.

METH abstinence or roflumilast was not associated with changes in cyclic AMP response element binding protein

Western blot of the whole nucleus accumbens tissue was used to determine whether there were differences in the activation of CREB protein in the nucleus accumbens following 14 days of METH self-administration and 7 days of abstinence and treatment. Figure 13 illustrates the quantifications of phospho-CREB (Figure 13a), total CREB (Figure 13b), and the relative expression of phospho-CREB compared to total CREB (13c). Figure 13d shows the representative Western blots of phospho-CREB, total CREB, and the actin loading control.

Two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. For phosphorylated CREB normalized to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,17)} = 1.421$, $p = 0.250$). Main factor analysis did not suggest an effect due to status ($F_{(1,17)} = 0.0328$, $p = 0.858$), nor treatment ($F_{(1,17)} = 0.0451$, $p = 0.834$). Power for performed test with $\alpha = 0.05$ for interaction was calculated at 0.050.

For total CREB normalized to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,17)} = 3.157$, $p = 0.093$). Main factor analysis did not suggest an effect due to status ($F_{(1,17)} = 2.835$, $p = 0.111$), nor treatment ($F_{(1,17)} = 0.867$, $p = 0.365$). Power for performed test with $\alpha = 0.05$ for interaction was calculated at 0.284, power for performed test for status was calculated at 0.248.

For phosphorylated CREB normalized to total CREB and Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,17)} = 0.497$, $p = 0.490$). Main factor analysis did not suggest an effect due to status ($F_{(1,17)} = 0.579$, $p = 0.457$), nor treatment ($F_{(1,17)} = 0.006$, $p = 0.938$). Power for performed test with $\alpha = 0.05$ for interaction was calculated at 0.0500.

Figure 13

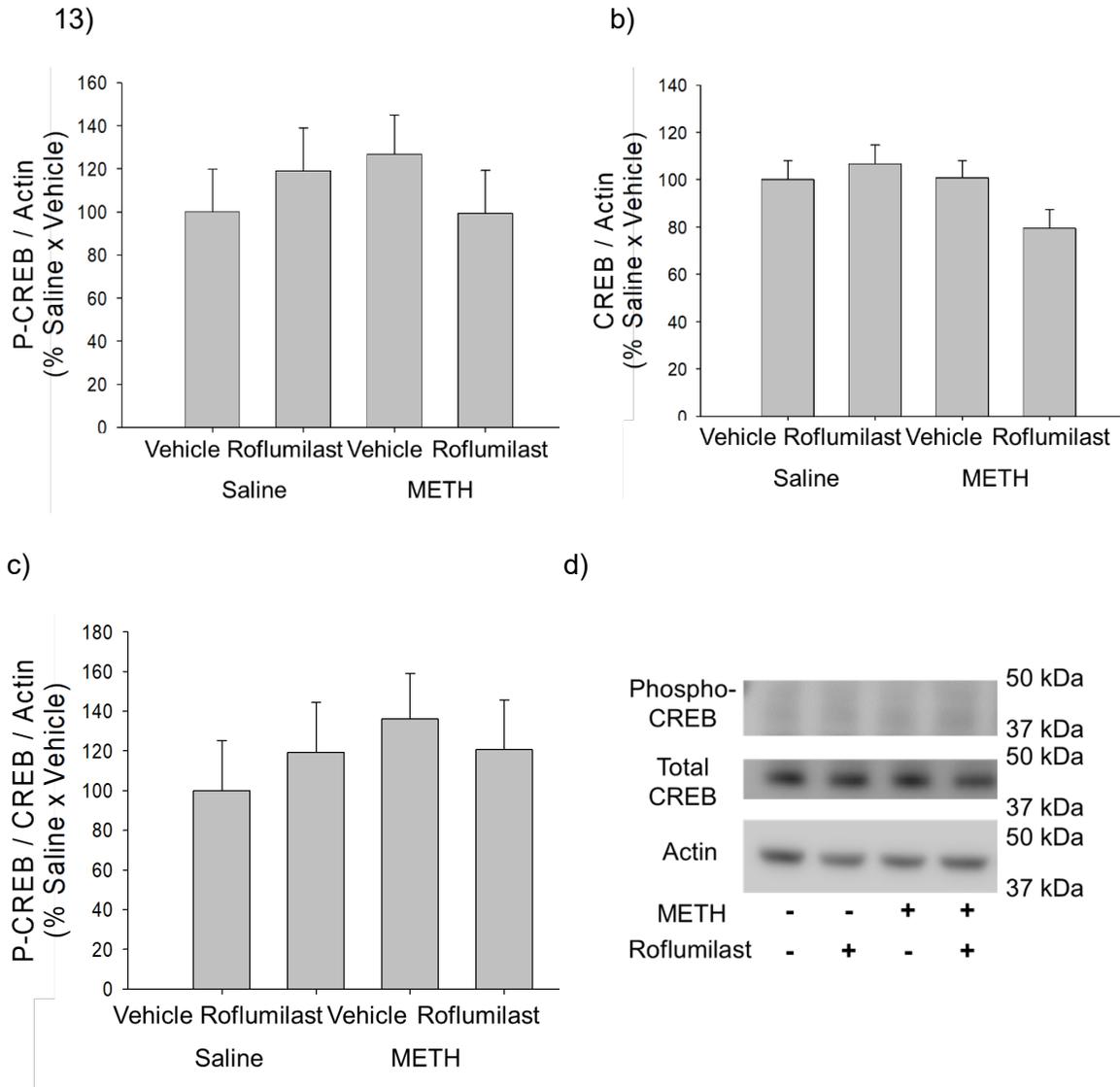


Figure 13: METH abstinence or roflumilast is not associated with changes in CREB expression or phosphorylation. (a) Phosphorylated CREB expression, (b) CREB expression, and (c) phosphorylated-CREB relative to total CREB in tissue homogenate. (d) A representative Western blot of phosphorylated CREB, total CREB, and actin. N=5/group.

Discussion:

The roles of proteins critical to glutamate and dopamine neurotransmission in the nucleus accumbens were investigated. After 14 days of METH self-administration and 7 days of abstinence and treatment with roflumilast, the expression of GluA1 AMPA receptors at the nucleus accumbens was not altered (Figure 10). In addition, proteins related to dopamine, specifically synaptosomal DAT and total tissue TH were analyzed. Neither METH self-administration nor treatment with roflumilast appeared to change synaptosomal levels of DAT (Figure 11), nor did they affect the phosphorylation or expression of TH (Figure 12). Additional analyses of the nucleus accumbens showed that neither METH self-administration and abstinence nor treatment with roflumilast were associated with changes in the phosphorylation or expression of CREB (Figure 13).

We examined whether the effect of roflumilast was mediated by the same neural substrate involved in the seeking behavior after abstinence of a related psychostimulant, cocaine. Because of evidence that a similar phenomenon may mediate relapse-like behaviors to METH seeking (X. Li, Rubio, et al. 2015; Scheyer et al. 2016), we hypothesized that the surface expression of GluA1 in the nucleus accumbens would be increased in the rats that underwent METH self-administration and abstinence of a similar time course as the rats in Li et al., (2015) and Scheyer et al., (2016). However, we found that the surface expression of GluA1 did not change with forced abstinence from METH (Figure 6). Subsequent to our findings, Murray et al. also reported that incubation of METH craving was not associated with changes in AMPA receptor subunits (Murray et al. 2019). Interestingly, Li et al., (2015) only observed increases in GluA1 mRNA transcript from cFos positive neurons METH abstinent rats that underwent extinction and cue-induced reinstatement, suggesting that changes induced by METH in the nucleus accumbens may be specific to neurons that activate in response to cues that drive METH seeking (X. Li, Rubio, et al. 2015). Additionally, this study only examined the

role of GluA1-type AMPA receptors, where many additional aspects of glutamate receptors may have implications for relapse. For instance, calcium permeability, and thereby AMPA-mediated calcium signaling is determined by the lack of GluA2-type AMPA receptors (Conrad et al. 2008; Man 2011), and therefore the role of GluA2-type AMPA receptors should also be explored. Furthermore, metabotropic glutamate receptors and NMDA receptors, glutamate transporters have also been implicated in mediating drug seeking in previous literature, suggesting these aspects of glutamatergic neurotransmission may also need to be explored (Simões et al. 2007; Scheyer et al. 2016; Siemsen et al. 2019).

Additionally, there are clear roles for dysregulation of neurotransmission in other brain regions in the mediation of relapse-like behaviors. It has been observed that extracellular levels of glutamate and dopamine increase in the dorsomedial prefrontal cortex during relapse-like behavior to METH (Parsegian and See 2014) and is necessary for the expression of the behavior (Mcfarland and Kalivas 2001). Furthermore, it has been observed that the incubation of METH craving was associated with increases in mRNA transcript for glutamate receptors, including GluA1, GluA3, and mGlu1, in the dorsal striatum, in neurons positive for Fos, a neural activity marker (X. Li, Rubio, et al. 2015). Inhibiting D1-type dopamine receptors with the infusion of an antagonist in the dorsal striatum prior to extinction training reduced relapse-like behaviors after 30-50 days of abstinence but not after only 2 days of abstinence. Given the theory that the dorsal striatum mediates habitual drug-seeking (Everitt et al. 2008; Harada et al. 2021), there is significant evidence suggesting that the prefrontal cortex as well as the dorsal striatum should also be investigated.

We next sought to characterize the expression of proteins involved in regulating dopamine neurotransmission, and whether they were affected by either METH self-administration or roflumilast. In the previous chapter, we showed that roflumilast affected

the maintenance dose of METH self-administered, suggesting an effect on either the pharmacokinetics or pharmacodynamics of METH. Given that the rate of the self-administration of amphetamines is most significantly dependent on their effects on the dopamine in the nucleus accumbens (Yokel and Wise 1976; Wise et al. 1995), and that D2R, DAT, and TH were decreased in humans that suffered from chronic use disorder suggesting a hypodopaminergic state (Nora D Volkow et al. 2001; McCann et al. 1998; N. D. Volkow, Chang, Wang, Fowler, Ding, et al. 2001; Moszczynska et al. 2004; Boileau et al. 2012), we investigated whether these proteins were also dysregulated by abstinence from chronic METH self-administration, and whether this was affected by roflumilast treatment.

Our results corroborated findings from Stefanski et al., (2002) and Schwendt et al., (2009), who reported that rat models of METH self-administration followed by 7 or more days of abstinence were not correlated with differences in DAT in the nucleus accumbens as measured by Western blot (Stefanski et al. 2002; Schwendt et al. 2009). However, we expanded on these findings by using a synaptosomal preparation to better isolate changes occurring to the nerve terminal (Riddle et al. 2002). Previously, increases in synaptosomal DAT in the nucleus accumbens 24 hours following 10 days of low-dose exposure to METH had been reported, and proposed to be a mediator of reduced extracellular dopamine measured by microdialysis (Broom and Yamamoto 2005). Taken together, these results suggest that differences in METH exposure conditions and duration of abstinence may play a role in the measurement of dopamine-related proteins in the nucleus accumbens. Furthermore, it did not appear that roflumilast treatment altered the levels of these proteins.

Additionally, decreases in TH had previously been seen as evidence of neurotoxicity to dopaminergic nerve terminals and also contribute to a hypodopaminergic state (Halpin, Collins, and Yamamoto 2014). Separately, PDE4 inhibition has been

shown to increase the synthesis of dopamine by TH, which correlated with increases in TH phosphorylation (Nishi et al. 2008). Given that roflumilast reduced relapse-like behavior to METH taking, it was thought that in the rats tested here, METH self-administration and 7 days of abstinence would be associated with decreases in TH, and roflumilast would replete TH and increase phosphorylated TH in the nucleus accumbens of these rats. However, neither METH self-administration nor abstinence or PDE4 inhibition by roflumilast during abstinence appeared to alter the phosphorylation or expression of TH, suggesting that levels of dopamine synthesis were not different between the groups. Taken together, these data suggest that alterations in proteins related to dopamine neurotransmission in the nucleus accumbens were not responsible for the effects of roflumilast on relapse to METH taking.

Similarly, the contribution of cAMP signaling and CREB to relapse-like behaviors remains unclear. The results of Lai et al., 2014 and Sun et al., 2015, in which abstinence from heroin was associated with decreases in phosphorylated CREB, and PDE4 inhibition reduced heroin-seeking behaviors and was correlated with increases in phosphorylated CREB (Lai et al. 2014; Sun et al. 2015). However, we observed that abstinence from METH self-administration and the effects of roflumilast on relapse behaviors were not associated with changes in CREB phosphorylation or expression. A limitation of this study was that levels of cAMP after METH abstinence or treatment with roflumilast were not directly measured, and cAMP is known to signal to alternative pathways that respond to drugs of abuse, including mitogen-activated protein kinases, MAPK, or extracellular signal-regulated kinases, ERK (Cadet et al. 2015). As such, how cAMP is affected by METH abstinence or roflumilast treatment remains to be explored.

Limitations of this study include not using yoked-METH rats, which may have revealed how METH affects unreinforced lever pressing behavior, as previous studies have suggested that 5 weeks of active but not passive METH self-administration can

have divergent effects on D1 and D2-type receptors in the rat when measured 1 day after the last self-administration session (Stefanski et al. 1999). However, it is questionable if the conclusions presented here would change with respect to METH's effects on dopaminergic proteins, as these changes did not appear to be persistent after 7 days of abstinence (Stefanski et al. 2002). This would beg the question as to whether or not differences in these markers would be present if measured at the 1 day time point. Given this consideration, it is not clear that protein changes present after 1 day of METH abstinence but are gone after 7 days of abstinence would mediate this behavior, as these protein changes are gone by the time that the behavioral phenotype is presented. Separately, the behavioral time course analyses presented in Chapter 2 suggest that relapse-like behaviors are dynamic, with relapse-like behaviors most strongly expressed within the first 15 minutes of the relapse tests (Figure 6c, 7b). This would suggest that more dynamic or temporally specific measurements may need to be considered in the investigation of relapse-like behaviors.

An additional limitation of this study includes not having direct measurements of neurotransmitters. However, as the extracellular concentration of dopamine is terminated primarily by the action of DAT (Sulzer et al. 2005) and we did not observe significant differences in synaptosomal DAT in the nucleus accumbens. Additionally, the rate limiting enzyme in the synthesis of dopamine, tyrosine hydroxylase, nor its activated phosphorylated state differed significantly between groups, suggesting that the overall levels of synthesized dopamine were similar between groups as well (Nishi et al. 2008). Finally, we did not observe that downstream changes in cAMP signaling, as measured by the phosphorylation of CREB (Heckman et al. 2018), was not different after abstinence from chronic METH self-administration, or roflumilast treatment. Taken together, this would suggest that overall dopaminergic signaling may not be changed after abstinence from chronic METH or by roflumilast treatment during abstinence,

However, techniques such as microdialysis and fast-scan cyclic voltammetry would be most appropriate to answer this question. Additionally, though there are specific drugs and genetic mouse models that can be used to more specifically examine the roles of dopamine-type receptors in drug self-administration, a commercially available and validated D1-type receptor antibody could have been used to expand our observations of any potential effect of roflumilast on the receptors themselves (Stojanovic et al. 2017). That being said, as D1R and D2R canonically signal through cAMP (Beaulieu, Espinoza, and Gainetdinov 2015), presumably producing the phosphorylation of CREB during drug self-administration (Self et al. 1998; Lai et al. 2014), the fact that CREB phosphorylation between the groups was not significantly different would suggest that the relative cAMP signaling affected by D1R and D2R in the nucleus accumbens may be similar across all experimental groups, though measurement of tissue cAMP would be required to clarify this statement.

Furthermore, it is worth noting that our examination of the nucleus accumbens was of the whole tissue rather than the subdivisions of core and shell. These two regions are anatomically distinct, with the core of the nucleus accumbens more anatomically connected to cortical regions, whereas the shell appearing to be an extension of basolateral amygdalar regions (Di Chiara 2002), though their functional significance is still being determined. Previous experiments have suggested that the core mediates craving after voluntary abstinence, whereas the shell may mediate more affective aspects of reward (Rossi et al. 2020). Previous studies examining the role of glutamate receptors in the nucleus accumbens mediating the incubation of METH seeking examined primarily the nucleus accumbens core, where they had not found changes in protein levels of GluA1 AMPA receptor subunits (Scheyer et al. 2016; Murray et al. 2019). These studies did not explicitly examine the nucleus accumbens shell, or dopaminergic proteins. However, a recent study in rats demonstrated that voluntary

abstinence from METH induced increases in the RNA encoding for DR1 and DR2 in the nucleus accumbens core and not the shell, suggesting that neural adaptations in response to METH and abstinence may be nucleus accumbens sub-region specific (Rossi et al. 2020). Given that inactivation experiments suggest there may be discrete roles and changes in subregions of the nucleus accumbens (Di Ciano, Robbins, and Everitt 2007), a finer dissection and analysis is warranted to determine whether the effects of METH abstinence or roflumilast were unable to be detected because they were restricted to a single subregion.

Finally, a principle limitation of the presented studies is limited n-size for groups. Given that power analyses presented by SigmaPlot suggested that power was often low, experiments may need to be repeated for certainty. However, given that the negative data presented here largely recapitulated findings from other literature, alternative methods of experimental inquiry and alternative hypotheses were considered more prudent in deciding future experiments as they were being performed.

It remains to be determined whether markers of inflammation are involved. Interestingly, previous studies on ibudilast, a non-specific phosphodiesterase inhibitor with anti-inflammatory action, reduced METH self-administration behavior in rats (Snider, Hendrick, and Beardsley 2013). Notably, it was shown that AV1103, a pharmacological analogue of ibudilast that possesses anti-inflammatory activity but negligible PDE inhibitory properties had the same effects as ibudilast in reducing METH self-administration behaviors (Y. Cho et al. 2010). This suggests inflammation itself is sufficient to drive the expression of METH-seeking behaviors, a conclusion also seen with METH-induced conditioned place preference (Narita et al. 2006). Given that chronic METH abuse may be associated with immune dysregulation (Sekine et al. 2008; Loftis et al. 2011; M. J. Li et al. 2020), and that immune dysregulation can impact neurotransmission (Beattie et al. 2002; Lewitus et al. 2014; Garcia-Keller et al. 2019).

The possibility that roflumilast attenuates an inflammatory mechanism that drives relapse-like behaviors remains to be explored.

Altogether, this study did not detect changes in dopaminergic proteins traditionally associated with METH-induced neurotoxicity, as well as changes in GluA1-type AMPA receptors that have previously been associated with METH seeking behaviors. As such, this would suggest that different mechanisms mediate the effect of roflumilast on reducing relapse-like behaviors to METH seeking and METH taking. Given that inflammation itself may drive relapse-like behaviors, the next chapter explores how roflumilast may also be affecting the immune system to reduce relapse to METH.

Chapter 4: Preliminary studies suggest METH self-administration and abstinence and treatment with roflumilast is not associated with changes in overt signs of inflammation

Introduction

METH has a number of immune consequences in the brain, including the activation of microglia and astrocytes (Thomas et al. 2004; Narita et al. 2006). Microglia and astrocytes are immune cells in the brain that mediate inflammation (Mander, Jekabsone, and Brown 2006). Microglia upregulate Iba-1 and CD68 in inflammatory states, whereas astrocytes upregulate GFAP, and these proteins can be detected by immunohistochemistry or by Western blot (He and Crews 2008; Eng, Ghirnikar, and Lee 2000; Hendrickx et al. 2017). Cadaveric studies of chronic METH users had found 50-60% reduced levels of dopamine and 30-40% reduced DAT protein, and ~20% reduced tyrosine hydroxylase in the caudate, putamen, and nucleus accumbens compared to control cohorts matched for age, sex, and postmortem death-to-autopsy interval (Wilson et al. 1996; Moszczynska et al. 2004). Additionally, histopathological study for microglia and astrocytes revealed that chronic METH users who died from complications related to METH intoxication had increased numbers of microglia in their caudate, putamen, and nucleus accumbens relative to controls (Kitamura et al. 2010). Preclinical models recapitulate those findings as METH has been shown to stimulate both astrocytic and microglial activation in the striatum within 3 days of exposure (Thomas et al. 2004; Narita et al. 2006), as well as the expression of pro-inflammatory cytokines (Sriram, Miller, and O'Callaghan 2006; Gonçalves et al. 2017). Blocking inflammation can reduce dopaminergic neurotoxicity induced by METH. For instance, high doses of investigator-administered METH (4 mg/kg x4 every 2 hours) in mice activated microglia in the dorsal striatum and correspondingly decreased DAT expression; however, the administration of ketoprofen, an anti-inflammatory COX-2 inhibitor, blocked these deficits (Asanuma et al.

2003). Taken together, these studies demonstrate inflammation as a contributing mechanism to METH toxicity.

Outside of mediating neurotoxicity, glial cells and the immune system may play a role in the establishment and persistence of relapse to drugs of abuse, including METH. One study found that dialysis of astrocyte-conditioned media into the nucleus accumbens of mice, but not the dorsal striatum, enhanced their METH-induced conditioned place preference. Pre-treatment with propentofylline, a non-specific PDE inhibitor that attenuates glial activation, was shown to decrease METH-induced conditioned place preference, suggesting a causal role for glial factors in the development of METH-seeking behaviors (Narita et al. 2006). A second study showed that both minocycline, and ibudilast, a non-specific phosphodiesterase inhibitor with anti-inflammatory action, reduced METH-induced locomotor activity and sensitization in mice (Snider et al. 2012), and METH self-administration behavior in rats (Snider, Hendrick, and Beardsley 2013). Additionally, it was shown that AV1013, a pharmacological analogue of ibudilast with negligible PDE inhibitory activity, but which inhibits pro-inflammatory cell recruitment and the catalytic activity of the proinflammatory cytokine macrophage inhibitory factor, had the same effects as ibudilast (Y. Cho et al. 2010). These behavioral studies suggest that neuroinflammation itself may play a role in the relapse-like behaviors to METH self-administration, and the activation of glial cells within the nucleus accumbens after METH or roflumilast should be assessed.

Cytokines can also have direct action on neuronal firing properties. Studies have shown that ex vivo application of tumor necrosis factor alpha (TNF α) from glial cells will induce the insertion of GluA2-lacking AMPA receptors in pyramidal neurons of the hippocampus, and TNF α blockade will inhibit AMPA surface expression, and these changes are associated with increases in the frequencies of miniature excitatory post-synaptic currents (Beattie et al. 2002; Stellwagen et al. 2005). This effect may be

dependent on the expression of a protein mediator of post-synaptic dopaminergic signaling, dopamine-and- cAMP-regulated phosphoprotein Mr 32 kDa (DARPP32), as TNF α has been shown to induce the opposite effect in medium spiny neurons of the dorsal striatum (Lewitus et al., 2014). Two additional pro-inflammatory cytokines, interleukin 1 beta (IL-1 β) and interleukin 6 (IL-6), have also been shown to respectively enhance and inhibit long-term potentiation and population excitatory post-synaptic potentials in hippocampal slices, suggesting that cytokine action on neurotransmission is cytokine and brain region dependent (Schneider et al. 1998; Tancredi et al. 2002; Pribiag and Stellwagen 2014). Given that cytokines can affect neurotransmission and that previous studies have shown anti-inflammatory drugs can influence drug seeking behaviors, cytokines should be explored as potential mediators of the effect of roflumilast on relapse-like behaviors for METH.

Additionally, the immune system's ability to mediate tissue remodeling at the level of the neural synapse has also been implicated in mediating the addictive properties of drugs including METH (Mizoguchi et al. 2007; Smith et al. 2014; Lasek, Chen, and Chen 2018; Garcia-Keller et al. 2019). In the body, after injury or in states of inflammation, leukocytes have been shown to release matrix metalloproteinases (MMP). MMP are tightly regulated proteases that degrade extracellular matrix components to allow for tissue remodeling, leukocyte migration, the release of growth factors stored in the extracellular matrix, and contribute to the local inflammatory reaction by cleaving regulatory subunits of cytokines (Yong, Power, and Edwards 2001; Yong 2005). Recently, it has been shown that MMP can mediate processes related to neuronal plasticity, including long term potentiation (LTP) in the hippocampus, prefrontal cortex, and specific nuclei of the amygdala (Nagy et al. 2006; Okulski et al. 2007; Gorkiewicz et al. 2015). Repeated treatment with METH increased MMP2 and MMP9 and activity in the frontal cortex and nucleus accumbens of rats, and mice deficient in MMP2 and

MMP9 exhibit blunted METH-induced conditioned-place preference and locomotor sensitization (Mizoguchi et al. 2007). Moreover, abstinence from cocaine self-administration produced increases in MMP9 activity in the nucleus accumbens of rats, and inhibition of MMP9 in the nucleus accumbens reduced cue-induced reinstatement of cocaine, nicotine, and heroin seeking behavior but not sucrose seeking (Smith et al. 2014). In addition, stimulation of MMP activity in the nucleus accumbens enhanced the reinstatement of cocaine-seeking behavior (Garcia-Keller et al. 2019). These studies suggest MMP proteins are causally involved in the expression of relapse to drug-seeking.

Roflumilast is clinically used to treat chronic obstructive lung disorder by reducing the inflammatory remodeling of the lung alveoli and subsequent airway changes associated with the lung disorder (Martinez et al. 2015). Roflumilast has been shown to inhibit the recruitment of immune cells, including macrophages, neutrophils, and eosinophils (Martorana et al. 2005), reduce the stimulated release of inflammatory cytokines in a variety of immune cells (Bundschuh et al. 2001; Kwak et al. 2005; Grootendorst et al. 2007), and inhibit the release of proteases such as elastase and MMP (N. A. Jones et al. 2005). These effects serve as the basis for its use to treat chronic obstructive pulmonary disorder (Calverley et al. 2009; Gross, Giembycz, and Rennard 2010). Because of the anti-inflammatory properties of roflumilast and the pre-clinical evidence suggesting inflammation itself may influence neurotransmission, we studied whether METH self-administration and abstinence or treatment with roflumilast affected markers of inflammation.

Given the previously established role of inflammation produced in response to METH, and that attenuation of inflammation has been previously demonstrated to reduce relapse-like behaviors to METH, we hypothesized that METH self-administration and abstinence was associated with increased activation of astrocytes and microglia, as

well as increased levels of the cytokines TNF α , IL-1 β , and IL-6, and increased MMP activation. Furthermore, roflumilast, in its action as an anti-inflammatory, would reduce the activation of glial cells and MMP, as well as the increases in cytokines (Figure 14).

Figure 14

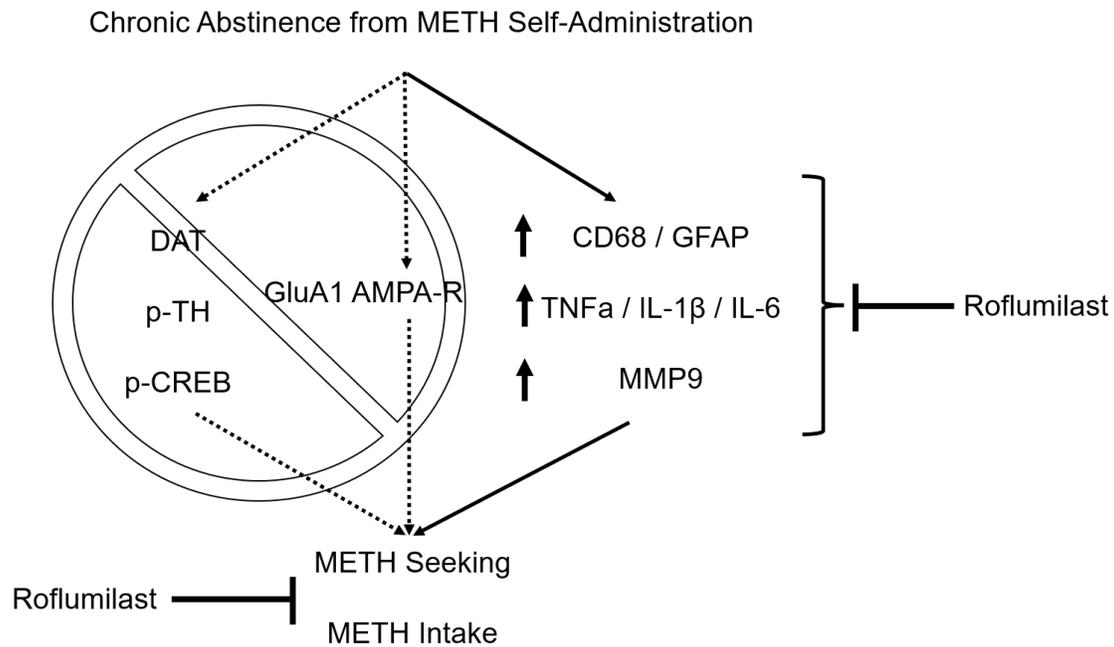


Figure 14: Diagram of hypothesis on how METH and roflumilast affect inflammation in the nucleus accumbens. METH and roflumilast were not associated with overt changes in dopaminergic proteins or GluA1 after 7 days of abstinence. Previous studies have demonstrated that METH is associated with inflammatory changes including activated glial cells, release of inflammatory cytokines, activation of MMP, and that attenuating inflammation can reduce relapse-like behaviors. Clinically, roflumilast is used to reduce inflammation associated with chronic obstructive lung disease. We hypothesized that METH and abstinence would be associated with increased levels of CD68 and GFAP, increased pro-inflammatory cytokines TNF α , IL-1 β , and IL-6, and increased activation of MMP9, which would be attenuated by roflumilast treatment.

Materials and Methods:

Subjects

Male Sprague-Dawley rats (225-250 g, Envigo, Indianapolis, IN) were used in all experiments. Rats were housed 2 per cage in a temperature and humidity controlled facility under a 12 hour light/dark cycle (7:00 AM – 7:00 PM) with a minimum 4 day acclimation period before any experimentation. Food and water were available ad libitum throughout the experiment. Experiments and surgical procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and approved by Indiana University Institutional Animal Care and Use Committee.

Rats underwent 14 days of METH self-administration followed by 7 days of forced abstinence and unreinforced METH seeking relapse test, as described in the previous chapter. Following the relapse test, rats were decapitated and their brains were extracted. An ice-cold brain matrix (ASI Instruments, Cat. RBM—4000S) was used to obtain coronal slices. Thin razor blades were inserted at the level of the optic chiasm, with two more blades inserted every 2 mm increments in the caudal direction to yield 2 contiguous coronal slices that were 2 mm thick. Nucleus accumbens was dissected from the face corresponding to approximately Bregma +0.5 mm using the anterior commissure as the landmark (Paxinos and Watson 3rd edition).

Tissue homogenization for protein analysis

Brain tissue for protein analyses was dissected from brains on the day of relapse. Brain regions were homogenized by sonication in lysis buffer (25mM HEPES pH 7.4, 500mM NaCl, 2mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 20mM NaF, 0.1% IGEPAL (v/v), and 1X HALT protease and phosphatase inhibitor cocktail (ThermoFisher, Cat. 78440), and twice for 5 s each on ice. The tissue homogenate was spun down at

20,000 RCF at 4 °C for 2 min and the supernatant was used for analysis. Total protein content was quantified using the Bradford assay before Western blot or ELISA.

ELISA

Sandwich-type colorimetric ELISA kits were run according to manufacturer instructions as follows. After overnight coating of the 96-well plate with the primary antibody diluted in PBS at room temperature. All solutions were aspirated, then plates underwent 3 wash cycles with PBS-Tween 20 (1x PBS with 0.05% Tween-20) with complete aspiration between washes. Plates were blocked in reagent diluent (1% BSA in PBS) for 1 hour at room temperature, after which they underwent 3 wash cycles as above. 50 µg of protein in 100 µL sample, diluted with reagent diluent as needed, was added to each well, along with a fresh 7-point standard curve of recombinant protein of known concentrations and a positive control, and incubated overnight at 4 °C with gentle agitation. After incubation, the plate wells were aspirated of all solution, then underwent 3 wash cycles before incubation with 100 µL of the detection antibody diluted in reagent diluent for 2 hours at room temperature with gentle agitation. Wells were again completely aspirated and underwent 3 wash cycles, followed by incubation in Streptavidin-HRP for 20 minutes at room temperature with gentle agitation while protected from light. Wells were again completely aspirated and underwent 3 wash cycles, then 100 µL of fresh HRP substrate was added to each well and allowed to react for 20 minutes at room temperature with gentle agitation while protected from light, followed by the addition of 50 µL of Stop solution (2 N H₂SO₄) to stop the reaction. Plates were gently tapped to ensure complete mixing before being read on the microplate reader at 450 nm, with subtraction of absorption of the 540 nm wavelength to correct for optical imperfections of the plate. Data from samples were calculated from a 4-parameter logistic curve fit against the known standards. Plates were covered with

adhesive strips to prevent evaporation in all incubations. All samples, standard curves, and positive controls were run in technical duplicates on the same plate. Each ELISA run had its own freshly prepared standard curve and positive control.

Kits used were as follows: Rat IL-1 beta/IL-1F2 Quantikine ELISA kit (R&D Systems, RLB00); Rat TNF alpha ELISA kit (abcam, ab100785); Invitrogen eBioscience Rat IL-6 Platinum ELISA kit (Invitrogen / ThermoFisher, BMS625).

Western Blot

Samples were loaded onto a 4-12% Bis-Tris NuPage Gel (ThermoFisher, Cat. NP0336) and were run in a MOPS running buffer at 150 V for 90 min. Proteins were transferred onto a PVDF membrane at 27 V for 120 min. Membranes were blocked at room temperature for 1 hour before being incubated in primary antibody overnight at 4 °C. The next day, membranes were washed 4 × 5 min in 0.5% Tris-buffered saline with Tween 20 (0.5% TBS-T), incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody. Membranes were again washed 4 × 5 min in 0.5% TBST and incubated for 2 min in chemiluminescence solution to allow visualization of antigen-antibody complexes (ThermoFisher, Cat. 34580). An LAS-4000 Image Analyzer System (FujiFilm) was used to image and quantify the optical density of proteins of interest. Membranes were then incubated in stripping buffer for 15 min (Fisher, Cat. 2502MI) before blocking and re-probing. Membranes were probed for a maximum of 4 proteins. Protein data were quantitated as relative optical density units.

Antibodies used were as follows. Anti-CD/SR D1 (ED1) Mouse monoclonal Ab, 1:500 (Novus, NB600-985); Anti-Glial Fibrillary Acidic Protein (GFAP) Antibody, 1:3000 (Millipore, AB5805); Anti-Actin, Clone C4, 1:2000 (Fisher, MAB1501); m-IgGk BP-HRP Mouse Secondary Antibody, 1:2000 (Santa Cruz, sc-516102); Goat anti-rabbit IgG Antibody, (H+L) HRP conjugate, 1:2000 (Millipore, AP307P).

Gelatin Zymography

Zymography is a technique used enzyme activity. Protein electrophoresis using a polyacrylamide gel with 10% gelatin allows for visualization of MMP2 and MMP9 activity after proteins are separated under non-reducing conditions, renatured, and incubated in a solution containing enzyme cofactors. In these conditions, MMP2 and MMP9 digest gelatin and their activity are visualized as bands of clearing within a stained background. Because the pro-domain of MMP is cleaved off to produce the active form, Pro and Active MMP have different weights, with the Pro-form measured at approximately 92 kDa and the active form measuring near 82 kDa (Christensen and Shastri 2015).

To perform this assay, brain regions were homogenized in a sterile microcentrifuge tube and tight-fitting plastic pestle in extraction buffer (0.35 M Sucrose; 10 mM HEPES Buffer, pH 7.4; 75 uL for bilateral nucleus accumbens). The sample was pelleted by centrifugation at 1000 RCF for 10 min. The supernatant was used for subsequent zymography analysis. The pellet is enriched in nuclear and plasmalemmal material and may be resuspended in 10 mM HEPES and 0.25 DTT for further analysis by Western blot. The supernatant protein concentration was equalized across samples with extraction buffer. Gelatin-sepharose beads were prepared by 3 cycles of wash and vortex in PBS of a volume 3 x the starting bead volume, followed by pelleting by centrifugation at 5000 RCF for 2 in at 4°C and discarding the supernatant. 100 ug of protein in a total 150 uL volume was combined with 50 uL gelatin-sepharose beads (Millipore-Sigm, Cat. GE17-0956-01) to isolate proteins containing fibronectin domains such as MMP and incubated overnight with agitation at 4°C. The suspension was then pelleted by centrifugation at 5000 RCF at 4°C for 2 min and the supernatant was discarded. Beads were then washed with 3 cycles of PBS wash, as previous, with care to decant all the supernatant after the last wash. Beads were then incubated with 15 uL

of 20 mM PBS (2x PBS) with 10% DMSO for 30 min at 37°C with constant agitation. The suspension was pelleted by centrifugation at 5000 RCF for 3 min at room temperature and the entire supernatant was decanted. Equal volumes of this supernatant was used for analysis. The supernatant was combined with 5x non-reducing SDS sample loading buffer and allowed to sit at room temperature for 5 minutes before loading. Equal sample volumes were loaded into a Novex 10% gelatin zymogram gel (ThermoFisher, Cat. ZY00100) and ran using 4°C chilled Tris-Glycine SDS running buffer at 125 V for 100 min at 4°C. After running, gels were washed with 2.5% Triton-100 twice, each for 30 min, and rinsed with ddH₂O 2 times, each for 15 min, all at 4 °C, to remove SDS and renature the proteins. Gels were then incubated with fresh developing buffer (50 mM Tris-HCl, pH 7.5; 5 mM CaCl₂; 0.15 M NaCl; 1 µM ZnCl₂; and 0.02% NaN₃) for 72 hours at 37°C. Gels were then fixed with a solution of 50% ethanol and 10% acetic acid for 1 hour at room temperature without agitation, then incubated with 50% methanol and 10% acetic acid for >1.5 hours at room temperature with agitation at room temperature.

To visualize MMP activity within the gel, gels were stained with 0.1% Coomassie R250, 50% Methanol and 10% acetic acid for 3 hours at room temperature with agitation, before being de-stained with 50% methanol and 10% acetic acid for 1 hour. Gels were then immediately transferred to a 5% acetic acid solution for re-hydration and storage. Gels were imaged after an overnight incubation in 5% acetic acid using a light-box and digital camera, and quantitated using ImageJ.

Statistical Analysis

Statistical analyses were performed using Sigma Plot 13. T-test was performed on IL-6 ELISA, two-way analyses of variance were performed on all other proteins according to the factors of status (METH or yoked-saline control) and treatment (vehicle or roflumilast). Significant differences were followed by Holm-Sidak's method for post-

hoc analysis. Data are reported in figures as means \pm standard error of the mean (SEM). For all experiments, statistical significance was set at $p < 0.05$. If a calculated p-value was under 0.50 but not significant, a power analysis was performed for the lowest p-values with alpha set to 0.05

Results:

METH abstinence or roflumilast are not associated with changes in GFAP and CD 68 in the nucleus accumbens

Western blot was used to detect the expression levels of proteins from astrocytes and microglia, to determine if there were differences in these protein levels in the nucleus accumbens after 14 days of METH self-administration followed by 7 days of abstinence and treatment with either vehicle or roflumilast (Figure 15). Figure 15 shows the expression of GFAP (Figure 15a) and CD 68 (Figure 15c). A representative Western blot image of GFAP and CD 68 are shown in Figure 15b and 15d, respectively.

For GFAP, a two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. For GFAP to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,16)} = 0.131$, $p = 0.722$). Main factor analysis did not suggest an effect due to status ($F_{(1,16)} = 0.789$, $p = 0.388$), nor treatment ($F_{(1,16)} = 0.443$, $p = 0.515$). Power for performed test with alpha = 0.05 for status, was calculated at 0.05.

For CD68, a two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. For CD68 to Actin, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,16)} = 0.0445$, $p = 0.836$). Main factor analysis did not suggest an effect due to status ($F_{(1,16)} = 0.169$, $p = 0.687$), nor treatment ($F_{(1,16)} = 0.455$, $p = 0.510$).

Figure 15

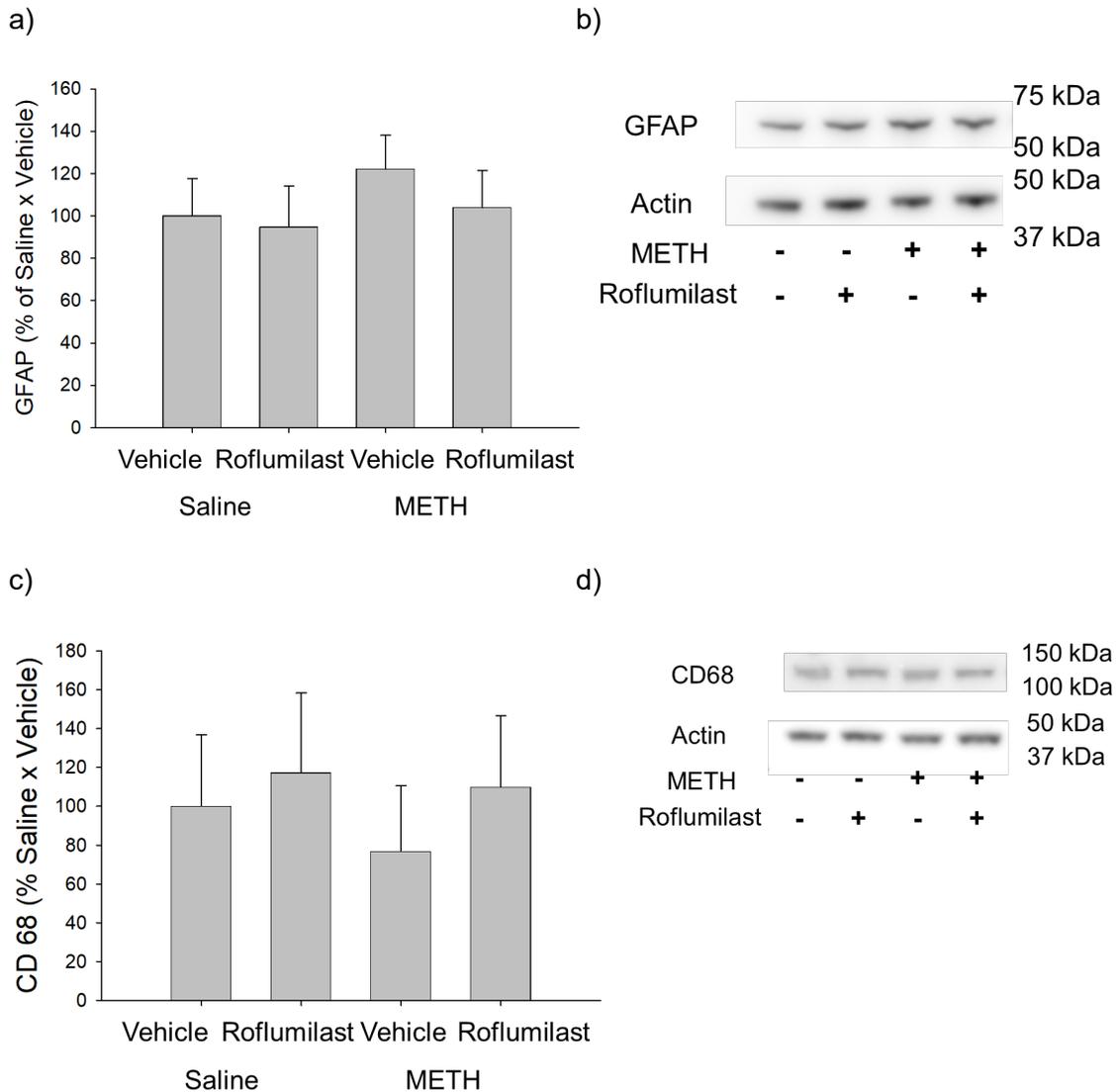


Figure 15: METH abstinence or roflumilast are not associated with changes in GFAP and CD 68 in the nucleus accumbens. (a) Expression of GFAP, (b) and a representative Western blot of GFAP and Actin. (c) Expression of CD 68, (d) and a representative Western blot of CD 68 and Actin (n=4-6 animals/group).

METH abstinence or roflumilast treatment are not associated with changes in TNF α , IL-1 β , or IL-6 in the nucleus accumbens

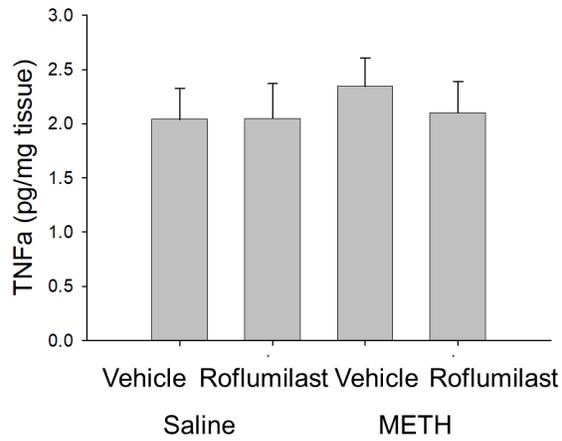
ELISA was used to quantitate the levels of inflammatory cytokines in the nucleus accumbens after 14 days of METH self-administration and 7 days of abstinence with treatment (Figure 16). Figure 16a illustrates the amount of TNF α , Figure 16b illustrates the amount of IL-1 β measured. For TNF α , a two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. The two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,16)} = 0.184$, $p = 0.673$). Main factor analysis did not suggest an effect due to status ($F_{(1,16)} = 0.164$, $p = 0.691$), nor treatment ($F_{(1,16)} = 0.372$, $p = 0.551$).

For IL-1 β , a two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. The two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,16)} = 0.764$, $p = 0.396$). Main factor analysis did not suggest an effect due to status ($F_{(1,16)} = 0.0598$, $p = 0.810$), nor treatment ($F_{(1,16)} = 0.1.666$, $p = 0.216$). Power of performed test with alpha = 0.05 for treatment was calculated to be 0.119.

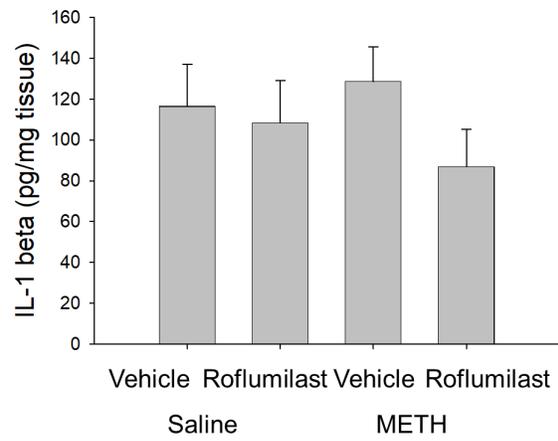
Figure 16c illustrates the amount of IL-6 measured. A t-test revealed no significant differences after METH self-administration or yoked-saline administration and 7 days of abstinence ($t = 1.141$, $p = 0.270$). The power of the performed test with alpha – 0.05 for status was calculated to be 0.190.

Figure 16

a)



b)



c)

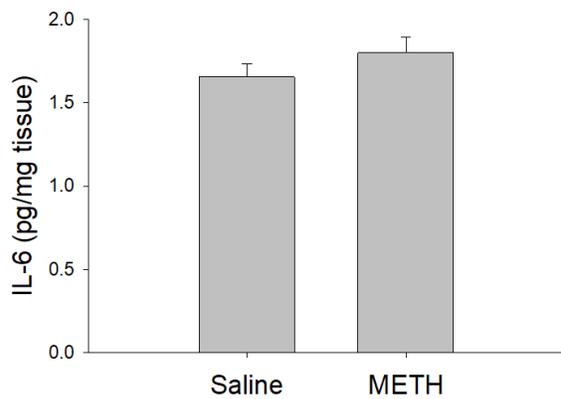


Figure 16: METH abstinence is not associated with changes in TNF α , IL-1 β , and IL-6 in the nucleus accumbens, and roflumilast did not affect TNF α and IL-1 β as measured by ELISA. (a) Expression of TNF α (n=4-6/group), (b) IL-1 β (n=4-6/group), (c) and IL-6 (n=9-11/group).

METH abstinence or roflumilast treatment are not associated with changes in nucleus accumbens MMP activity

Gelatin zymography was used to measure and MMP9 activity in the nucleus accumbens after 14 days of METH self-administration and 7 days of abstinence with treatment (Figure 17). Figure 17a illustrates the quantification of MMP activity, and Figure 17b illustrates a representative zymogram image. MMP2 activity was not significantly detected.

A two-way ANOVA was run using status (Saline vs METH) and Treatment (Vehicle vs Roflumilast) as main factors. For active MMP9, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,39)} = 0.667$, $p = 0.419$). Main factor analysis did not suggest an effect due to status ($F_{(1,39)} = 0.665$, $p = 0.420$), nor treatment ($F_{(1,39)} = 0.665$, $p = 0.430$). Power of performed test with alpha = 0.05 for the interaction was calculated to be 0.251.

For pro MMP9, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,39)} = 0.455$, $p = 0.504$). Main factor analysis did not suggest an effect due to status ($F_{(1,39)} = 0.00795$, $p = 0.922$), nor treatment ($F_{(1,39)} = 0.693$, $p = 0.332$). Power of performed test with alpha = 0.05 for the treatment was calculated to be 0.534.

For the active to pro MMP9 ratio, the two-way ANOVA did not suggest an interaction between status and treatment ($F_{(1,39)} = 0.119$, $p = 0.732$). Main factor analysis did not suggest an effect due to status ($F_{(1,39)} = 1.011$, $p = 0.321$), nor treatment ($F_{(1,39)} = 0.450$, $p = 0.506$). Power of performed test with alpha = 0.05 for the status was calculated to be 0.489.

Figure 17

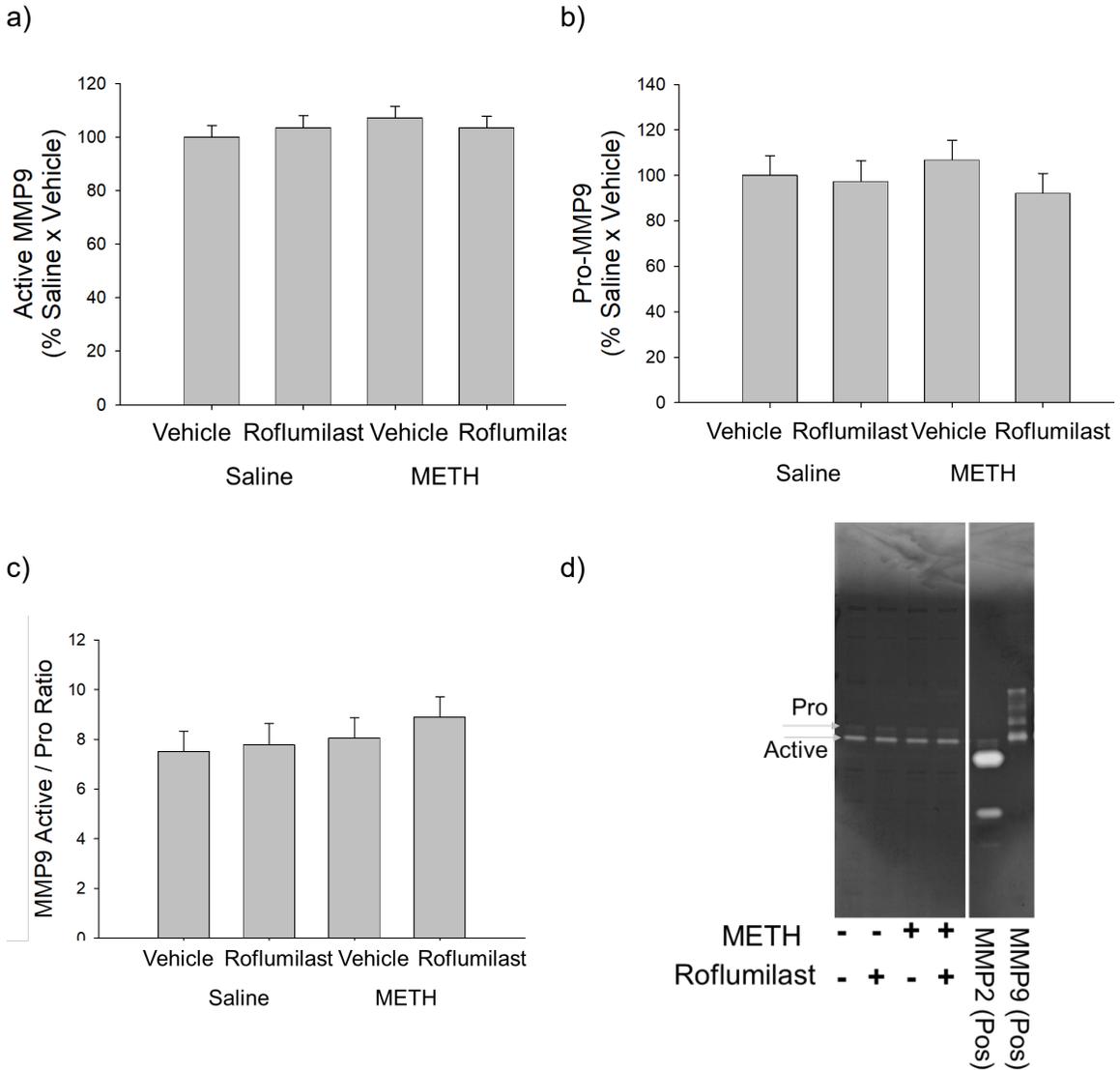


Figure 17: METH abstinence or treatment with roflumilast is not associated with differences in MMP activity in the nucleus accumbens as measured by gelatin zymography. (a) Activity of active MMP9, (b) activity of pro-MMP9, and (c) the ratio of active MMP9 to pro-MMP9, with (d) a representative zymogram with purified MMP9 used as positive controls (n=10-11/group).

Discussion:

The involvement of the immune system in METH self-administration followed by abstinence, and treatment with roflumilast during abstinence was investigated. METH self-administration and abstinence was not associated with changes in the protein levels of CD68 and GFAP in the nucleus accumbens, suggesting that microglia or astrocytes were not activated or affected by roflumilast treatment (Figure 15). The proinflammatory cytokines TNF α , IL-1 β , and IL-6 were not different between treatment groups after 14 days of METH self-administration and abstinence with treatment (Figure 16). MMP9 activity did not appear to differ between treatment groups (Figure 17).

Microglia and astrocytes are two non-neuronal cell types that have been shown to coordinate many physiological processes in the brain including synaptic development and remodeling (Vainchtein and Molofsky 2020). CD68 and GFAP are protein markers used to label microglia and astrocytes, respectively, and are known to be upregulated by stimuli that drive inflammatory processes, such as lipopolysaccharide, hypoxia, and brain parenchymal damage (Guttenplan and Liddelow 2019). In our study, it did not appear that METH self-administration and abstinence were associated with changes in the expression of microglia or astrocyte markers in the nucleus accumbens (Figure 15), going against the hypothesis that METH and abstinence are associated with increases in inflammation. Our results are in concordance with Schwendt et al. (2009), who found that a similar paradigm of METH self-administration did not alter the protein expression levels of GFAP, or Iba-1, another microglial marker, in both the prefrontal cortex and the nucleus accumbens (Schwendt et al. 2009).

However, absence of changes in glial markers in this study as well as Schwendt et al., (2009) may be due to the inability of Western Blot to detect changes in morphology. For instance, Siemsen et al, (2019) utilized a technique to visualize astrocytes in exceptional detail by tagging their membranes with green fluorescent

protein using an astrocyte selective adeno-associated viral vector, with GFAP as a promoter (Siemsen et al. 2019). This allowed astrocyte processes in the nucleus accumbens to be visualized to the resolution of their confocal microscope, approximately 150 nm, which is larger than a synapse. Using this technique, they performed immunohistochemistry to also label synapsin-1, a protein that coats synaptic vesicles, and quantified the co-localization of GFP and synapsin-1 within a voxel. As the resolution of this technique was measured to be 150 nm, Siemsen et al., assumed that co-localization of synapsin-1 with GFP represented an astrocyte process in close association with, but not invading, a synapse tagged with synapsin-1. Using this method, they found that rats that underwent METH self-administration and extinction did not have overt changes to astrocyte number in the nucleus accumbens compared to yoked saline controls. However, METH self-administration and extinction reduced astrocyte-synapse association in the nucleus accumbens by 50% (Siemsen et al. 2019), with similar reduced co-localization with synapsin-1 seen in a cocaine self-administration model (Scofield et al. 2016). This is all to say that Western Blot may not capture glial changes in response to METH self-administration or roflumilast treatment, and that techniques that consider glial morphology may be more appropriate to evaluate effects (Hendrickx et al. 2017).

The cytokines TNF α , IL-1 β , and IL-6 are classic pro-inflammatory cytokines produced by glial cells within the brain and induced by immune insult (McAfoose and Baune 2009). Though METH-induced increases in TNF α mRNA and protein in the nucleus accumbens have been reported in investigator-administered METH paradigms, we were unable to detect changes in TNF α protein (Nakajima et al. 2004; Gonçalves et al. 2010). The lack of changes with regards to microglia and astrocytes in response to METH self-administration and abstinence was also reflected in the absence of detected changes in TNF α , IL-1 β , and IL-6, nor did they appear to be affected by roflumilast

treatment (Figure 16). Although overt changes in inflammation in response to neurotoxic stimuli such as binge-doses of METH can be measured with Western blot or ELISA (Gonçalves et al. 2010), that low physiological concentrations of cytokines play a role in mediating plasticity (McAfoose and Baune 2009; Civciristov and Halls 2019)(Civciristov and Halls 2019). For instance, on rat hippocampal slice preparations, bath applications of IL-1 β inhibited field excitatory potential recordings taken from hippocampal CA1 neurons, in a reversible and dose-dependent fashion, with the effective concentrations ranging from the nano-molar to the sub-femtomolar scale (Luk et al. 1999). As the sensitivity and variance of standard ELISA or Western blot cannot produce as precise measurements, this may be a reason why no changes were detected in the experiments presented. Alternative approaches where experimenters can have such precise control over immune-related variables may be necessary to better define a physiological role for cytokines in the nucleus accumbens. This would include techniques such as bath applications of brain slices or microdialysis of immune modulators. It is of note that these approaches were used in Narita et al., (2008), to first define a causal role for glial modulation in the production of rewarding behaviors related to METH and morphine (Narita et al. 2006).

Finally, matrix metalloproteinases (MMP) in the nucleus accumbens play a role in the reinstatement of cocaine, nicotine, and heroin seeking behaviors (Smith, Scofield, and Kalivas 2015). The efficacy of MMP inhibition to reduce relapse to METH stems from their critical role in the structural modification of dendrites at the synapse of hippocampal neurons (X. Bin Wang et al. 2008). They are also acutely increased and necessary for the reinstatement of cocaine, nicotine, and heroin seeking behaviors (Smith et al. 2014). Moreover, investigator-administered injections of METH increased the activity of MMP9 in the frontal cortex, hippocampus, and nucleus accumbens core (Mizoguchi et al. 2007; Conant et al. 2011). Furthermore, pharmacological interventions

that have been shown to reduce the expression of METH seeking behaviors – propentofylline, ibudilast, rolipram, and minocycline – have also separately been shown to reduce either the expression or activity of MMP (Golub et al. 1984; Sánchez et al. 2005; V. L. Jacobs et al. 2012; J. Y. Lee et al. 2012). However, any differences in the activation of MMP9 do not appear to be present in the nucleus accumbens after 14 days of METH self-administration followed by 7 days of abstinence and a relapse test, or affected by treatment with roflumilast during abstinence (Figure 17).

The transient nature of MMP activation may explain the lack of effect in our paradigm. Smith et al. (2014) showed that MMP activity was only correlated briefly with the reinstatement of cocaine-seeking behavior and returned to levels comparable to yoked-saline rats by less than 2 hours after the beginning of the reinstatement test (Smith et al. 2014). Given that our relapse test paradigm is 6 hours in duration, it may be that the MMP activity has returned to basal levels by the time the MMP activity was measured in our animals. Of note, the time course of the MMP activation reported by Smith et al., (2014) roughly correlates with time course of the relapse to METH seeking behavior that we observed in the time course analyses performed (Figure 6c). As MMP activity has been shown to dynamically mediate neurotransmission in the hippocampus (X. Bin Wang et al. 2008; Michaluk et al. 2009), if relapse to METH self-administration behaviors and the effect of roflumilast are found to be associated with MMP activity, this may suggest that the MMP activity may be a neural substrate that directly correlates with drug-seeking behavior in a temporally specific manner.

A principle limitation of the presented studies is, again, limited n-size for analysis. Given that power analyses presented by SigmaPlot suggested that power was often below the desired 0.80, experiments may need to be repeated with increased sample size for certainty. However, as previously explored it may be that inherent technical limitations of western blot or ELISA would make it difficult to detect effects of METH or

roflumilast. Also, given the lack of positive findings with regards to inflammation, and because inflammation is known to occur along specific time courses (Thomas et al. 2004; Loftis et al. 2011). Given this, inflammation may have been stimulated and measurable at earlier time points than when these experiments were conducted. For instance, rats exposed to a neurotoxic regimen of METH had increased levels of microglia in the striatum that peaked 48 hours after exposure, and declined to baseline by approximately 7 days. However, as before, changes present at earlier time courses of METH abstinence but are gone after 7 days of abstinence would mediate behavior present at the 7 day time point, as it would be assumed that those differences would be present when comparing the effects of vehicle and roflumilast. However, the behavioral time course analyses presented in Chapter 2 suggest that relapse-like behaviors are dynamic, with relapse-like behaviors most strongly expressed within the first 15 minutes of the relapse tests (Figure 6c, 7b). This would suggest that more dynamic or temporally specific measurements may need to be considered in the investigation of relapse-like behaviors. Finally, there must also be consideration that the unreinforced METH-seeking relapse test itself may be inducing effects that mask biological differences. For instance, it has been observed that extracellular levels of both dopamine and glutamate arise in response to both cues as well as METH intake in the nucleus accumbens from baseline during relapse tests after extinction (Parsegian and See 2014), suggesting that the relapse test itself is sufficient to produce biological effects, and investigations of tissues both before and after relapse testing are required to fully assess the effects of the test itself, apart from the effect of METH abstinence or roflumilast treatment.

Altogether, this study did not detect changes in upregulation in glial markers associated with inflammation after METH and abstinence, did not detect changes in the pro-inflammatory cytokines $\text{TNF}\alpha$, $\text{IL-1}\beta$, or IL-6 , nor did we detect differences in MMP activity after METH self-administration and 7 days of abstinence. Furthermore, no

markers of inflammation appeared to be overtly affected by roflumilast at this time point. Taken together, this would suggest that METH self-administration and abstinence are not associated with overt signs of inflammation, and are not affected by roflumilast treatment, suggesting alternative mechanisms that drive relapse-like behaviors to METH after abstinence that are responsive to roflumilast treatment may need to be explored. The next and final chapter will explore this possibility and future directions for these findings.

Chapter 5: Summary and Discussion

Summary of results

The experiments described in this dissertation report the use of roflumilast, a phosphodiesterase 4 inhibitor, to reduce relapse to both methamphetamine seeking and taking behaviors in a rat model of methamphetamine use disorder. Experiments to uncover the mechanism of action were performed, but the exact mechanism by which roflumilast reduces relapse-like behaviors to METH remain to be explored (Figure 18).

Chapter 2 describes that roflumilast reduces relapse to both METH seeking and METH taking behaviors when taken during abstinence. To enhance the clinical relevance of experiments investigating treatments for reducing relapse to METH, roflumilast treatment was given during the 7 days of forced abstinence from METH self-administration. Roflumilast decreased the relapse to METH seeking behaviors, as well as decreased the relapse to METH taking, reducing both the self-administered loading dose and maintenance dose of METH, while leaving resumption of sucrose taking behaviors intact.

Chapter 3 demonstrated that 14 days of METH self-administration and abstinence was not associated with the accumulation of GluA1 AMPA receptors at the nucleus accumbens surface as seen in models of cocaine self-administration. Additionally, changes in dopaminergic proteins associated with chronic METH use and a hypodopaminergic state were not observed in the nucleus accumbens. Roflumilast treatment did not appear to modulate these measured proteins, nor did it affect levels of phosphorylated CREB.

Chapter 4 showed that 14 days of METH self-administration and abstinence was not associated with changes signaling overt inflammation in the nucleus accumbens, nor did it appear that roflumilast affected the markers measured in the nucleus accumbens.

The activity of MMP9 in the nucleus accumbens was also measured the rats underwent the unreinforced relapse test, and it did not appear that there were differences between rats that had self-administered METH and yoked-saline controls, or differences due to treatment with roflumilast or vehicle.

Altogether, these results demonstrated the efficacy of using an FDA-approved PDE4 inhibitor during abstinence to treat relapse, though further experiments uncovering how roflumilast produces this effect remain.

Figure 18

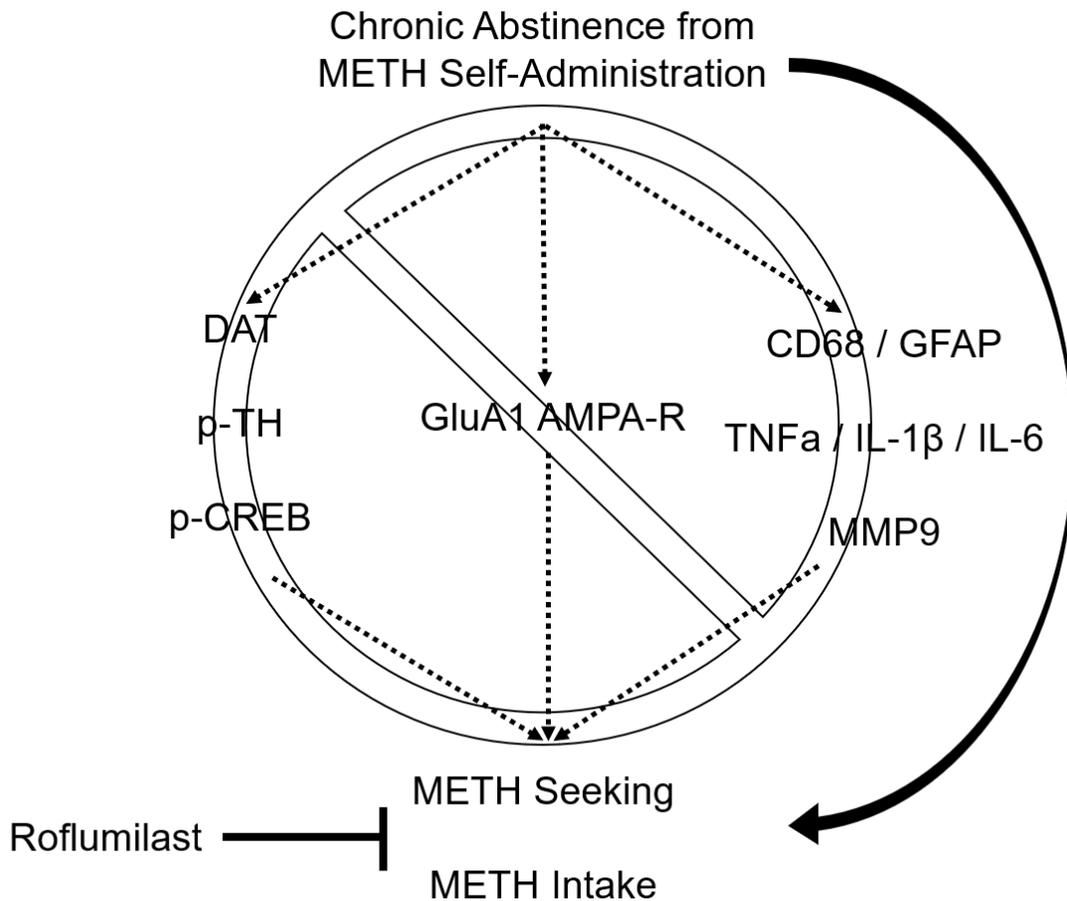


Figure 18: Diagram summarizing results. Roflumilast inhibited relapse-like behaviors to METH self-administration, including METH seeking and METH taking. METH had been

observed to be produce dopaminergic nerve terminal toxicity associated with reductions in DAT and TH, including in the nucleus accumbens. However, METH self-administration and abstinence was not associated with overt changes in proteins related to dopamine synthesis and neurotransmission. CREB, a canonical mediator of cAMP signaling induced by dopamine, was also unchanged. Additionally, though surface expression of GluA1-subtype AMPA receptors in the nucleus accumbens had previously been suggested to mediate relapse-like behaviors to METH seeking after 7 days of abstinence, the expected changes were not observed. Furthermore, roflumilast did not produce measurable effects on these measured proteins. Finally, METH has been associated with a pro-inflammatory state that has been suggested to mediate relapse-like behaviors. However, METH self-administration was not associated with overt changes in inflammation including the activation of microglia or astrocytes, measured by increased CD68 or GFAP respectively, or increased pro-inflammatory cytokines TNF α , IL-1 β , and IL-6, or increased activation of MMP9. Additionally, roflumilast did not produce measurable effects on these markers of inflammation.

General discussion and future directions

Results presented in Chapter 2 demonstrated that a pharmacological intervention could be effective if applied during forced abstinence instead of as a pre-treatment before a relapse test. The implementation of roflumilast during the abstinence period was made so that our model more accurately reflected a human patient seeking pharmacological treatment for METH use disorder (Shoptaw et al. 2009; Chan et al. 2019). Two distinct relapse tests were used to determine the effect of roflumilast, a relapse test where operant behavior was not rewarded to assess METH seeking behavior, and a relapse test where operant behavior was rewarded with METH to assess METH intake upon relapse. We found that treatment with roflumilast during

abstinence from METH self-administration reduced METH seeking as well as reduced METH intake. In addition to the benefits in reducing METH seeking behavior, reducing METH intake in relapse may also assist to reduce harms associated with relapse, as mortality associated with METH use has been increasing over the past 10 years (M. P. Paulus and Stewart 2020). The work presented here is one of the first reported preclinical studies of a pharmaceutical intervention implemented during abstinence before the relapse test (Chan et al. 2019). The fact that roflumilast is already approved by the Food and Drug Administration for use in humans reduces barriers in advancing its use in the treatment of METH use disorder (Morley et al. 2017; *Orange Book : Approved Drug Products with Therapeutic Equivalence Evaluations*. 2020).

To determine the effect of roflumilast on behavior, experiments were constructed based on the following observations from the literature. It has been shown that cocaine and METH self-administration by rats results in increased glutamate and dopamine efflux in the nucleus accumbens that correlated with drug seeking behavior, and was necessary for the reinstatement of drug seeking behaviors (McFarland, Lapish, and Kalivas 2003; Parsegian and See 2014). In a rat model of METH self-administration, whole cell patch clamp electrophysiological recordings of medium spiny neurons of the nucleus accumbens after 7 days of abstinence showed an enhanced decrease in evoked excitatory post-synaptic current (EPSC) in response to Nasp, a calcium-permeable AMPA receptor blocker (Twomey et al. 2018), suggesting that calcium-permeable AMPA receptors developed a larger contribution to glutamatergic neurotransmission to the nucleus accumbens in response to METH abstinence (Scheyer et al. 2016). In models of cocaine self-administration, calcium-permeable GluA1 AMPA receptors at the surface of the nucleus accumbens accumulated during abstinence, and inhibition of these receptors decreased relapse-like behaviors to cocaine seeking (Conrad et al. 2008), suggesting that this may be the mechanism explaining the

increased potential glutamatergic neurotransmission seen in the nucleus accumbens (Scheyer et al. 2016). Separate experiments also showed METH abstinence was accompanied by an increase in GluA1 transcript in the dorsal striatum (X. Li, Rubio, et al. 2015).

To determine if the surface expression of GluA1 AMPA receptors at the nucleus accumbens regulated relapse-like behaviors to METH seeking, we used the same biotinylation-based method utilized in Conrad et al., (2008) to fractionate extracellular surface proteins and intracellular proteins in Chapter 3. However, we found that changes in the expression of GluA1 AMPA receptors was not affected during abstinence from METH self-administration, nor by treatment with roflumilast. Soon thereafter, these results were corroborated by Murray et al., (2019), who also observed a lack of change in GluA1, GluA2, and GluA3 AMPA receptors subunits, or metabotropic glutamate receptor 1 (Conrad et al. 2008; Murray et al. 2019). It may be important to note that in Li et al., 2015, the increase in GluA1 transcript after METH self-administration and abstinence was only observed in C-fos positive neurons of the dorsal striatum that responded to cue-induced reinstatement after extinction, and not in whole dorsal striatal tissue preparation (X. Li, Rubio, et al. 2015). Additionally, it may be that the contribution of GluA1 AMPA receptors to nucleus accumbens neurotransmission seen in Scheyer et al., (2016) are much more dynamic than can be detected by biotinylation of surface proteins, as changes in calcium-permeable AMPA receptor mediated neurotransmission were detected using electrophysiology, a technique that specifically assess neuron action potentials (Scheyer et al. 2016).

The positioning and distribution of AMPA receptors may also play functional roles (Roth, Zhang, and Huganir 2017). AMPA receptors are rapidly trafficked in and out of synapses by mechanisms regulating their insertion from endosomes as well as by lateral diffusion along the plasma membrane surface on timescales that are as short as

seconds to minutes (Lopez et al. 2015). The position of AMPA receptors has significance, as clustered AMPA receptors can generate greater amplitude EPSC (MacGillavry et al. 2013), and the inhibition of lateral immobilization of AMPA receptors can ablate short-term potentiation in hippocampal neurons and inhibit freezing in experiments of fear conditioning in mice (Penn et al. 2017). The clustering of AMPA receptors that modulate EPSC is thought to be due to protein-protein interactions by synaptic adhesion molecules present within dendritic spines anchored by post-synaptic density protein 95 (Jang, Lee, and Kim 2017). In light of how the relative position of AMPA receptors can significantly impact neurotransmission, it may be that METH self-administration and abstinence or roflumilast treatment produces their effects through changes in AMPA receptor clustering and dynamics without affecting surface expression. This may occur by increasing affinity of AMPA protein-protein interactions at the post-synaptic density to enhance clustering at existing synapses, or by increasing the number of dendritic spines such that lateral diffusion of AMPA receptors have an increased likelihood of clustering at post-synaptic densities. There is evidence supporting both these theories, as self-administration of several different classes of drugs have increased dendrite spine head area (Smith et al. 2014), and repeated amphetamine administration and abstinence can increase dendritic sprouting (Robinson and Kolb 1997).

We also studied proteins related to dopaminergic transmission and whether they were affected by METH self-administration and abstinence or treatment with roflumilast. The rationale was that rodents self-administering psychostimulants will alter their rate of psychostimulant self-administration in response to changes in dopaminergic transmission in the nucleus accumbens (Yokel and Wise, 1976; Wise et al., 1995). In addition, chronic METH abuse is associated with significant losses in proteins related to dopaminergic neurotransmission, including dopamine transporter (DAT), dopamine type-

2 receptors (D2R), and tyrosine hydroxylase (TH) (Wilson et al., 1996; Moszczynska et al., 2004; Volkow et al., 2001). We did not observe differences in these proteins but there is a growing body of evidence that PDE4 in complex with the scaffolding enzyme beta-arrestin exert significant effects on G-coupled protein receptor (GPCR) actions and should be considered (Shenoy and Lefkowitz, 2011). For example, PDE4 and beta-arrestin are binding partners, and stimulation with the beta-2 adrenergic receptor (B2R) agonist isoprenaline induced co-recruitment of beta-arrestin and PDE4D (Baillie et al., 2003) in cardiac myocytes and human embryonic kidney 293 cells overexpressing B2R. The co-recruitment induced a switch in the coupling of the B2R from its cognate Gs G-protein to a Gi G-protein. This Gs to Gi switch was prevented by competitive antagonism of the PDE4 enzyme, or inhibition by the PDE4 inhibitor rolipram (Baillie et al., 2003; Baillie and Houslay, 2005). Similarly, prolonged dopamine 1-type receptor (D1R) agonism by catechols induces the recruitment of beta-arrestin to the receptor and subsequent D1R endocytosis, resulting in receptor desensitization and tachyphylaxis (Goulet et al., 1996; Gray et al., 2018). It was speculated that this tachyphylaxis is what induces the euphoria associated with METH to dissipate long before METH is eliminated from the body, and drives METH users to adopt a pattern of METH use where individuals smoke small doses of METH in intervals as short as 30 minutes (Perez-Reyes et al., 1991; Cho and Melaga, 2001).

Interestingly, beta-arrestin 2 has been shown to be modulate the response to cocaine in beta-arrestin 2 knockout mice (Porter-Stransky et al., 2019), and the complexing of beta-arrestin to PDE4 has been shown to be necessary for the development of fear learning in these beta-arrestin 2 knockout mice (Li et al., 2009). Furthermore, the development of fear learning mediated by the complexing of beta-arrestin 2 and PDE4 was sensitive to PDE4 inhibition by rolipram. As such, it is an open question as to what the effects of roflumilast is on beta-arrestins. Given that the rate of

METH self-administration in rats is influenced by inhibiting either D1 or D2-type dopamine receptors (Yokel and Wise, 1976; Wise et al., 1995; John et al., 2015) and that dopaminergic neurotransmission in the nucleus accumbens is necessary for METH seeking after abstinence (Rossi et al., 2020), further investigations into the potential action of roflumilast on D1 and D2-type receptors by beta-arrestin is warranted.

A previous study utilizing a phosphodiesterase inhibitor analogue suggested that inhibiting inflammation mediates a reduction in METH seeking behaviors independent of phosphodiesterase inhibition (Snider et al., 2012). Given that the primary clinical indication of roflumilast is to reduce inflammatory remodeling of lung alveoli (Martorana et al., 2004; Martinez et al., 2015), we also explored whether METH self-administration and abstinence, as well as treatment with roflumilast was associated with changes in inflammation. Our working definition of inflammation was defined as the activation of immune cells to produce cytokines that are canonically known to mediate the cardinal signs of inflammation (Scott et al., 2004). The primary immune cells in the brain are astrocytes and microglia that secrete cytokines such as tumor necrosis factor alpha (TNF α), interleukin 1 beta (IL-1 β) and interleukin 6 (IL-6) (Bellinger et al., 1998; McAfoose and Baune, 2009; Vainchtein and Molofsky, 2020). These pro-inflammatory cytokines have been shown to influence the development of long-term potentiation in the hippocampus (Schneider et al., 1998; Tancredi et al., 2002; Pribiag and Stellwagen, 2014). There has also been some evidence suggesting that chronic METH use and abstinence are associated with inflammation (Sekine et al., 2008; Loftis et al., 2011; Cheng et al., 2019; Li et al., 2020).

Based on the above findings, we explored whether these cytokines and proteins related to the activation of astrocytes and microglia changed in response to METH self-administration and abstinence in the nucleus accumbens. None of the cytokine markers examined were changed in the nucleus accumbens in response to METH self-

administration and 7 days of abstinence in the rat when measured by Western blot or ELISA. Additionally, roflumilast did not appear to affect inflammatory markers in the nucleus accumbens. However, given that femtomolar differences in interleukin concentrations can induce physiological effects (Civciristov and Halls 2019), and that differences in astrocyte-synapse interaction can occur without overt changes in astrocyte number and morphology (Siemsen et al. 2019), it is unclear if the techniques used were sensitive enough to detect differences in our samples. Alternative approaches such as using microinjection of immune modulators into the nucleus accumbens may be required to rigorously test the role of inflammation in the nucleus accumbens.

Recent evidence suggests that matrix metalloproteinases (MMP) in the nucleus accumbens are causally involved in the expression of drug-seeking behaviors. MMP are thought to mediate glutamate neurotransmission by changing dendritic spine morphology and glutamate neurotransmission (X. Bin Wang et al. 2008). For instance, MMP activation allows the expansion of the dendritic head, as well as the lateral diffusion of AMPA and NMDA receptors (Michaluk et al. 2009). Furthermore, MMP activation appears necessary to induce long term potentiation (LTP), as inhibition of MMP appears to prevent LTP in the hippocampus, prefrontal cortex, and certain nuclei of the amygdala (Nagy et al. 2006; Okulski et al. 2007; Gorkiewicz et al. 2015). It has been shown that MMP activity can mediate the reinstatement of drug seeking, as MMP inhibition in the nucleus accumbens reduced the reinstatement of cocaine, nicotine, and heroin seeking behaviors (Smith et al. 2014), and conversely, the enhancement of MMP activation with tissue-plasminogen activator in the nucleus accumbens potentiated the reinstatement of cocaine-seeking behaviors (Garcia-Keller et al., 2019). It is also of note that several pharmaceutical interventions that reduced METH-induced relapse behaviors also inhibits MMP (Golub et al. 1984; Sánchez et al. 2005; V. L. Jacobs et al. 2012; J. Y. Lee et al. 2012).

Roflumilast is used in clinic to inhibit protease-induced remodeling of lung alveoli in inflammatory lung diseases (Jones et al., 2005). We explored whether MMPs are activated in the nucleus accumbens in our model, and whether that activation was sensitive to roflumilast. We did not observe differences in MMP9 activation in the nucleus accumbens after METH self-administration followed by 7 days of abstinence and treatment and an unreinforced relapse test. However, a previous study demonstrating that MMP activity is necessary for the reinstatement of cocaine and heroin seeking behavior found that MMP activity was stimulated with the reinstatement of drug seeking but declined to baseline within 2 hours (Smith et al. 2014). Therefore, it is possible that the MMP activity occurred prior to when we examined MMP, so earlier timepoints should be examined.

Finally, though the nucleus accumbens is strongly implicated in mediating relapse-like behaviors to METH (Koob and Volkow 2009), there are certainly other brain regions that have been involved in mediating the relapse to drugs of abuse, including the dorsal striatum (Fuchs, Branham, and See 2006), prefrontal cortex (Rocha and Kalivas 2010), and extended amygdala (Pelloux et al. 2018). Though the presented work was limited to the nucleus accumbens, given the lack of biochemical results, these other regions should also be explored as potential regions affected by METH and roflumilast to mediate relapse-like behaviors (Nestler and Lüscher 2019).

Based on the presented data and the review of the literature, there is still significant support for the hypothesis that roflumilast acts to reduce relapse-like behaviors to METH seeking by reducing inflammation in the nucleus accumbens. Our data demonstrated that roflumilast reduced relapse-like behaviors to METH seeking predominantly in the first 90 minutes of the unreinforced relapse test, with the largest difference occurring within the first 15 minutes.

Recent reports found that MMP activation mediates relapse to drug seeking in the nucleus accumbens in cocaine, nicotine, and heroin on a similar time course before declining to levels comparable to control within 120 minutes (Smith et al. 2014), and it has been demonstrated that MMP activity is necessary for the facilitate glutamatergic neurotransmission, with the current theory being that it allows for the lateral diffusion of AMPA receptors (Michaluk et al. 2009). This is well before the 6+ hour timepoint at which the MMP activation was measured in Figure 17. It is known that glutamatergic neurotransmission in the nucleus accumbens is necessary for the reinstatement of relapse-like behavior (McFarland and Kalivas 2001; McFarland, Lapish, and Kalivas 2003). Given that one of the principle mechanisms by which roflumilast reduces COPD exacerbations is by reducing protease-induced remodeling of lung alveoli (N. A. Jones et al. 2005), the next tested hypothesis is that MMP are maximally activated in rats that had previously self-administered METH during the first 15 minutes of the unreinforced METH-seeking relapse test, and that this activation is attenuated by roflumilast. Though technically challenging, the acute in vivo activity of MMP can be measured using quenched fluorescent gelatin acutely injected into the nucleus accumbens core before the relapse test, the same technique used to measure the in vivo activity of MMP by Smith et al (Smith et al. 2014). Should it be seen that animals treated with roflumilast have correlatively reduced levels of acutely activated MMP in the nucleus accumbens, microinjection of MMP activators such as tissue plasminogen activator into the nucleus accumbens should be performed (Garcia-Keller et al. 2019). If reactivation of MMP restores relapse-like behaviors to METH seeking in rats that were treated with roflumilast, this would support a mechanistic link between MMP and the effect of roflumilast in reducing relapse-like behaviors. Further experiments beyond that would include determining the cellular origin of MMP, given that both microglia and neurons have been shown to release MMP (Nagy et al. 2006; V. L. Jacobs et al. 2012). Due to the fact

that there still remains no promising treatment for stimulant use disorder, these experiments further examining the mechanism by which roflumilast reduced relapse-like behaviors to METH maintain their significance (C. M. Jones, Compton, and Mustaquim 2020).

Overall, the current findings are the first to report the use of a pharmacological agent during abstinence to reduce relapse to METH. The increases in METH overdoses and the critical lack of treatment options for patients struggling with METH use disorder make identifying an effective intervention of great clinical importance. Roflumilast effectively reduced METH seeking as well as METH taking while leaving natural rewards intact. Though the mechanism behind the effects of roflumilast remains unclear, the strong behavioral results suggest further studies investigating roflumilast's mechanism of action on neurotransmission are warranted.

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Nucleus Accumbens Glutamatergic Transmission." *Cold Spring Harbor Perspectives in Medicine* 10 (12): a039255.
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<https://doi.org/10.1111/j.1360-0443.2010.03066.x>.

Curriculum Vitae

James Jaewoo Baek

Education

Indiana University, Indianapolis, IN

M.D., Matriculated in 2015

Ph.D, Matriculated in 2015, Achieved Graduate Candidacy in 2018, Completed in 2022

Thesis: Treatment During Abstinence from Methamphetamine in a Rat Model of Methamphetamine Use Disorder

Washington University in St. Louis, St. Louis, MO

B.A., Matriculated in 2011, Completed in 2014

Major: Neuroscience; Minor: Philosophy-Neuroscience-Psychology

Publications

1. Baek, J. J., Kline, H., Deveau, C. M., & Yamamoto, B. K. (2022). Roflumilast treatment during forced abstinence reduces relapse to methamphetamine seeking and taking. *Addiction biology*, 27(1), e13082.
2. Paek, S. B., Min, H. K., Kim, I., Knight, E. J., Baek, J. J., Bieber, A. J., ... & Chang, S. Y. (2015). Frequency-dependent functional neuromodulatory effects on the motor network by ventral lateral thalamic deep brain stimulation in swine. *Neuroimage*, 105, 181-188.

Conferences & Presentations:

1. Rusk, D.S., Wilson, J., Baek, J.J., "Effects of COVID-19 on Career Exploration: Furthering the Role of the Surgery SIG Cluster," Annual IUSM Education Day. Indianapolis, IN, April 2021
2. "Effect of the Phosphodiesterase Inhibitor Roflumilast on Methamphetamine Self-Administration," Stark Summer Science Symposium, speaker. Indiana University School of Medicine, Indianapolis, IN, October 2020

3. "Using PDE4 Inhibitors in the Treatment of Methamphetamine Addiction," IU MSTP Student Seminar Series, speaker. Indiana University School of Medicine, Indianapolis, IN, April 2020
4. Sharp, S., Rusk, D., Rodriguez, V., Chiu, M., Baek, J.J., Mercho, R., "Clustering Student Interest Groups: Re-imagining Early Career Exploration," Learn Serve Lead 2019: The AAMC Annual Meeting, Phoenix, AZ, November 2019
5. Baek, J.J., Mitchell, C.M., Natarajan, R., Yamamoto, B.K. "Phosphodiesterase inhibitor roflumilast attenuates relapse to methamphetamine self-administration after forced abstinence." Society for Neuroscience Annual Conference. San Diego, CA, November 2018
6. "The Neurobiology of Addiction, and Understanding the Current Opioid Crisis," Practice in Healthcare Settings SWK-S 693, invited speaker, Indiana University School of Social Work, Indianapolis, IN, June 2018
7. Baek, J.J., Paek, S.B., Chang, S.Y. "Adenosine evoked by high-frequency DBS reduces harmaline-induced tremor." Mayo Clinic SURF Poster Symposium. Mayo Clinic, Rochester, MN, August 2013.

Honors and Awards

2018 Christopher Hrovj Travel Award Recipient

Previous Research Experiences

Indiana University Stark Neurosciences Research Institute Indianapolis, IN

with Dr. Eri Hashino

Graduate Student Research Rotation June 2016 to July 2016.

- Research focused on inner ear sensory epithelia derived from pluripotent stem cells in 3D cultures
- Differentiated mouse embryonic stem cells into otic vesicles with functional inner ear hair cells, passaged and maintained cell lines, generated and cloned guide DNA plasmids for use with the CRISPR-eSpCas9 system, and assisted with laboratory upkeep

with Dr. Bryan Yamamoto

Graduate Student Research Rotation May 2016 to June 2016.

- Research focused on the mechanism behind the neurotoxicity caused by chronic stress
- Induced rodent stress using a 10-day stress paradigm, performed Western Blotting and data analysis, and assisted with laboratory upkeep

with Dr. Fletcher White

Graduate Student Research Rotation June 2015 to August 2015.

- Research focused on the mechanism of opioid-induced hyperalgesia and neuropathic pain
- Set up Reverse Transcription-qPCR protocol, performed RNA extraction and RT-qPCR, cryostat sectioning, staining and fluorescence microscopy, and assisted with laboratory upkeep

Washington University in St. Louis School of Medicine, St. Louis, MO

with Dr. Joseph Corbo

Research Technician February 2015 - May 2015.

- Research focused on the expressed genetic profile of cone cells involved in the enhanced color vision of avians
- Performed retinal extraction dissections, single-cell dissociation and harvesting, single-cell mRNA profiling, qPCR, staining and fluorescence microscopy, and assisted with laboratory upkeep

with Dr. Henry Han

Research Assistant January 2012 - May 2013; September 2013 - May 2014.

- Research focused on the cerebrovascular pathology of Alzheimer's, cerebral amyloid angiopathy, and ischemic stroke in mouse models
- Performed mouse brain extraction and microtome slicing, genotyping, staining and fluorescence microscopy, PCR, and assisted with laboratory upkeep

Mayo Clinic, Rochester, MN

with Dr. Su-youne Chang

Summer Undergraduate Research Fellow, May 2013 - August 2013.

Research Intern, August 2013 - December 2013; May 2014 - August 2014.

- Research focused on developing animal model of essential tremor
- Performed rodent brain surgeries, assisted swine brain surgeries, processed swine deep brain stimulation fMRI data, ran behavioral tests monitoring rodent tremor, used fast-scan cyclic voltammetry and microdialysis to observe experimental changes in neurotransmitter levels, assisted with laboratory upkeep

Technical Skills

Research:

Versed in Experimental Design, Statistical Analyses, and Scientific Writing, Rodent Behavioral Testing for Drug Self-Administration, Jugular Vein Catheterization Surgery, Stereotactic Surgery for Implantation of Microdialysis Probe, Probe Construction, Immunohistochemistry, Gelatin Zymography, Tissue Fractionation and Western Blot.

Familiar with Rodent Behavioral Testing for Tremor, Stereotactic Surgery for Rodents for Deep Brain Stimulation, Optogenetics, Fast-Scan Cyclic Voltammetry, and Electric Field Potential Recording; Fluorescence Microscopy; Differentiation, Maintenance, and Passaging of 3D Human and Rodent Pluripotent Stem Cell Cultures; Designing and Cloning of Plasmids; RNA Extraction; Reverse Transcription-qPCR; Performed Microdialysis, fMRI Data Processing

Computer:

Experienced in Microsoft Office, Statistical Analyses. Familiar with Programming in R and MATLAB

Extracurricular Experiences

IUSM Surgery Student Interest Group Cluster (Surgery SIG Cluster), Indianapolis, IN

Founding Co-Chair, August 2019 - April 2021.

Neurosurgery Student Interest Group Representative, August 2018 - August 2019.

- Served as the Founding Co-chair following a pilot event in 2018 and wrote its constitution and vision statement, 2018-2019

- Hosted Surgery SIG Cluster Suture Workshops, an annual teaching and networking event where residents from multiple surgical subspecialties taught basic suturing and knot-tying to medical students, 2018-2019
- Developed a statewide network of representatives to extend extracurricular career development opportunities to medical students at satellite campuses, 2019-2020
- Created a Resident Panel Case Series, where residents from multiple surgical subspecialties presented standard cases from their specialty to allow medical students to efficiently compare careers, 2020-2021

IUSM Neurosurgery Student Interest Group, Indianapolis, IN

Co-Chair, 2017-2019.

Research Chair, 2016-2017.

Member, 2015-2016.

- Helped create the Indiana University School of Medicine Medical Student Chapter of the national professional organization, American Association of Neurological Surgeons, 2017
- Created a new Student Interest Group volunteer opportunity with the Goodman Campbell Brain and Spine Neurosurgery Department for the BrainBolt 5k, a local charity race, and led the coordination with IUSM's Student Interest Group in Neurology (SIGN) for NS-SIG's first collaborative event, 2018
- Revived membership by changing Student Interest Group focus from exclusively hosting events targeting students interested in neurosurgery to engaging the broader audience and local community, 2017-2019

Washington University in St. Louis Alumni and Parent Admission Program, Indianapolis, IN

Co-Chair, 2018-Present.

Member, 2015-2018.

- Organized and hosted alumni networking events
- Served as a mentoring resource for recent alumni as well as incoming students
- Hosted college fairs and interviewed high school students for admission

Wheeler Mission Homeless Shelter and Addiction Recovery Center, Indianapolis, IN

March 2016 - Present.

- Provided primary care services at the Addiction Recovery and Homeless Shelter
- Served meals to those who needed at the downtown Indianapolis Shelter

Indiana University Student Outreach Clinic, Indianapolis, IN

August 2015 - Present.

- Provide free healthcare to underserved populations of Indianapolis in coordination with the graduate schools of Medicine, Nursing, Pharmacy, Physical Therapy, Optometry, Dentistry, Law, and Social Work

Courses Taken

Medical

Biochemistry; Cellular & Molecular Biology; General Pathology; Gross Anatomy; Histology; Human Physiology; Introduction to Clinical Medicine; Medical Microbiology; Medical Genetics; Medical Immunology; Neuroscience & Clinical Neurology; Pharmacology

Graduate

Advanced Cytoplasmic & Nuclear Signaling; Biostatistics; Distilling Your Message: Communicating Science; Experimental Design & Research; Intracellular Signal Transduction; Principles of Pharmacology; Principles of Toxicology; Responsible Conduct of Translational Research; Reagent Validation for Research
Reproducibility