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EXAMINING THE CHRONIC EFFECTS OF INDIRECT AND DIRECT  
CANNABINOID RECEPTOR AGONISTS ON DOPAMINE TRANSMISSION IN THE  
NUCLEUS ACCUMBENS OF MICE

by

Kevin Honeywell

A Thesis

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## Abstract

A major problem with current anxiolytic medications is abuse liability; thus, new pharmaceutical targets are being explored. Cannabinergic and vanilloidergic signaling is of interest in the modulation of anxiety through cannabinoid type 1 receptor (CB<sub>1</sub>R) activation and transient vanilloid type 1 channel (TRPV<sub>1</sub>) inhibition. Arachidonoyl serotonin (AA-5-HT), a dual fatty acid amide hydrolase (FAAH) and TRPV<sub>1</sub> inhibitor, and arachidonyl-2-chloro-ethylamide (ACEA), a direct CB<sub>1</sub>R agonist, are drugs of interest in modulating these two systems. The current study explored the addictive potential of chronic AA-5-HT or ACEA administration in the open field (OF), during *in vivo* fixed potential amperometry (FPA), during conditioned place preference (CPP), and during saccharin preference. AA-5-HT did not alter locomotor activity in the OF, dopamine efflux in the NAc, CPP, and saccharin preference. ACEA altered dopamine dynamics in the NAc, but did not alter locomotor activity in the OF, CPP, or saccharin preference. These results suggest these drugs present little addictive potential.

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Examining the Chronic Effects of Indirect and Direct Cannabinoid Receptor  
Agonists on Dopamine Transmission in the Nucleus Accumbens of Mice

Uncertainty plays an integral role in human and non-human animal behaviors. Anxiety is an anticipatory process by which animals predict aversive events or fail to recognize safety signals, causing disinhibition of the default neurobiological stress response (Freels, Lester, & Cook, 2019; Brosschot, Verkuil, & Thayer, 2016). Anxiety is the anticipation of future threats or danger, leading to muscle tension and vigilance and is typically associated with an overestimation of the danger of possible, future threats (American Psychological Association, 2013). Anxiety-related behaviors and their central nervous system underpinnings are not inherently maladaptive. Correctly responding to a threatening situation with anxiety through aversion or other means is paramount to the safety of any individual. However, anxiety behaviors become problematic when such responses occur in situations that are objectively neutral or of a lower objective threat level than perceived (Brosschot, Verkuil, & Thayer, 2016). Anxiety disorders stem from excessive anxiety, typically lasting more than 6 months and can have symptoms including restlessness, muscle tension, fatigue, difficulty concentrating, irritability, incapacitation, and sleep disturbance all of which can disrupt positive relations and performance in work, school, and everyday life (American Psychological Association, 2013).

In the United States of America, anxiety disorders have become expensive and prevalent health issues. In 2009 and 2010, the adult population of the United States of America paid an estimated \$33 billion annually for anxiety-symptom-related treatment (Shrineshan et al., 2013). From interviews between 2001 and 2003, it was found that anxiety symptoms affected around 18.1% of the United States of America's adult population annually, of which 22.8% of those suffering or 4.1% of this country's adult population, had symptoms classified as severe (Kessler,

Chiu, Demler, & Walters, 2005). Alleviation of these symptoms in a non-addictive way is paramount to helping those who suffer from anxiety.

Benzodiazepines are a group of conventional anxiolytics, which are typically prescribed for acute and chronic anxiety. Two recognizable trade name drugs that fall into this category are Xanax and Valium. Between July 2007 and June 2008, pharmacies filled 112.8 million prescriptions for benzodiazepines (Cascade & Kalali, 2008). Benzodiazepines work by binding to benzodiazepine binding pockets on  $\gamma$ -Aminobutyric acid receptors (GABA<sub>A</sub>Rs), causing inhibition of neuronal transmission in the amygdala, which is thought to mediate brain circuits associated with the appraisal of threat (Starcevic, 2012). Additionally, these drugs positively modulate GABA<sub>A</sub>Rs on interneurons in the ventral tegmental area (VTA) causing dopamine neurons to fire by increasing the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor/*N*-methyl-D-aspartate (NMDA) receptor ratio mediated frequency of miniature excitatory synaptic currents (Braestrup & Squires, 1977; Heikkinen, M ykkynen, & Korpi, 2009; Tan et al., 2010). The associated changes in neurotransmission may be indicative of abuse potential. People utilizing chronic benzodiazepine treatment for anxiety are at risk for developing dependence, which can lead to abuse, overdose, and even death (Farach et al., 2012). Between 1996 and 2013, the rate of fatal overdoses involving benzodiazepines increased over 4-fold to 3 per 100,000 adults, which is faster than the increased rate of prescriptions filled (Bachhuber, Hennessy, Cunningham, & Starrels, 2016). Such problems limit the use of benzodiazepines for treating chronic anxiety and have physicians seeking alternate pharmaceuticals.

Antidepressants including selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs) are another group of conventional anxiolytics often

used as an alternative treatment for chronic anxiety. However, this class of drugs has been associated with high non-response rates (such as 44%) and long periods (e.g., four months) between drug onset and therapeutic benefits (Montgomery, Sheehan, Meoni, Haudiquet, & Hackett, 2002). Additionally, anxiety symptoms can worsen in the first two weeks of treatment with an SSRI for patients with co-morbid major depressive disorder (Gollan et al., 2012). Such issues lend support for the investigation of new pharmacotherapies for anxiety. The cannabinergic system is a promising target, which could provide anxiolytic effects without the drawbacks of benzodiazepines and antidepressants.

### **Cannabinoid system**

The cannabinergic system consists of two primary types of cannabinoid receptors (CBRs), type 1 (CB<sub>1</sub>Rs) and type 2 (CB<sub>2</sub>Rs), with CB<sub>1</sub>Rs being the main target for anxiolytic effects. CB<sub>1</sub>Rs are predominately expressed in the central nervous system (CNS) where they are found at significantly higher levels on GABAergic than glutamatergic neurons in various brain regions (Howlett et al., 2002; Kano, Ohno-Shosaku, Hashimoto-dani, Uchigashima, & Watanabe, 2009). CB<sub>1</sub>Rs are concentrated in high densities in brain regions associated with the regulation of emotional processing and behaviors related to anxiety such as the prefrontal cortex, basolateral amygdala, and ventral hippocampus (Katona et al., 1999; Rubino et al., 2008). Activation of glutamate receptors causes Ca<sup>2+</sup> influx and postsynaptic neuron depolarization, which then triggers the release of the endocannabinoids (eCBs). Specifically, N-arachidonyl-ethanolamide (AEA) that binds to CB<sub>1</sub>R and 2-arachidonylglycerol (2-AG) that binds to CB<sub>1/2</sub>R through retrograde transmission are released (Batista et al., 2014; Howlett et al., 2002; Kano et al., 2009). AEA and 2-AG then inhibit voltage activated Ca<sup>2+</sup> channels, inhibiting pre-synaptic neurotransmission before being trafficked by fatty acid binding protein-5 (FABP5) and fatty acid



binding protein-7 (FABP7) to the endoplasmic reticulum and metabolized by fatty acid amide hydrolase (FAAH) in presynaptic terminals and monoacylglycerol lipase (MAGL) in postsynaptic terminals respectively (Blankman & Cravatt, 2013; Dinh et al., 2002; Giang & Cravatt, 1997; Gulyas et al., 2004; Haj-Dahmane et al., 2018; Kaczocha, Glaser, & Deutsch, 2009; Ohno-Shosaku, Maejima, & Kano, 2001; Maccarrone, 2017; Maione et al., 2007). The modulation of cannabinergic signaling can alter behaviors and may be a target for the development of new pharmaceuticals.

The pharmacological manipulation of the cannabinoid system can be used as a target for anxiolytic treatment. Rodent studies indicate that pharmacologically stimulating CB<sub>1</sub>Rs or increasing eCB levels (via inhibition of FAAH or eCB transporters) promote anxiolytic-like behaviors in the elevated plus maze (EPM), open field (OF), and light/dark box (LDB) (Fogaça, Aguiar, Moreira, & Guimarães, 2012; Freels, Lester, & Cook, 2019; Hakimzadeh, Oryan, Moghaddam, Shamsizadeh, & Roohbakhsh, 2012; Zaitone, El-Wakeil, & Abou-El-Ela, 2012). Thus, activation of CB<sub>1</sub>Rs may be a target for the treatment of anxiety.

It is important to note that CB<sub>1</sub>R agonists act in a dose dependent manner where lower doses are anxiolytic and higher doses can be anxiogenic (Patel & Hillard, 2006). This biphasic effect can be attributed in part to the dose-dependent modulation of glutamatergic and GABAergic signaling. Low doses of cannabinoids inhibit glutamatergic neurons, producing anxiolytic effects, and high doses inhibit GABAergic neurons, producing anxiogenic effects (Rey, Purrio, Viveros, & Lutz, 2012). These effects may be associated with the distribution or sensitivity of the CB<sub>1</sub>Rs on each of these neuron types. CB<sub>1</sub>Rs on glutamatergic presynaptic neuron terminals display an around 30-fold lower sensitivity to CBR agonists than those on GABAergic presynaptic neuron terminals (Ohno-Shosaku et al., 2002; Rey, Purrio, Viveros, &

Lutz, 2012). Additionally, downstream effects may play an integral role in this modulation. In the amygdala, CB<sub>1</sub>R activation inhibited glutamatergic signaling (leading to depolarization induced suppression of excitation: DSE) and GABAergic signaling (leading to depolarization induced suppression of inhibition: DSI), both of which may be modulating factors in anxiety-like behaviors (Azad et al, 2003; Kamprath et al., 2011). Therefore, in finding potential pharmacological treatments for anxiety, it is paramount to ascertain the correct dosage in order to prevent the inverse of the desired effect. The neuropharmacology of eCBs are further complicated when considering other receptor types activated by eCBs.

### **TRPV<sub>1</sub> channel activity**

The transient receptor potential cation channel subfamily V member 1 (TRPV<sub>1</sub>) is another target for altering neurotransmission and potentially alleviating anxiety as eCB activation of these receptors is anxiogenic. TRPV<sub>1</sub>s are Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup> permeable ion channels, which are located on both presynaptic and postsynaptic neurons and are predominately localized on glutamatergic neurons (Caterina et al., 2000; Iannotti et al., 2004; Kaur & Gibson, 2009; Marinelli et al., 2007). AEA enters the cell via the AEA membrane transporter (AMT) where it functions as an agonist at an intracellular pocket on TRPV<sub>1</sub>, which causes an influx of Ca<sup>2+</sup> ions (De Petrocellis et al., 2001; Ross, 2003). This activation of TRPV<sub>1</sub> induces cellular depolarization (Rosenbaum & Simon, 2007). This causes an excitatory response in the brain that may induce anxiety and anxiety-like behaviors.

Antagonists of TRPV<sub>1</sub>s are potentially anxiolytic. For example, capsazepine, a TRPV<sub>1</sub> antagonist, induced anxiolytic-like behaviors in the EPM (Aguiar, Terzian, Guimarães, & Moreira, 2009; Hakimzadeh et al., 2012; Terzian, Aguiar, Guimarães, & Moreira, 2009), suggesting the TRPV<sub>1</sub> plays the opposite role of the CB<sub>1</sub>R in anxiety. Additionally, TRPV<sub>1</sub>

knockout mice expressed more anxiolytic-related behaviors in LDB and EPM (Marsch et al., 2007). Therefore, the TRPV<sub>1</sub> may be a target for antagonism in the treatment of anxiety.

Though, the modulation of TRPV<sub>1</sub> is complicated due to some agonists at this receptor also agonizing CB<sub>1</sub>R. Importantly, the eCB AEA is an agonist for both CB<sub>1</sub>Rs and TRPV<sub>1</sub>s (Kaur & Gibson, 2009; Smart et al., 2000). Additionally, exogenous CBs such as cannabidiol (CBD) can also activate TRPV<sub>1</sub> (Iannotti et al., 2014). Furthermore, the endogenous villanoid N-arachidonoyl-dopamine (NADA) can bind to CB<sub>1</sub>Rs and TRPV<sub>1</sub>s located in midbrain regions housing dopamine neurons, thus modulating dopaminergic neurotransmission important for both anxiety and addiction (de la Mora, Gallegos-Cari, Arizmendi-Gracia, Marcellino, & Fuxe, 2010; Marinelli et al., 2007). Additionally, CB<sub>1</sub>Rs and TRPV<sub>1</sub>s are co-localized in the nucleus accumbens (NAc) shell (predominately) and core of mice (Micale et al, 2009). Therefore, eCBs can modulate activity at these receptors in areas implicated in reward. This crosstalk lends support for the utilization of TRPV<sub>1</sub> antagonism as well as CB<sub>1</sub>R agonism as a means for reducing anxiety-like behaviors and the development of novel anxiolytic drugs.

### **AA-5-HT**

N-Arachidonoyl-serotonin (AA-5-HT) is a potential drug of interest in treating anxiety. AA-5-HT is an endogenous inhibitor of FAAH catalyzed hydrolysis of AEA and antagonist of TRPV<sub>1</sub>s (Maione et al., 2007). AA-5-HT administration in male C57BL/6J (B6) mice elicited anxiolytic effects in the EPM and was more potent than URB597 (FAAH blocker) or SB3667791 (TRPV<sub>1</sub> blocker) when used individually, suggesting inhibiting both the enzyme and the receptor simultaneously is more efficacious (Micale et al., 2009). Additionally, the co-administration of AA-5-HT and SB3667791 (TRPV<sub>1</sub> blocker) enhanced the anxiolytic effect, which may suggest the antagonists work in concert (Micale et al., 2009). These results were mitigated by

administration of either a selective CB<sub>1</sub>R antagonist or a selective TRPV<sub>1</sub> agonist; although, it should be noted that these effects were strain specific to B6 mice and varied for Swiss mice (Micale et al., 2009). For example, administration of 2.5 mg/kg of AA-5-HT via intraperitoneal (i.p.) injections in Swiss mice did not significantly alter anxiolytic behaviors in the EPM when injected either once 30 mins before testing or sub-chronically, every other day for seven days but did induce anxiolytic behaviors in the EPM when injected chronically, every day for seven days (Micale et al., 2009). In a study done using Sprague-Dawley rats, the administration of AA-5-HT into the intra-basolateral amygdala (BLA) also produced anxiolytic effects in the EPM (John & Currie, 2012). Furthermore, previous studies from our lab have shown that BALB/cJ (BCJ) mice, a relatively more anxious strain, expressed moderate anxiolytic-like behaviors in the LDB and OF after acute i.p. injections of AA-5-HT (Freels, Lester, & Cook, 2019). *In vivo* Fixed potential amperometry (FPA) in B6 mice found decreased dopamine release in the NAc following an injection of AA-5-HT, suggesting AA-5-HT is not highly rewarding, which may mitigate the potential for abuse (Freels, Lester & Cook, 2019). The chronic administration of AA-5-HT may alter the dopamine dynamics in the NAc via CB<sub>1</sub>R indirect agonism in a different way than acute administration, and, thus, it is important to discern how chronically increasing the activation of CB<sub>1</sub>Rs indirectly via AA-5-HT administration alters anxiety-related behaviors and dopamine dynamics.

## **ACEA**

Arachidonyl-2-chloro-ethylamide (ACEA) is another drug that increases CB<sub>1</sub>R activation. ACEA is a selective agonist for this receptor but does have some affinity in high concentrations for TRPV<sub>1</sub> as a partial agonist (Casarotto et al., 2012; Price, Patwardhan, Akopian, Hargreaves, & Flores, 2004). Administration of ACEA has been shown to moderate

depression and anxiety-related behaviors (Freels, Lester, & Cook, 2019; Rutkowska & Jachimczuk, 2004; Simone, Malivoire, & McCormick, 2015). ACEA administration produced anxiolytic-like behaviors in Wistar rats in the EPM and Vogel conflict tests (VCT) (Fogaça, Aguiar, Moreira, & Guimarães, 2012). Additionally, ACEA increased the threshold of electric stimulation of the midbrain dorsal periaqueductal gray (PAG) required to induce panic-like behaviors in male Wistar rats (Casarotto et al., 2012). The mechanism for this drug is related to the regulation of CB<sub>1</sub>R signaling and glutamate transmission. ACEA agonism of CB<sub>1</sub>R depressed evoked glutamate release (Fawley, Hofmann, & Anderson, 2014). Additionally, the administration of ACEA prevented stress induced upregulation of CB<sub>1</sub>R mRNA and restored excitatory amino acid transporter-2 (EAAT-2) protein expression, which prevented stress induced reductions in glutamate uptake (García-Bueno, Caso, Pérez-Nievas, Lorenzo, & Leza, 2007; Zoppi et al., 2011). *In vivo* FPA in B6 mice found decreased dopamine release in the NAc following an injection of ACEA, suggesting ACEA is not highly rewarding (Freels, Lester & Cook, 2019). The chronic administration of ACEA may alter the dopamine dynamics in the NAc in a different way than acute administration. Therefore, ACEA plays a role in modulating CB<sub>1</sub>R expression, depressing glutamate and dopamine activity, and decreasing anxiety-like behaviors.

### **Dopamine system**

The dopamine system is comprised of morphologically and functionally differing receptors. The main receptors for the dopamine system are divided into two categories comprised of D1-like receptors (D1 and D5), which stimulation increases cyclic adenosine monophosphate (cAMP), and D2-like receptors (D2, D3, and D4), which stimulation decreases cAMP (Neve, Seamans, & Trantham-Davidson, 2004; Stoof & Keabian, 1981). The dopamine

D2 receptor (D<sub>2</sub>R) can be subdivided into two isoforms with distinct morphology and function, which are the long (D<sub>2</sub>L) a postsynaptic receptor and the short (D<sub>2</sub>S) a presynaptic autoreceptor forms (Usiello et al., 2000). The dopamine autoreceptor (DAR) on dopamine neurons are comprised of this subdivision and modulate dopamine transmission through binding dopamine, which cascades into regulating the expression of tyrosine hydroxylase (TH) and the plasma membrane dopamine transporter (DAT) in the VTA (Ford, 2014). The DAT can transport dopamine into and from the presynaptic neuron. The DAT typically transports dopamine into the cell via a Na<sup>+</sup>/Cl<sup>-</sup> dependent mechanism (Khalig & Galli., 2005). Dopamine receptors predominately exist in the brain along pathways associated with cognition (mesocortical dopamine pathway), lactation (tuberoinfundibular pathway), locomotion (nigrostriatal pathway, hypothalamospinal projection, and incertohypothalamic pathway), and reward (mesolimbic pathway).

The mesolimbic dopamine pathway has been implicated in anxiety. This pathway is comprised of dopamine cell bodies located in the VTA that project to the limbic system, most notably the NAc (Wise & Bozarth, 1984). Stress-related events alter signaling in the mesolimbic dopamine pathway. Acute, forced, and, cold swim test stress increased the AMPA/NMDA ratio of EPSCs and thus enhanced the strength of excitatory synapses on midbrain dopamine neurons (Saal, Dong, Bonci, & Malenka, 2003). Restraint stress increased dopamine levels in the NAc for 40 min post-stress before returning to baseline, and release from restraint increased dopamine levels in the NAc (Imperato, Angelucci, Casolini, Zocchi, & Puglisi-Allegra, 1992; Imperato, Puglisi-Allegra, Casolini, & Angelucci, 1991). Social isolation stress increased anxiety-like behaviors in the EPM and increased dopamine release and DAT activity but not DAR activity in

the NAc core (Yorgason, España, Konstantopoulos, Weiner, & Jones, 2013). Therefore, stress alters signaling in this pathway in a type and time-course dependent manner.

Additionally, the mesolimbic dopamine pathway has been implicated in reward and abuse liability for posited drugs. Naturally and pharmacologically increasing dopamine transmission in this pathway has been implicated in reward and addiction. Acute drug administration including cocaine, amphetamine, morphine, nicotine, and ethanol enhanced the strength of excitatory synapses on midbrain dopamine neurons like acute stress did (Saal, Dong, Bonci, & Malenka, 2003). The mesolimbic dopamine pathway's signals are associated with the salience of predicted cues for a reward including drugs of abuse (Berridge & Robinson, 1998; Day, Roitman, Wightman, & Carelli, 2007; Everitt & Robbins, 2005). Seeking and taking addictive drugs activate this pathway, and the modulation of this pathway has been implicated in the rewarding nature of cannabinoids (Gardner, 2011; Ginovart et al., 2012). Therefore, discerning the alterations in dopamine signaling in the mesolimbic pathway can signify the abuse liability of a drug, which is additionally prevalent to anxiolytics as anxiety also alters dopamine signaling in this pathway.

### **Cross-modulation of cannabinoid and dopamine systems**

Neurotransmitters and drugs of specific receptors can alter the sensitivity, expression, and activation of other receptors. This cross-modulation between systems plays an integral role in the efficacy and liability of potential pharmaceuticals including anxiolytics. Therefore, discerning and evaluating the changes in various neurotransmitter pathways grants a better understanding of the potential off-target, especially undesired, effects from the administration of a given drug.

Cannabinoid administration can alter dopaminergic signaling. Chronic  $\Delta^9$ -tetrahydrocannabinol (THC) administration upregulated  $D_3R$  mRNA and binding in the NAc and  $D_{2/3}R$  in the VTA, leading to super-sensitization, which may play a role in the rewarding and addictive nature of this drug (Ginovart et al., 2012). On the contrary, THC desensitizes  $CB_1Rs$  via removal by an arrestin (Morgan et al., 2014). Therefore,  $CB_1R$  activation sensitizes  $D_{2/3}Rs$  and desensitizes  $CB_1Rs$ .  $CB_1R$  agonist administration attenuated dopamine release in the NAc of mice and rats (Cheer, Wassum, Helen, Philips, & Wightman, 2004; Freels, Lester, & Cook, 2019; O'Neill, Evers-Donnelly, Nicholson, O'Boyle, & O'Connor, 2009). In male Swiss Webster mice, rimonabant ( $CB_1R$  antagonist) administration before cocaine decreased locomotor activity and percent change in dopamine in the NAc core but not in the shell, and URB597 (FAAH blocker) administration before cocaine increased locomotor activity and percent change in dopamine in the NAc core (Mereu et al., 2013). However, in male Sprague-Dawley rats, WIN-55,212-2 ( $CB_1R$  agonist) reduced dose-dependent increases in cocaine-induced locomotor activity (Vlachou, Stamatopoulou, Nomikos, & Panagis, 2008). These contrary results may be due to the biphasic effects associated with cannabinoid administration where the FAAH blocker may have led to the inhibition of glutamatergic signaling and the direct  $CB_1R$  agonist may have led to the inhibition of GABAergic signaling. Thus, effects may differ when comparing indirect eCB enhancers (such as FAAH blockers) and direct  $CB_1R$  agonists. Cross-sensitization of the cannabinergic system with drugs such as AA-5-HT with other neurotransmitter systems warrants further investigation.

The cannabinoid system may still be a target for treating anxiety utilizing drugs, which are not direct agonists of this system and may not increase dopamine in the mesolimbic dopamine pathway.  $TRPV_1$  and FAAH have implications in dopamine modulation. Agonism



and antagonism of TRPV<sub>1</sub> may modulate dopamine signaling. TRPV<sub>1</sub> agonist administration in the VTA increases dopamine firing and concentrations in the NAc, TRPV<sub>1</sub> antagonism co-administration prevented this increase, and these changes are caused via alterations in glutamatergic transmission onto these neurons (Marinelli, Pascucci, Bernardi, Puglisi-Allegra, & Mercuri, 2005). Inhibition of FAAH via URB597 alone did not increase basal levels of dopamine or peak dopamine response, but URB597 administration followed by AEA increased the peak dopamine response (Solinas, Justinová, Goldberg, & Tanda, 2006). This suggests the dopaminergic effect requires the combination of the FAAH blocker and eCB AEA administration. Previous research in our lab has shown that acute administration of AA-5-HT decreased stimulation-evoked dopamine in the NAc of mice (Freels, Lester, & Cook, 2019). Similarly, using microdialysis, Murillo-Rodríguez and colleagues (2017) recently showed that AA-5-HT administration decreased the extracellular content of dopamine and reduced the increase in extracellular dopamine caused by the administration of CBD in male Wistar rats. Therefore, drugs that do not directly activate the CB<sub>1</sub>R but instead act in indirect ways such as FAAH inhibition or TRPV<sub>1</sub> antagonism may not increase dopamine release and thus may not be rewarding.

Drugs that act on the cannabinergic system may have potential as anxiolytics but also may carry an abuse potential. Indirect and direct modulation of this system has been posited as a potential target for the treatment of anxiety (Freels, Lester, & Cook, 2019; Micale et al., 2009). However, little is known about the effects of chronic administration of indirectly modulating drugs. Investigating indirect and direct modulation of the cannabinergic system on addiction-related behaviors and dopamine signaling, especially when chronically administered, would determine the addictive potential of these posited pharmaceuticals.

## **Cannabinoids and anhedonia**

When exploring the anxiolytic potential of posited pharmaceuticals, anhedonia is of concern. Anhedonia is a reduction in the ability to experience pleasure or a diminished interest in pleasurable activities (American Psychological Association, 2013). A two-bottle choice test for a sweet liquid can be utilized to explore the role of drug administration on anhedonia (Liu et al., 2018). Dopamine signaling plays a role in anhedonia. D<sub>1</sub>R antagonist administration attenuated the acquisition of a sucrose or fructose preference (Azzara, Bodnar, Delamater, & Sclafani, 2001; Bernal et al., 2009; Fazilov et al., 2018; Kraft et al., 2015; Muscat & Willner, 1989). CB<sub>1</sub>R signaling also plays a role in anhedonia. CP55940 (a CB<sub>1</sub>R agonist), URB597, and rimonabant had no effects on saccharin preference alone, but chronic mild stress-induced reductions in sucrose preference were attenuated by CP55940 or URB597 and enhanced by rimonabant (Bortolato et al., 2007; Rademacher & Hillard, 2007). CB<sub>1</sub>R-knockout on D<sub>1</sub>R expressing neurons mildly reduced sucrose preference (Terzian, Drago, Wotjak, & Micale, 2011). Anhedonia-related behaviors may be associated with reductions in dopaminergic signaling as research has shown that CB<sub>1</sub>R agonist administration attenuated dopamine release in the NAc of mice and rats (Cheer, Wassum, Helen, Philips, & Wightman, 2004; Freels, Lester, & Cook, 2019; O'Neill, Evers-Donnelly, Nicholson, O'Boyle, & O'Connor, 2009). Therefore, reducing the activation of D<sub>1</sub>Rs through antagonism or through synaptic dopamine reductions by CB<sub>1</sub>R indirect or direct agonism may lead to anhedonia.

The cannabinergic system and the dopaminergic system are linked to anxiety and reward, and the cross-modulation of these systems require further exploration in order to assess the anxiolytic potential of drugs that indirectly or directly agonize the cannabinergic system and to evaluate the associated addiction- and anhedonia-related behaviors. The length of drug

administration, the size of the dosage, the pharmacology of the posited anxiolytic, and the type of stress all impact the interactions between these systems and their associated behaviors.

### **Current study**

The current study explored the addictive potential of the posited anxiolytics AA-5-HT and ACEA. Specifically, the project determined the effect of repeated AA-5-HT or ACEA administration on NAc dopamine transmission. Mice were pretreated with a chronic, seven-day dose of either AA-5-HT, ACEA, or vehicle (negative control). Locomotor activity and behaviors related to addiction were assessed using the open field, and aspects of dopamine transmission were assessed by *in vivo* FPA. As mentioned above, Freels and colleagues from our lab (2019) have done similar experiments with AA-5-HT and ACEA, using only acute injections, finding both drugs decrease NAc dopamine release. The current study is more generalizable compared to our previous study by using dosing more applicable to the human population (chronic use). Chronic administration of these drugs also allows us to address the aforementioned issues of sensitization. To determine cross-sensitization effects between the cannabinoid and dopamine systems, a subset of mice was administered the dopamine reuptake blocker cocaine during amperometric recordings. Overall, this study examines the effects of chronic cannabinoid activation (by both indirect and direct agonists) on the mesolimbic dopamine system and the way this pathway responds to a dopamine agonist.

### **Methods**

All procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Memphis and were also aligned with those outlined in The Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health 2012) and the Guidelines for the Care and Use of Mammals in Neuroscience and

Behavioral Research (National Research Council 2003). The current study was split into two major experiments. In the first, mice underwent chronic, seven-day drug treatment, the OF on day one and day seven, and then *in vivo* FPA on day eight. In the second, mice underwent CPP for eight days followed by two-bottle choice test between saccharin and water for five days.

## **Animals**

Fifty-four, male C57BL/6J mice ( $n = 54$ ) for the first experiment and 27, male C57BL/6J mice ( $n = 27$ ) for the second experiment were obtained from Jackson Laboratory (Bar Harbor, ME) and were housed 2-5 per cage in 18.5 x 29.5 cm polycarbonate Generic State Microisolators with Sani-Chips bedding (P.J. Murphy Forest Products, Montville, New Jersey). Food and water were available ad libitum. Mice were housed in a temperature-controlled room ( $21 \pm 1^\circ \text{C}$ ) with a 12 h light: 12 h dark cycle. At the time of drug treatments and experiments, mice were 3 to 5 months old (adults).

## **Chronic Drug Treatments**

For the first experiment, mice were randomly selected to receive one of three chronic treatments. Chronic drug treatments consisted of one i.p. injection per day for seven days of either AA-5-HT (2.5 mg/kg,  $n = 19$ ), ACEA (1.0 mg/kg,  $n = 15$ ), or vehicle ( $n = 20$ ) all acquired from Sigma Aldrich (St. Louis, MO). This amount of AA-5-HT reflects the dose that was utilized by Micale et al. (2009) and caused behavioral changes related to anxiety in both B6 and Swiss mice. This amount of ACEA reflects the dose that was utilized by Rutkowska and Jachimczuk (2004) and Freels, Lester, and Cook (2019), which trended to or moderately caused behavioral changes related to stress. On the eighth day, during *in vivo* FPA, mice received an i.p. injection of either AA-5-HT (2.5 mg/kg,  $n = 13$ ), ACEA (1.0 mg/kg,  $n = 9$ ), cocaine (10.0 mg/kg,  $n = 17$ ) or vehicle ( $n = 15$ ). As can be seen in *Table 1*, the ten total groups encompassed AA-5-

HT-AA-5-HT ( $n = 7$ ), AA-5-HT-vehicle ( $n = 6$ ), AA-5-HT-cocaine ( $n = 6$ ), ACEA-ACEA ( $n = 5$ ), ACEA-vehicle ( $n = 5$ ), ACEA-cocaine ( $n = 5$ ), vehicle-AA-5-HT ( $n = 6$ ), vehicle-ACEA ( $n = 4$ ), vehicle-vehicle ( $n = 4$ ), and vehicle-cocaine ( $n = 6$ ) where the first drug represents that given during behavioral testing and the second drug represents that given during *in vivo* FPA. For, the second experiment, mice were also randomly selected to receive one of three chronic treatments. Chronic drug treatments consisted of one i.p. injection every other day for six days, two days off, then five days of either AA-5-HT (2.5 mg/kg,  $n = 9$ ), ACEA (1.0 mg/kg,  $n = 9$ ), or vehicle ( $n = 9$ ) all acquired from Sigma Aldrich (St. Louis, MO). To minimize outside anxiogenic variables, drug treatments were administered by the same person, at the same time each day.

### **Open Field**

For the first experiment, mice were tested in the OF chamber twice, once on the first day of the chronic drug treatment and again on the seventh day of treatment. On test day, each mouse was placed in a single holding cage inside a sound attenuated cabinet in the testing room for 45 minutes in order to habituate the mouse. At the beginning of each OF test, the mouse was placed in the center of the OF chamber. The OF apparatus was a HamiltonKinder SmartFrame™ (HamiltonKinder, Poway, CA) with a clear Plexiglass insert with dimensions of 24.13 cm x 45.72 cm, a 4 x 8 photo beam strip, and a 4 x 8 photo beam rearing attachment. During the session, software (MotorMonitor version 4.14, HamiltonKinder, Poway, CA) tracked the time spent in the central area, time spent outside of the central area, rearing, and total distance travelled. The central area specified in the software's zone map function was a of 9 cm x 10 cm space positioned 4.5 cm from the left and right walls and 15 cm from the front and back walls of the chamber. After a 20 min baseline movement assessment, the mouse received an i.p. injection of the assigned chronic treatment (AA-5-HT, ACEA, or vehicle) and testing continued

for 90 min post-injection. At the end of the test, each mouse was returned to its home cage in the mouse colony. The OF chamber was then cleaned with 10% isopropyl alcohol and allowed to dry after each trial.

### **Dopamine Recordings**

For the first experiment, on the eighth day of the chronic drug treatments, all mice underwent stereotaxic surgery for the measurement of dopamine transmission using *in vivo* FPA. Mice were permanently anaesthetized with urethane (1.5 g/kg, i.p.). The dose of which was split into two i.p. injections separated by 10 min. Fifteen min following the second injection, mouse anesthesia was assessed through eye blink, mild tail pinch, and mild foot pinch reflexes. Mice were mounted into a stereotaxic frame, and their body temperature was kept at approximately 37°C. A stimulating electrode (Rhodes Medical Co., Summerland, CA) was placed into the left VTA (coordinates in mm from bregma: AP -3.3, ML +0.3, and DV -4.0 from dura; Paxinos & Franklin, 2001). A Ag/AgCl reference and stainless-steel auxiliary electrode combination was placed on contralateral cortical tissue -2.0 mm to bregma, and a carbon fiber recording electrode (500  $\mu$ m length x 7  $\mu$ m o.d.; Union Carbide, North Seadrift, TX) was positioned in the left NAc (coordinates in mm from bregma: AP +1.5, ML +1.0, and DV -4.0 from dura; Paxinos & Franklin 2001). A fixed +0.8V current was continuously applied to the recording electrode, which oxidized the dopamine. The change in current due to the oxidation of dopamine was monitored by the electrometer (filtered at 50 Hz) 10,000 times per second.

Stimulation parameters varied depending on the aspect of dopamine transmission being measured. Initially, while establishing a baseline response, the stimulation protocol consisted of 20 monophasic 0.5 ms duration pulses (800  $\mu$ Amps) at 50 Hz. DAR sensitivity was assessed by applying a pair of test stimuli (T1 and T2, each 10 pulses at 50 Hz with 10 sec between T1 and

T2) to the VTA every 30 sec (Fielding et al., 2013, Holloway et al., 2018, and Mittleman et al., 2011). Six sets of conditioning pulses (1, 5, 10, 20, 40, and 80; 0.5 ms pulse duration at 15 Hz) were delivered prior to T2 such to leave 0.3 s between the end of the conditioning pulse train and initiation of T2. DAR-mediated inhibition of evoked dopamine efflux was expressed in terms of the change in the amplitude of T2 with respect to T1 for each set of conditioning pulses; low-to-high DAR sensitivity was represented as low-to-high percent inhibition of evoked dopamine efflux (i.e. high sensitivity results in lower amplitude of T2 relative to T1).

Upon completion of the autoreceptor sensitivity test, stimulation parameters were reset to 20 pulses at 50 Hz every 30 sec. Following 5 min of baseline dopamine efflux recording, each mouse was given a drug challenge via an i.p. injection of AA-5-HT (2.5 mg/kg), ACEA (1.0 mg/kg), cocaine (10 mg/kg) or vehicle. See *Table 1* or the chronic drug treatments subsection for the experimental groups. Dopamine recordings continued for 90 min post drug challenge for AA-5-HT, ACEA, and vehicle groups and for 60 min post drug challenge for cocaine groups. After the recordings were complete, direct anodic current of 100  $\mu$ Amps was applied to the stimulating electrode for 10 s to create an iron deposit, which marked the electrode's position.

Mice were euthanized via an intracardial injection of urethane (0.345 g/mL). Brains were removed and stored in 30% sucrose / 10% formalin solution with 0.1% potassium ferricyanide. Coronal sections of each brain were sliced at  $-30^{\circ}\text{C}$  using a cryostat, and electrode placements were identified using a light microscope and marked on coronal diagrams (Paxinos & Franklin, 2001). Following the experiment, *in vitro* electrode calibration occurred by recording in solutions of dopamine (0.2  $\mu\text{M}$  – 1.2  $\mu\text{M}$ ) via a flow injection system (Dugast, Suaud-Chagny, & Gonon, 1994), which allowed for the conversion of current measurements to dopamine concentrations.

## Conditioned Place Preference

For the second experiment, mice were tested in conditioned place preference (CPP) chambers that were counterbalanced across conditions. On test day, each mouse was placed in a single holding cage inside a sound attenuated cabinet in the testing room for 45 min in order to habituate the mouse. On the first day, the mouse was placed into the front part of the HamiltonKinder SmartFrame™ (HamiltonKinder, Poway, CA) with an insert to divide the OF into two halves measuring 21.84 cm x 22.23 cm each and a doorway between them measuring 6.99 cm (width) x 8.89 cm (height). The walls of the chamber were covered with either vertical or horizontal black and white bars each measuring 2.54 cm thick. The vertical bars side was paired with Sani-chips bedding (P.J. Murphy Forest Products, Montville, New Jersey), and the horizontal bars side was paired with So Phresh Natural Softwood bedding (Petco Animals Supplies, San Diego, CA). The beddings were covered by a black grating. The test occurred for 30 min. On day two to seven, the mouse was given an i.p. injection of either the assigned drug treatment or vehicle, starting with drug treatment and alternating each day. The mouse was then placed into a specific side of the CPP chamber where the other side was blocked off by a black, opaque insert over the doorway. The side in which the mouse was placed was alternated depending on whether the mouse was receiving the drug treatment or vehicle on that day. The test occurred for 30 min. On the eighth day, the mouse was returned to the CPP chamber with the door insert removed for 30 min. At the end of each test, the mouse was returned to its home cage in the mouse colony. The OF chamber was then cleaned with 10% isopropyl alcohol and allowed to dry after each trial.



## **Two-Bottle Choice Test**

Additionally, for the second experiment, one day following CPP testing, mice underwent two-bottle choice test for saccharin or water preference. Saccharin is a non-satiating sweetener for which animals develop a preference (Collier & Novell, 1967; Hausmann, 1933; Sheffield & Robey, 1950). A reduction in the preference ratio for saccharin during this test is indicative of anhedonia (Liu et al., 2018). Mice were weighed and then deprived of water for 12 hours. Following water deprivation, mice were injected with AA-5-HT (2.5 mg/kg,  $n = 9$ ), ACEA (1.0 mg/kg,  $n = 9$ ), or vehicle ( $n = 9$ ). One bottle of saccharin (0.001 g/ml water) and one bottle of water were weighed and fixed onto a second cage with the same dimensions of the home cage. Then, mice were placed individually into the cages for 2 h to examine drinking preference. After the 2 h test, mice were returned to the home cage, and the bottles were weighed again. The first day was considered a trial, days two to six were utilized for examining group differences.

## **Design and Statistical Analyses**

For the first experiment, data was analyzed in two separate sets, one for behavioral data and one for electrochemical data. OF data was analyzed using three-way ANOVAs for day x block x treatment effects, which includes distance travelled, rears, and percent time in center. Significant treatment differences indicated by  $p < 0.05$  were further explored using Games-Howell post-hoc tests when appropriate. Electrochemical data were broken down into autoreceptor sensitivity, baseline dopamine recordings, and drug challenge recordings. Dopamine oxidation current recordings were used to quantify VTA stimulation-induced dopamine release in the and NAc by extracting data points occurring between 0.25 s pre- and 10 s post-stimulation at 10 min intervals (11 total intervals for AA-5-HT, ACEA, and vehicle and 7 total intervals for cocaine). Dopamine autoreceptor sensitivity was assessed by applying a pair

of test stimuli (T1 and T2), which were each 10 pulses at 50 Hz with 10 s between them to the VTA every 30 s. In between the two test stimuli, for every other set of test stimuli, sets of 1, 5, 10, 20, 40, and 80 conditioning pulses of .5 ms at 15 Hz were delivered prior to T2 such that there was 0.3 s between the end of the conditioning pulse train and initiation of T2. These stimulation parameters are like previous studies exploring dopamine dynamics (Fielding et al., 2013; Holloway et al., 2018; Mittleman et al., 2011). Dopamine half-life (in seconds) was calculated using the formula: (peak release time - baseline return time) / 2 (Mittleman et al., 2011). Data from *in vitro* calibrations of recording electrodes were used to convert the mean change in dopamine oxidation current (nA) to a mean concentration ( $\mu\text{M}$ ). Change in the concentration of dopamine release was expressed as an average percent change relative to baseline release (pre-drug = 100%). For the autoreceptor sensitivity test, a two-way repeated measures ANOVA was used to assess the impact of pretreatment and amount of pre-pulses on percent inhibition. For the baseline and drug challenge, a two-way repeated measures ANOVA was used to assess the impact of treatment and time point on percent change in dopamine release and percent change in dopamine half-life in the NAc of B6 mice. A one-way ANOVA was used to test the effects of treatment on percent change in dopamine release at each time point. Significant treatment differences indicated by  $p < .05$  were further explored using Tukey's HSD post-hoc or Games-Howell post-hoc tests when appropriate.

For the second experiment, the two sets of behavioral data were analyzed separately. For CPP, bias on the first day was analyzed by a one samples *t*-test compared to 900 s. Time spent on the drug paired side on day one was subtracted from the time spent on the drug paired side on day eight, and the difference was analyzed by a one-way ANOVA. Entries into the drug paired side on day one was subtracted from entries into the drug paired side on day eight, and the

difference was analyzed by a one-way ANOVA. Distance travelled (cm) on drug paired days was analyzed by a two-way ANOVA. For the two-bottle choice test, saccharin consumed (g) was divided by mouse weight (kg), and the quotient was analyzed by a two-way ANOVA. Saccharin consumed (g) as a percent of total liquid consumed (g) was analyzed by a two-way ANOVA.

## Results

In this study, the OF and FPA were utilized to explore the profiles of indirectly and directly agonizing the cannabinergic system through differences in locomotor activity and dopaminergic responses in the NAc via stimulation in the VTA. Then, CPP and two-bottle choice test were utilized to explore the profiles of indirectly and directly agonizing the cannabinergic system with regards to addiction potential and anhedonia respectively.

### Open Field

Locomotor activities assessed in the OF included distance travelled (cm), rearing, and percent time spent in center. Baseline locomotor activity was assessed in two 10 min blocks per day. As can be seen in *Figure 1A*, there was not a main effect of pretreatment on distance travelled,  $F(2,51) = 0.46$ ,  $p = .67$ ,  $\eta_p^2 = .02$ . There was not a day x pretreatment ( $F(2,51) = 0.10$ ,  $p = .91$ ,  $\eta_p^2 = .004$ ), block x pretreatment ( $F(2,51) = 1.05$ ,  $p = .357$ ,  $\eta_p^2 = .04$ ), or day x block x pretreatment ( $F(2,51) = 2.69$ ,  $p = .078$ ,  $\eta_p^2 = .10$ ) interaction effect on distance travelled. As can be seen in *Figure 1B*, there was not a main effect of pretreatment on rears,  $F(2,51) = 0.57$ ,  $p = .57$ ,  $\eta_p^2 = .02$ . There was not a day x pretreatment ( $F(2,51) = 0.64$ ,  $p = .53$ ,  $\eta_p^2 = .02$ ), block x pretreatment ( $F(2,51) = 2.69$ ,  $p = .077$ ,  $\eta_p^2 = .10$ ), or day x block x pretreatment ( $F(2,51) = 0.92$ ,  $p = .41$ ,  $\eta_p^2 = .04$ ) interaction effect on rears. As can be seen in *Figure 1C*, there was not a main effect of pretreatment on percent time in the center,  $F(2,51) = 0.89$ ,  $p = .42$ ,  $\eta_p^2 = .03$ . There was

not a day x pretreatment ( $F(2,51) = 1.08, p = .35, \eta_p^2 = .04$ ), block x pretreatment ( $F(2,51) = 1.37, p = .26, \eta_p^2 = .05$ ), or day x block x pretreatment ( $F(2,51) = 0.29, p = .75, \eta_p^2 = .01$ ) interaction effect on percent time in the center.

During the drug challenge in the OF on the first and seventh day, locomotor activities were recorded in 10 min blocks. Distance travelled (cm) in the OF was analyzed across the three pretreatment conditions. As can be seen in *Figure 2A*, the effect of pretreatment on distance travelled approached significance,  $F(2,51) = 2.75, p = .07, \eta_p^2 = .10$ . Games-Howell post-hoc test revealed that ACEA pretreated mice ( $M = 129.95, SE = 15.25$ ) travelled significantly less distance than vehicle pretreated mice ( $M = 208.14, SE = 23.52$ ),  $p = .02$ . There was not a day x pretreatment interaction effect (Pillai's trace = 0.02,  $F(2,51) = 0.64, p = .53, \eta_p^2 = .02$ ) on distance travelled. There was not a block x pretreatment interaction effect (Pillai's trace = 0.32,  $F(16,90) = 1.05, p = .41, \eta_p^2 = .16$ ) on distance travelled. There was not a day x block x pretreatment interaction effect (Pillai's trace = 0.226,  $F(16,90) = 1.47, p = .13, \eta_p^2 = .21$ ) on distance travelled.

Rearing behavior in the OF was analyzed across the three pretreatment conditions. As can be seen in *Figure 2B*, there was a main effect of pretreatment on rears,  $F(2,51) = 4.53, p = .02, \eta_p^2 = .15$ . Games-Howell post-hoc test revealed that ACEA pretreated mice ( $M = 5.50, SE = 0.04$ ) reared significantly less than vehicle pretreated mice ( $M = 11.51, SE = 0.05$ ),  $p = .01$ . There was not a day x pretreatment interaction effect (Pillai's trace = 0.03,  $F(2,51) = 0.90, p = .41, \eta_p^2 = .03$ ) on rears. There was not a block x pretreatment interaction effect (Pillai's trace = .03,  $F(16,90) = 1.03, p = .43, \eta_p^2 = .15$ ) on rears. There was not a day x block x pretreatment interaction effect (Pillai's trace = 0.30,  $F(16,90) = 0.99, p = .48, \eta_p^2 = .15$ ) on rears.

Percent time in the center was analyzed across the three pretreatment conditions. As can be seen in *Figure 2C*, there was not a main effect of pretreatment on percent time spent in the center,  $F(2,51) = 2.01, p = .14, \eta_p^2 = .07$ . There was not a day x pretreatment interaction effect (Pillai's trace = 0.07,  $F(2,51) = 1.96, p = .15, \eta_p^2 = .07$ ) on percent time in center. There was not a block x pretreatment interaction effect (Pillai's trace = 0.18,  $F(16,90) = 0.51, p = .92, \eta_p^2 = .09$ ) on percent time in center. There was not a day x block x pretreatment interaction effect (Pillai's trace = 0.35,  $F(16,90) = 1.18, p = .30, \eta_p^2 = .17$ ) on percent time in center.

### **Dopamine Autoreceptor Sensitivity**

DAR sensitivity was analyzed across pretreatment conditions as previously described. As seen in *Figure 3*, there was not a main effect of pretreatment on autoreceptor sensitivity,  $F(2,50) = 1.66, p = .20, \eta_p^2 = .06$ . There was a main effect of amount of pre-pulses on autoreceptor sensitivity, Pillai's trace = 0.77,  $F(6,45) = 25.30, p < .001, \eta_p^2 = .77$ . There was not an amount of pre-pulses x pretreatment interaction effect (Pillai's trace = 0.18,  $F(12,92) = 0.76, p = .70, \eta_p^2 = .09$ ) on autoreceptor sensitivity.

### **Baseline Dopamine Recordings**

Baseline dopamine release ( $\mu\text{M}$ ) was analyzed across the three pretreatment conditions. As can be seen in *Figure 4*, there was a main effect of pretreatment on baseline dopamine release,  $F(2,51) = 3.21, p = .049, \eta^2 = .11$ . Tukey's post-hoc test revealed that ACEA pretreated mice's baseline dopamine release ( $M = 0.18, SE = 0.01$ ) was diminished in comparison to vehicle pretreated mice's baseline dopamine release ( $M = 0.29, SE = 0.01$ ),  $p = .04$ . Baseline dopamine half-life was analyzed across the three pretreatment conditions. There was not a main effect of pretreatment on baseline dopamine half-life,  $F(2,51) = 0.66, p = .52, \eta^2 = .02$ .

## Dopamine Recordings Following Drug Challenge

### Drug Challenge: AA-5-HT

During dopamine recordings, a subset of the mice pretreated with AA-5-HT or vehicle received an i.p. drug challenge of either AA-5-HT ( $n = 7$  and  $6$ , respectively per pretreatment) or vehicle ( $n = 5$  and  $4$ , respectively per pretreatment). Percent change of dopamine release and half-life (with baseline dopamine release and half-life being 100%) were analyzed at 10 min intervals for 90 min post injection. As can be seen in *Figure 5A*, there was not a main effect of pretreatment on percent change in dopamine release,  $F(1,19) = 0.67, p = .42, \eta_p^2 = .03$ . There was not a main effect of drug challenge on percent change in dopamine release,  $F(1,19) = 0.00024, p = .99, \eta_p^2 = .00$ . There was not a pretreatment x drug challenge interaction effect ( $F(1,19) = 0.12, p = .73, \eta_p^2 = .01$ ) on percent change in dopamine release. There was not a time point x pretreatment interaction effect (Pillai's trace =  $0.57, F(9,11) = 1.62, p = .22, \eta_p^2 = .57$ ) on percent change in dopamine release. There was not a time point x drug challenge interaction effect (Pillai's trace =  $0.45, F(9,11) = 0.99, p = .50, \eta_p^2 = .45$ ) on percent change in dopamine release. There was not a time point x pretreatment x drug challenge interaction effect (Pillai's trace =  $0.45, F(9,11) = 0.23, p = .98, \eta_p^2 = .16$ ) on percent change in dopamine release.

Percent change in dopamine half-life was analyzed across AA-5-HT and vehicle pretreated mice. As can be seen in *Figure 5B*, there was not a main effect of pretreatment on percent change in dopamine half-life,  $F(1,19) = 0.39, p = .54, \eta_p^2 = .02$ . There was not a main effect of drug challenge on percent change in dopamine half-life,  $F(1,19) = 3.25, p = .09, \eta_p^2 = .15$ . There was not a pretreatment x drug challenge interaction effect ( $F(1,19) = 0.10, p = .75, \eta_p^2 = .01$ ) on percent change in dopamine half-life. There was not a time point x pretreatment

interaction effect (Pillai's trace = 0.38,  $F(9,11) = 0.75$ ,  $p = .66$ ,  $\eta_p^2 = .38$ ) on percent change in dopamine half-life. There was not a time point x drug challenge interaction effect (Pillai's trace = 0.39,  $F(9,11) = 0.77$ ,  $p = .65$ ,  $\eta_p^2 = .39$ ) on percent change in dopamine half-life. There was not a time point x pretreatment x drug challenge interaction effect (Pillai's trace = 0.60,  $F(9,11) = 1.82$ ,  $p = .17$ ,  $\eta_p^2 = .60$ ) on percent change in dopamine half-life.

### **Drug Challenge: ACEA**

During dopamine recordings, a subset of the mice pretreated with ACEA or vehicle received an i.p. drug challenge of either ACEA ( $n = 5$  and  $4$ , respectively per pretreatment) or vehicle ( $n = 5$  and  $4$ , respectively per pretreatment). Percent change of dopamine release and half-life (with baseline dopamine release and half-life being 100%) were analyzed at 10 min intervals for 90 min post injection. Percent change in dopamine release was analyzed across ACEA and vehicle pretreated mice. As can be seen in *Figure 6A*, there was not a main effect of pretreatment on percent change in dopamine release,  $F(1,14) = 2.09$ ,  $p = .17$ ,  $\eta_p^2 = .13$ . The main effect of drug challenge on percent change in dopamine release ( $F(1,14) = 4.59$ ,  $p = .05$ ,  $\eta_p^2 = .25$ ) approached significance. There was not a pretreatment x drug challenge interaction effect ( $F(1,14) = 1.55$ ,  $p = .23$ ,  $\eta_p^2 = .10$ ) on percent change in dopamine release. There was not a time point x pretreatment effect (Pillai's trace = 0.54,  $F(9,6) = 0.79$ ,  $p = .64$ ,  $\eta_p^2 = .54$ ) on percent change in dopamine release. There was not a time point x challenge drug interaction (Pillai's trace = 0.78,  $F(9,6) = 2.41$ ,  $p = .15$ ,  $\eta_p^2 = .78$ ) on percent change in dopamine release. There was a time point x pretreatment x challenge drug interaction (Pillai's trace = 0.90,  $F(9,6) = 6.07$ ,  $p = .02$ ,  $\eta_p^2 = .90$ ) on percent change in dopamine release.

Percent change in dopamine half-life was analyzed across ACEA and vehicle pretreated mice. As can be seen in *Figure 6B*, there was not a main effect of pretreatment on percent change in dopamine half-life,  $F(1,19) = 0.65, p = .44, \eta_p^2 = .04$ . There was not a main effect of drug challenge on percent change in dopamine half-life,  $F(1,19) = 3.77, p = .073, \eta_p^2 = .21$ . There was not a pretreatment x drug challenge interaction effect ( $F(1,19) = 0.57, p = .46, \eta_p^2 = .04$ ) on percent change in baseline dopamine half-life. There was not a time point x pretreatment interaction effect (Pillai's trace = 0.60,  $F(9,6) = 1.00, p = .52, \eta_p^2 = .60$ ) on percent change in baseline dopamine half-life. There was not a time point x drug challenge interaction effect (Pillai's trace = 0.76,  $F(9,6) = 0.78, p = .19, \eta_p^2 = .76$ ) on percent change in dopamine half-life. There was not a time point x pretreatment x drug challenge interaction effect (Pillai's trace = 0.61,  $F(9,6) = 1.04, p = .50, \eta_p^2 = .61$ ) on percent change in baseline dopamine half-life.

### **Drug Challenge: Cocaine**

During dopamine recordings, a subset of the mice pretreated with AA-5-HT, ACEA, or vehicle received an i.p. drug challenge of cocaine ( $n = 6, 5, \text{ and } 6$  per pretreatment group respectively). Percent change of dopamine release (with baseline dopamine release being 100%) was analyzed at 10 min intervals for 60 min post injection. As can be seen in *Figure 7A*, there was not a main effect of pretreatment on percent change in dopamine release,  $F(2,14) = 1.21, p = .33, \eta_p^2 = .15$ . There was not a time point x pretreatment interaction effect (Pillai's trace = 0.98,  $F(12,20) = 1.61, p = .17, \eta_p^2 = .49$ ) on percent change in dopamine release.

Percent change in dopamine half-life was analyzed across mice receiving the drug challenge of cocaine. As can be seen in *Figure 7B*, there was not a main effect of pretreatment on percent change in dopamine half-life,  $F(2,14) = 2.99, p = .08, \eta_p^2 = .30$ . There was a



significant time point x pretreatment interaction effect (Pillai's trace = 1.20,  $F(12,20) = 2.50$ ,  $p = .034$ ,  $\eta_p^2 = .60$ ) on percent change in dopamine half-life. There was a near significant main effect of pretreatment (*Welch's*  $F(2,6.68) = 4.77$ ,  $p = .05$ ,  $\omega^2 = 0.31$ ) on percent change in dopamine half-life at 10 min post-injection. Games-Howell post-hoc test revealed ACEA pretreated mice's percent change in dopamine half-life ( $M = 273.32$ ,  $SE = 28.11$ ) was significantly increased in comparison to vehicle ( $M = 170.81$ ,  $SE = 18.43$ ),  $p = .04$ . There was a near significant main effect of pretreatment (*Welch's*  $F(2,8.13) = 4.42$ ,  $p = .05$ , est.  $\omega^2 = 0.29$ ) on percent change in dopamine half-life at 20 min post-injection. Games-Howell post-hoc test revealed ACEA pretreated mice's percent change in dopamine half-life ( $M = 346.19$ ,  $SE = 26.77$ ) was significantly increased in comparison to vehicle ( $M = 240.04$ ,  $SE = 21.68$ ),  $p = .04$ .

### **Stereotaxic Placement of Electrodes**

As can be seen in *Figure 8*, following amperometry experiments, the placement of stimulating electrodes ( $n = 54$ ) and recording electrodes ( $n = 54$ ) were determined by examining lesioned regions in sectioned mouse brains. The positions of stimulating electrodes were localized within the anatomical region of the VTA spanning -3.08 to -3.52 mm AP from bregma and -4.0 to -5.0 mm DV from dura, and the positions for the recording electrodes were localized within the anatomical region of the NAc spanning +1.54 to + 1.34 mm AP from bregma and -4.0 to -5.0 mm DV from dura.

### **Conditioned Place Preference**

During CPP, mice could explore the entire chamber for 30 min on the first day. The mice travelled a similar amount of time on the drug paired side ( $M = 929.98$  s,  $SE = 19.11$ ) as compared to the expected for each side (900 s),  $t(26) = 1.57$ ,  $p = .05$ . On the eighth day, mice

could explore the entire chamber again to ascertain a preference for either side. Time spent on the drug paired side for day one was subtracted from time spent on the drug paired side for day eight. As can be seen in *Figure 9A*, there was not a main effect of pretreatment ( $F(2,26) = 1.10$ ,  $p = .35$ ,  $\eta_p^2 = .08$ ) on time spent on the drug paired side. Additionally, entries to the drug paired side were recorded. Entries into the drug paired side for day one was subtracted from entries into the drug paired side for day eight. As can be seen in *Figure 9B*, there was not a main effect of pretreatment ( $F(2,26) = 0.02$ ,  $p = .98$ ,  $\eta_p^2 = .00$ ) on entries into the drug paired side.

Distance travelled was recorded on the drug paired days where mice were given either an i.p. injection of AA-5-HT, ACEA, or vehicle and confined to a single side of the chamber. As can be seen in *Figure 10*, there was not a main effect of drug on distance travelled on drug paired days,  $F(2,24) = 1.36$ ,  $p = .28$ ,  $\eta_p^2 = .10$ . There was not a day x drug treatment interaction effect ( $F(4,48) = 0.74$ ,  $p = .57$ ,  $\eta_p^2 = .06$ ) on distance travelled on drug paired days.

### **Two-Bottle Choice Test**

For the two-bottle choice test, saccharin consumed (g) was divided by mouse weight (kg). There was not a main effect of treatment on body weight,  $F(2,24) = 0.05$ ,  $p = .95$ ,  $\eta_p^2 = .00$ . There was no day x treatment interaction effect (Pillai's trace = 0.46,  $F(8,44) = 1.65$ ,  $\eta_p^2 = .23$ ) on body weight. As can be seen in *Figure 11A*, there was not a main effect of treatment on saccharin consumed over body weight,  $F(2,24) = 0.18$ ,  $p = .83$ ,  $\eta_p^2 = .02$ . There was not a day x treatment interaction effect (Pillai's trace = 0.77,  $F(8,44) = .54$ ,  $p = .84$ ,  $\eta_p^2 = .09$ ) on saccharin consumed over body weight. Additionally, preference for saccharin was calculated as saccharin consumption (g) as a percent of total liquid consumed (g). As can be seen in *Figure 11B*, there was not a main effect of treatment on saccharin as a percent of total liquid consumed,  $F(2,24) =$

.004,  $p = .99$ ,  $\eta_p^2 = .00$ . There was not a day x treatment interaction effect ( $F(8,96) = .68$ ,  $p = .71$ ,  $\eta_p^2 = .05$ ) on saccharin as a percent of total liquid consumed.

## Discussion

The cannabinergic system is a target for the development of potential anxiolytic drugs. The mesolimbic dopamine pathway plays integral roles in addiction and anxiety. Therefore, the development of anxiolytic pharmaceuticals necessitates exploring the dopaminergic alterations in response to their administration. The current study aims to explore the addictive potential of indirectly or directly modulating cannabinergic signaling via AA-5-HT or ACEA administration respectively.

In the OF, AA-5-HT administration did not significantly alter locomotor activities including distance travelled, rearing, or percent time in the center. The lack of change in distance travelled and rearing may be indicative of a non-addictive profile for this drug. Previous research has shown that AA-5-HT and AACOCF<sub>3</sub> (an FAAH inhibitor) did not alter locomotor activity in the OF (Freels, Lester, & Cook, 2019; Rutkowska, Jamontt, & Gliniak, 2006). However, with no change in percent time in center, this drug also did not appear to have anxiolytic potential in this study. This may have occurred due to the choice of strain (B6) over a more anxious strain and due to the choice of test for anxiety as moderate anxiolytic effects were found in BCJ mice and in the EPM (Freels, Lester, & Cook, 2019; Micale et al., 2009). Further research could explore the anxiolytic potential of this drug in more anxious mice or more anxiogenic tests. ACEA significantly decreased the rearing frequency of mice but did not alter distance travelled or percent time in center. Previous research has shown that CB<sub>1</sub>R agonists do not alter locomotor activities in the OF except for suppressing rearing behavior (Freels, Lester, & Cook, 2019; Järbe, Andrzejewski, & DiPatrizio, 2002; Rutkowska, Jamontt, & Gliniak, 2006).

Future research could address the length of the OF test as it may have been too long as the mice moved infrequently between 70 and 90 mins in the OF. An altered paradigm such as a shorter OF test or altered design such as the EPM or forced swim test may reveal anxiolytic effects. However, the mouse strain and OF contexts of the current study did not provide behaviors related to anxiety or addiction.

During FPA, AA-5-HT did not significantly alter dopamine dynamics at baseline, in autoreceptor sensitivity, or during drug challenge. Previous research has shown AA-5-HT to attenuate evoked dopamine release (Freels, Lester, & Cook, 2019). Though, these results also support a lack of reinforcing effects of AA-5-HT administration via not increasing dopamine in the NAc, which coincides with previous studies exploring FAAH inhibition (Freels, Lester, & Cook, 2019; Gamage et al., 2015; Justinová et al., 2015; Murillo-Rodríguez, Palomero-Rivero, Millán-Aldaco, & Di Marzo, 2013; Valchou, Nomikos, & Pangais, 2006). AA-5-HT pretreatment did not alter dopamine dynamics in response to a cocaine drug challenge. Previous research has shown cocaine and URB597 pretreatment followed 24 h later by a cocaine drug challenge increased percent change in dopamine release in the NAc core (Mereu et al., 2013). Thus, the dual FAAH inhibition and TRPV<sub>1</sub> blocking action of AA-5-HT, the chronic administration, or the lack of DAT sensitization due to a single dose of cocaine, may have attenuated the alterations in dopamine signaling previously shown during a cocaine drug challenge in the NAc of mice. Therefore, AA-5-HT treatment did not elicit a dopaminergic profile associated with abuse and addiction.

ACEA significantly reduced baseline dopamine release, altered drug challenge evoked dopamine release in a pretreatment- and time-dependent manner, and, through pretreatment, enhanced cocaine drug challenge induced increases in dopamine half-life. A previous study

from our laboratory found ACEA administration attenuated evoked dopamine release in the NAc utilizing *in vivo* FPA (Freels, Lester, & Cook, 2019). Additional studies found similar results utilizing direct CB<sub>1</sub>R agonists with regards to the reduction in dopamine efflux utilizing fast-scan cyclic voltammetry (Cheer, Wassum, Heien, Phillips, & Wightman, 2004; O'Neill, Evers-Donnelly, Nicholson, O'Boyle, & O'Connor, 2009). ACEA pretreatment increased dopamine half-life in the NAc during the cocaine drug challenge. This may be mediated through cannabinoid driven alterations in DAT populations or binding affinities. WIN55,212-2 administration decreased dopamine uptake and increased dopamine clearance time (Price et al., 2006). Additionally, chronic, 20-day administration of WIN55,212-2 reduced DAT protein levels in the striatum and DAT mRNA levels and binding in the VTA (Perdikaris, Tsarouchi, Fanarioti, Natsaridis, Mitsacos, & Giompres, 2018). CB<sub>1</sub>R antagonism decreased cocaine induced dopamine transient frequency and dopamine transients' amplitude in the NAc in a 2-AG-dependent manner, suggesting an eCB- and CB<sub>1</sub>R-dependent inhibition of dopamine signaling (Cheer et al., 2007; Wang, Treadway, Covey, Cheer & Lupica, 2015). Cocaine and rimonabant pretreatment followed 24 h later by a cocaine drug challenge blocked the percent increase in dopamine release following a cocaine drug challenge in the NAc core (Mereu et al., 2013). Thus, CB<sub>1</sub>R agonist pretreatment alters baseline dopamine release, DAT expression and, in response to a cocaine drug challenge, NAc dopamine dynamics. Chronic administration of direct CB<sub>1</sub>R agonists may interfere with the efficacy of other drugs that work on the dopamine system such as amphetamines that bind to and reverse DAT function and alter DAT trafficking (Robertson, Matthies, & Galli, 2009). Further research could explore DAT expression in response to a CB<sub>1</sub>R agonist pretreatment and cocaine drug challenge through Western blot, radioligand binding assay, and autoradiography. Therefore, ACEA treatment also does not elicit

a dopaminergic profile associated with abuse and addiction but may sensitize the mesolimbic dopamine pathway to the effects of cocaine and other dopamine modulating drugs of abuse.

During CPP, mice did not develop a CPP or CPA for either AA-5-HT or ACEA.

Previous research has shown that Wistar rats, Sprague-Dawley rats and B6 mice in standard housing conditions do not have a CPP for AM404 (an inhibitor of AMT), AM404 except at 10.0 mg/kg, or SBFI26 (an inhibitor of FABP5 and FABP7) respectively (Bortolato et al., 2006; Scherma et al., 2012; Thanos et al., 2016). Direct CB<sub>1</sub>R agonist administration can produce CPP, no preference, or CPA in a drug and dose dependent manner where THC typically causes a CPP under 5.0 mg/kg and a CPA at 5.0 mg/kg or higher but WIN55,212-2 has variable results where a CPP occurred under 1.0 mg/kg, no preference occurred from 1.0 mg/kg to 3.0 mg/kg, and a CPA occurred from 0.25 mg/kg to 2.5 mg/kg (Murray & Bevins, 2010; Panagis, Mackey, & Vlachou, 2014). Therefore, indirectly and directly agonizing the cannabinergic system can lead to CPP, but the doses in the current study, which were chosen due to their anxiolytic potential, did not, which suggests these doses are not rewarding or aversive in the current paradigm.

During the two-bottle choice test, mice treated with either AA-5-HT or ACEA did not develop a preference or aversion for saccharin. CP55940, URB597, and rimonabant had no effects on saccharin preference alone, but chronic mild stress-induced reductions in sucrose preference were attenuated by CP55940 or URB597 and enhanced by rimonabant (Bortolato et al., 2007; Rademacher & Hillard, 2007). This suggests that indirectly and directly agonizing the cannabinoid system does not alter preference for sweet rewards alone but can modify stress-induced alterations in preference. Therefore, exploring preference for saccharin following both cannabinoid administration and stress may further the potential use of these drugs as anxiolytics.

However, CP55940 increased progressive ratio responding for Ensure where rimonabant reduced it, suggesting the cannabinergic system still plays a role in the wanting of sweet rewards (Ward & Dykstra, 2005). AA-5-HT and ACEA administration did not alter saccharin preference, indicating these drugs do not elicit reward- or anhedonia-related behaviors.

Cross-modulation between neurotransmission systems can impact the efficacy and off-target effects of posited drugs. The current study explored the interplay between the cannabinergic system and dopaminergic system through the administration of AA-5-HT and ACEA. Through this study's paradigms, AA-5-HT did not modulate mesolimbic dopamine neurotransmission, but ACEA decreased baseline dopamine release, altered dopamine transmission during the drug challenge, and enhanced cocaine induced increases in dopamine half-life in the NAc. Cannabinoid induced increases in DRs and decreases in CB<sub>1</sub>Rs could lead to a system primed for dysregulation and super-sensitization of the dopamine system due to increasing DR populations and decreasing GABA<sub>A</sub>R modulation, which could prime the brain, especially the mesolimbic dopamine pathway, for addiction to drugs that act on this pathway (Ginovart et al., 2012; Morgan et al., 2014). The current study indicates this potential via enhanced cocaine induced dopamine-half in the NAc following ACEA pretreatment. Therefore, the dosage as well as other pharmaceuticals affecting the cannabinergic and dopaminergic systems must be monitored to prevent deleterious off-target and side effects.

The cannabinergic system remains a target for the development of anxiolytic pharmaceuticals. The current study found no evidence for an abuse potential for AA-5-HT or ACEA with regards to alterations in locomotor activity in the OF or VTA stimulation evoked dopamine efflux in the NAc. Overall, the current study did not find an abuse potential from

indirectly or directly agonizing the cannabinergic system and suggests, with future research, that the cannabinergic system may still be a potential target for the treatment of anxiety.



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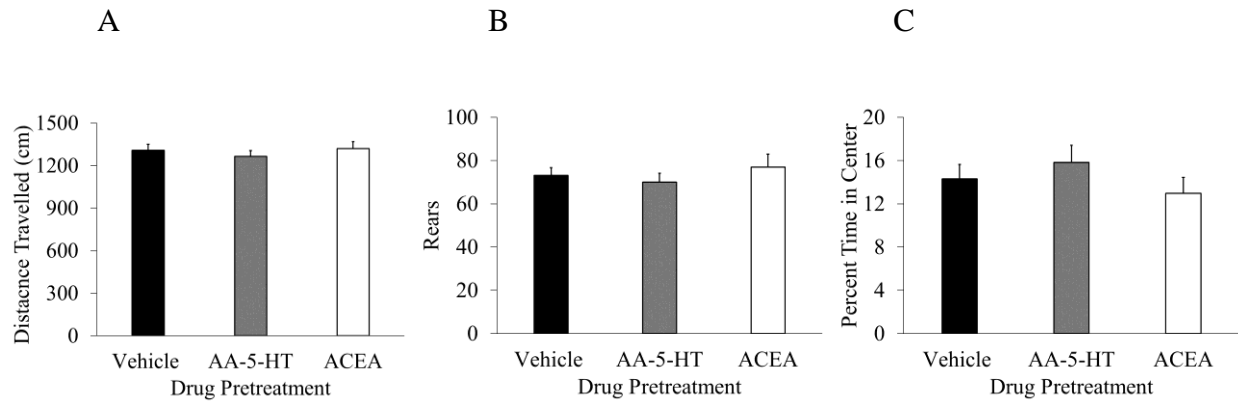
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## Appendix A

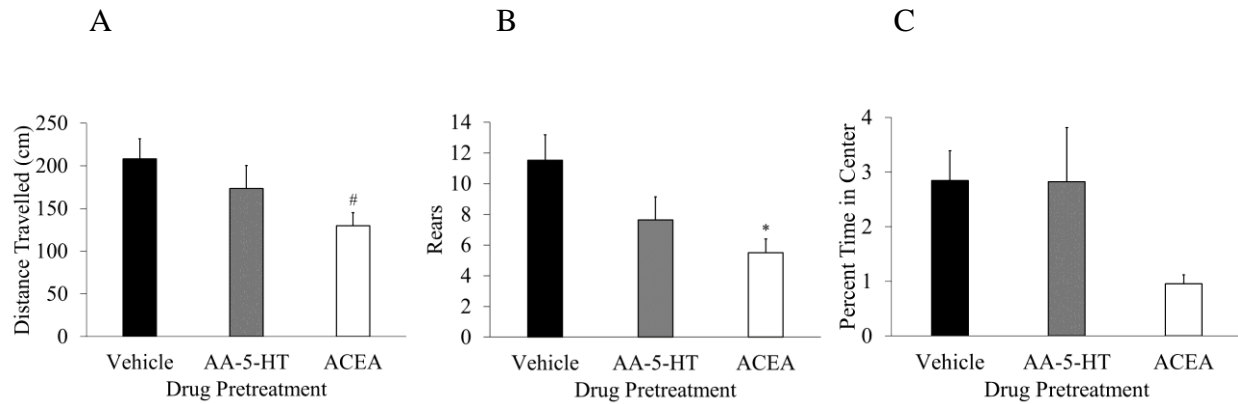
Table 1  
*Dopamine Recording Drug Groups*

Drug Pretreatment	FPA Drug Challenge	<i>n</i>
Vehicle	Vehicle	4
	AA-5-HT	6
	ACEA	4
	Cocaine	6
AA-5-HT	Vehicle	6
	AA-5-HT	7
	Cocaine	6
ACEA	Vehicle	5
	ACEA	5
	Cocaine	5

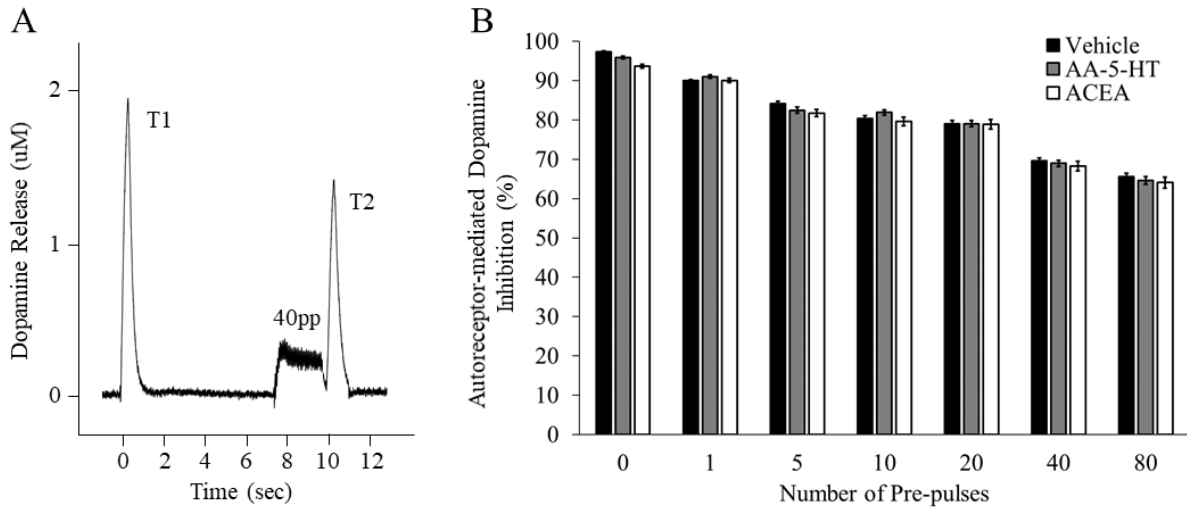
*Note.* AA-5-HT = arachidonoyl serotonin; ACEA = arachidonoyl-2'-chloroethylamide.



*Figure 1.* Baseline locomotor activities in the OF averaged into 10 min blocks. There were no baseline significant differences between vehicle, AA-5-HT (arachidonoyl serotonin), or ACEA arachinoyl-2'-chloroethylamide) on (A) mean distance travelled  $\pm$  SEMs, (B) mean rears  $\pm$  SEMs, or (C) mean percent time in center  $\pm$  SEMs.



**Figure 2.** Drug challenge locomotor activity in the OF averaged into 10 min blocks. (A) There was a near significant difference in mean distance travelled  $\pm$  SEMs. (B) There was a significant difference in mean rears  $\pm$  SEMs. (C) There was not a significant difference in percent time in center  $\pm$  SEMs. AA-5-HT = arachidonoyl serotonin, ACEA = arachidonoyl-2'-chloroethylamide, # indicates near significance at  $p < .075$  and \* indicates significance relative to vehicle pretreated mice at  $p < .05$ .



*Figure 3.* Dopamine autoreceptor sensitivity. A representative response (A) and dopamine autoreceptor sensitivity  $\pm$  SEMs (B) following pre-pulses. Chronic pretreatment with either AA-5-HT (arachidonoyl serotonin) or ACEA (arachidonyl-2'-chloroethylamide) did not alter dopamine autoreceptor functioning in the NAc.



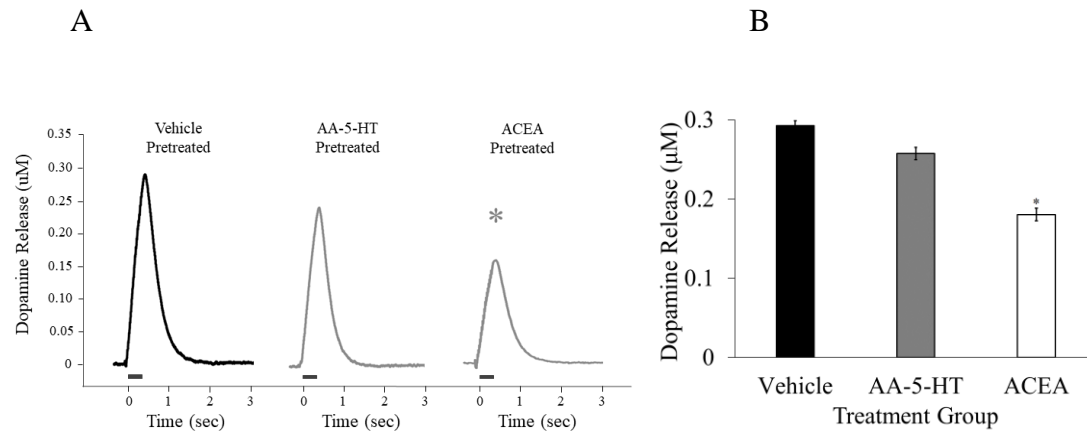
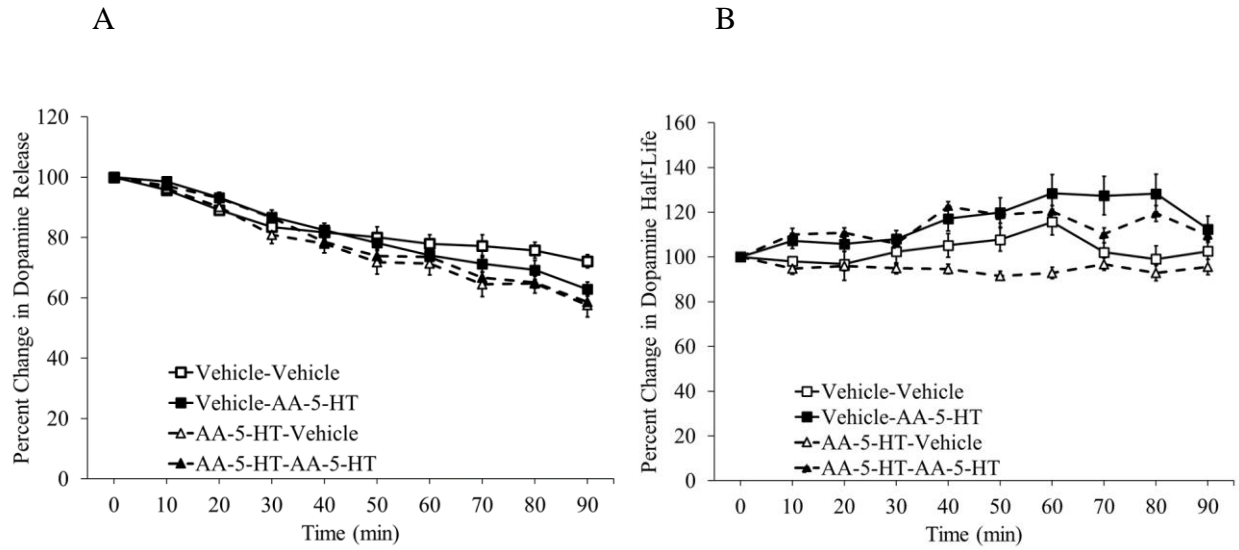
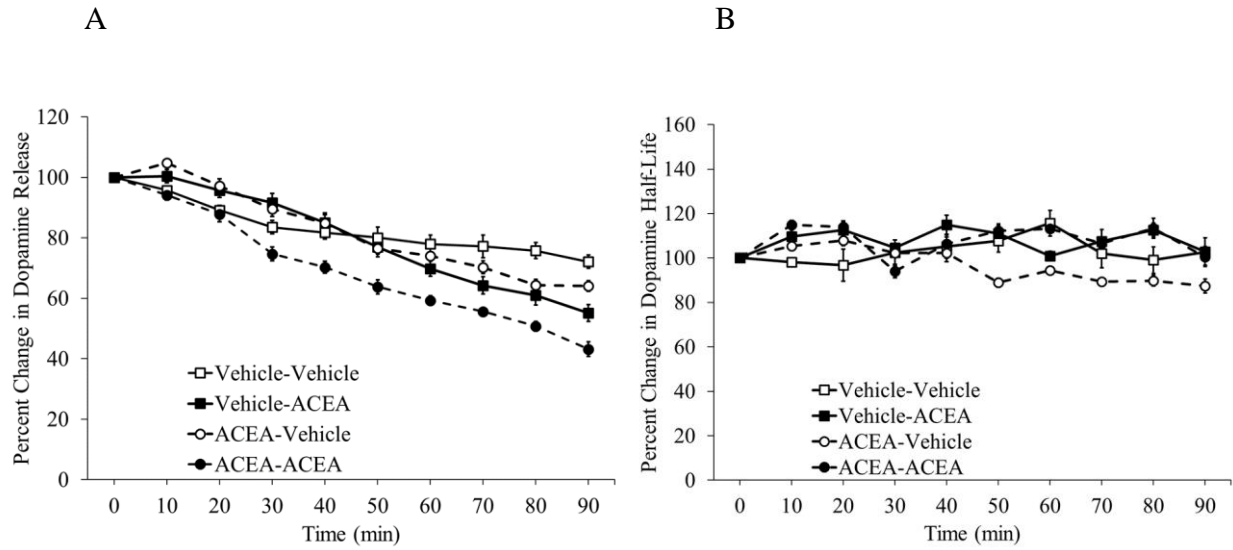


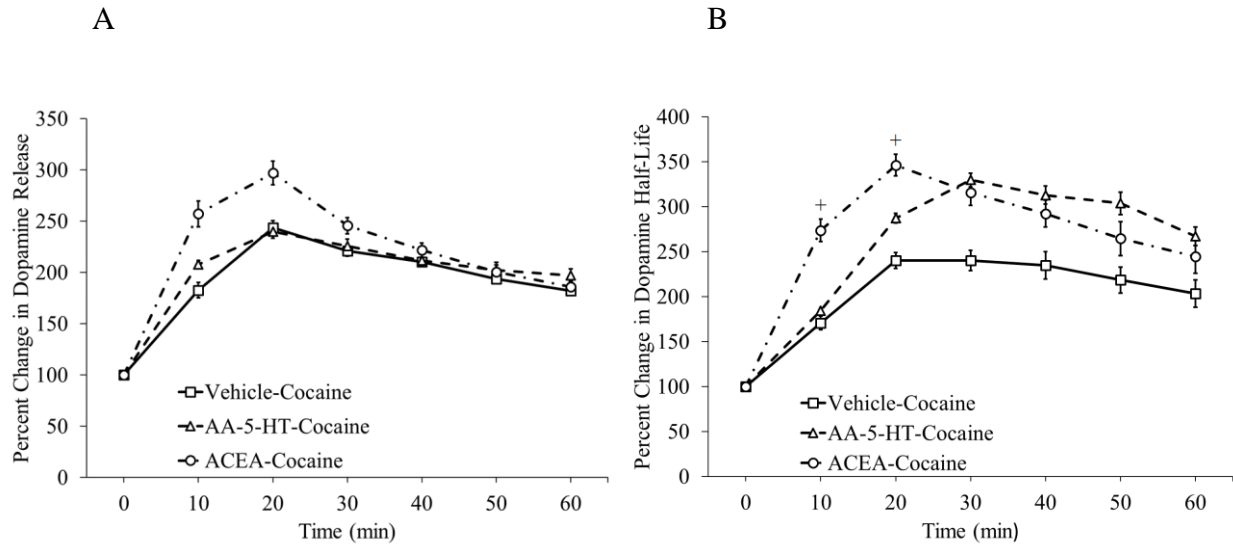
Figure 4. Baseline dopamine results. Representative responses (A) and means  $\pm$  SEMs (B) of stimulation evoked dopamine release in the NAc following vehicle, AA-5-HT, or ACEA pretreatment. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide. \* indicates a significant decrease relative to vehicle pretreatment at  $p < .05$ .



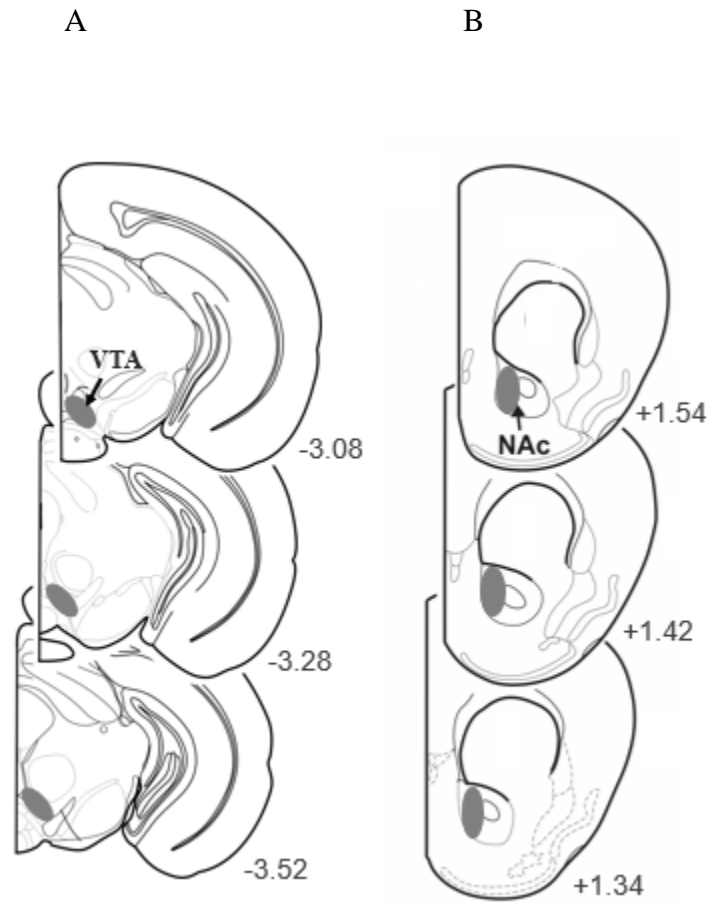
*Figure 5.* Percent baseline dopamine release and half-life for AA-5-HT. (A) Pretreatment of vehicle or AA-5-HT on percent baseline dopamine release presented as means  $\pm$  SEMs. The figure legend indicates the pretreatment drug then the drug challenge such that vehicle-vehicle represents mice that received vehicle pretreatment and vehicle drug challenge. (B) Pretreatment and drug challenge of vehicle or AA-5-HT on percent baseline dopamine half-life presented as means  $\pm$  SEMs. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide.



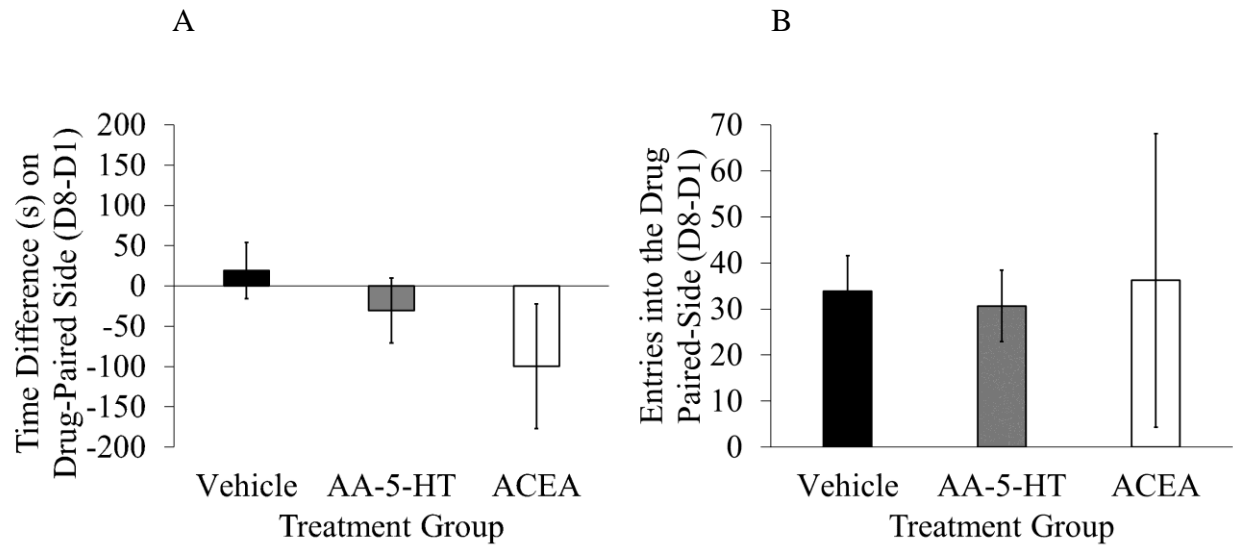
*Figure 6.* Percent baseline dopamine release and half-life for ACEA. (A) Pretreatment of vehicle or ACEA on percent baseline dopamine release presented as means  $\pm$  SEMs. The figure legend indicates the pretreatment drug then the drug challenge such that vehicle-vehicle represents mice that received vehicle pretreatment and vehicle drug challenge. (B) Pretreatment and drug challenge of vehicle or ACEA on percent baseline dopamine half-life presented as means  $\pm$  SEMs. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide.



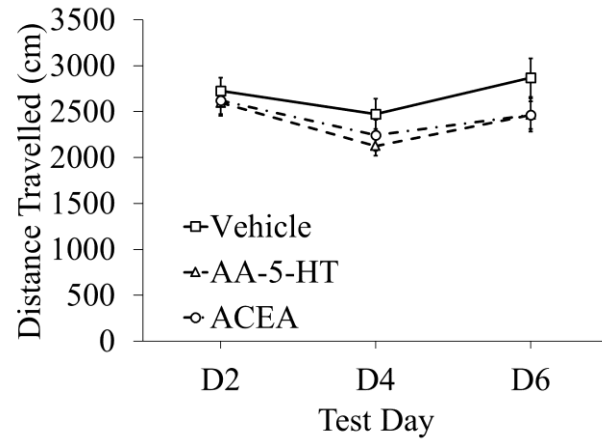
*Figure 7.* Percent change in dopamine (A) release and (B) half-life following cocaine administration (with baseline dopamine release set at 100%). Figure legends indicate the pretreatment drug then the drug challenge such that vehicle-cocaine represents mice that received vehicle pretreatment and cocaine drug challenge. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide. + indicates  $p < .06$ .



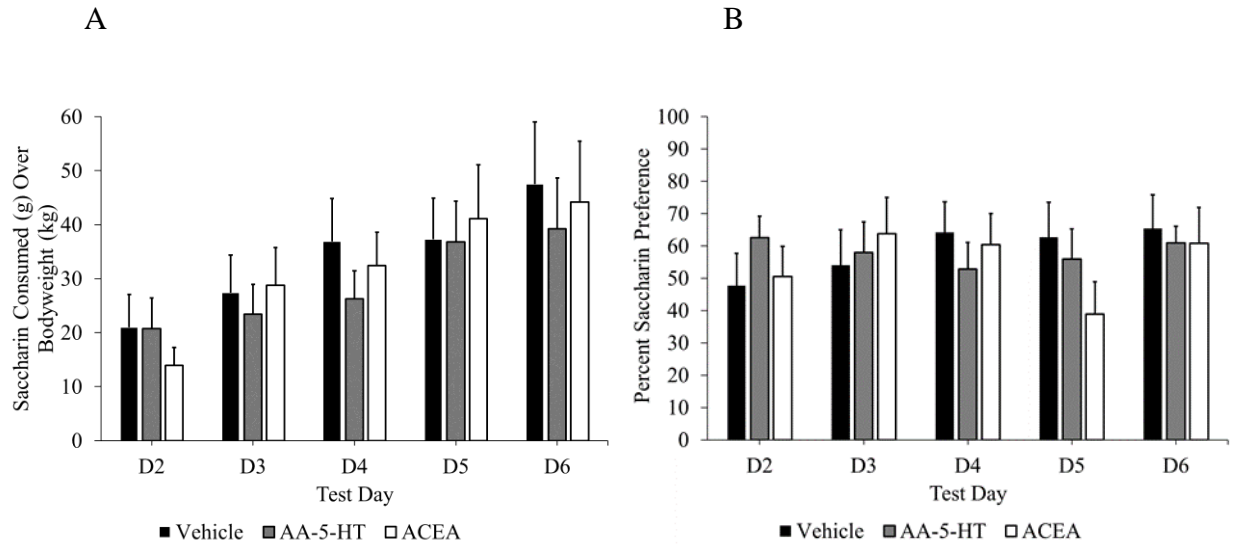
*Figure 8.* Representative coronal sections of the mouse brain (adapted from the atlas of Paxinos & Franklin, 2001), with gray-shaded areas indicating the placements (A) stimulating electrodes in the ventral tegmental area (VTA) and amperometric recording electrodes in the (B) nucleus accumbens (NAc). Numbers correspond to mm from bregma



*Figure 9.* Time differences within and entries into the drug paired side. (A) Drug challenge on time spent in the drug-paired side presented as day one subtracted from day eight. (B) Drug challenge on entries into the drug paired side presented as day one subtracted from day eight. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonyl-2'-chloroethylamide.



*Figure 10.* Drug challenge on distance travelled on drug-paired days. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide.



*Figure 11.* Drug challenge on saccharin preference when presented with one bottle of saccharin and one of water. (A) Drug challenge on saccharin consumed over body weight. (B) Drug challenge on saccharin consumed as a percent of total liquid consumed. AA-5-HT = arachidonoyl serotonin and ACEA = arachidonoyl-2'-chloroethylamide.



Appendix B



IACUC PROTOCOL ACTION FORM

To:	Deranda Lester
From:	Institutional Animal Care and Use Committee
Subject:	Animal Research Protocol
Date:	November 10, 2016

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No.

0794 Part 2 Arachidonoyl Serotonin (AA-5-HT) as a Putative Cannabinoid-Based Anxiolytic

Your protocol is approved for the following period:

From:  To:

Your protocol is not approved for the following reasons (see attached memo).|

Your protocol is renewed without changes for the following period:

From:  To:

Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated  for the following period:

From:  To:

Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated