MECHANISMS OF SYNTHETIC CANNABINOIDS ON CARDIOVASCULAR HEALTH

Madeleine A. Nelson
University of South Dakota

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MECHANISMS OF SYNTHETIC CANNABINOIDs
ON CARDIOVASCULAR HEALTH

By
Madeleine A. Nelson

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
University Honors Program

Department of Basic Biomedical Sciences
The University of South Dakota
May 2021
The members of the Honors Thesis Committee appointed
to examine the thesis of Madeleine A. Nelson
find it satisfactory and recommend that it be accepted.

Dr. Doug Martin
Professor of Basic Biomedical Sciences
Director of the Committee

Dr. Hong Zheng
Assistant Professor
Department of Basic Biomedical Sciences

Dr. Lisa McFadden
Assistant Professor
Department of Basic Biomedical Sciences
Cannabinoids encompass natural cannabis and synthetic cannabinoids. While the synthetic cannabinoids interact with the same endogenous system as cannabis, their effects are quite different and poorly understood. In addition to psychological effects that trigger their use, these substances are linked to cardiovascular morbidity. To assess the cardiovascular effect of synthetic cannabinoids, we first tested the hypothesis that intravenous administration of a synthetic cannabinoid would increase blood pressure in conscious rats. Second, we tested the hypothesis that the sympathetic nervous system is involved by injecting a ganglion blocker to see if the cardiovascular response from synthetic cannabinoids would be blocked. Third, we tested the hypothesis that the hypothalamic paraventricular nucleus is involved by doing immunohistochemistry to check for cFos fluorescence.

We found that synthetic cannabinoid use peaked with 7,000 calls to poison control centers in 2011 and has stabilized between 1,000-2,000 calls per year in the US. In humans, cardiovascular effects account for approximately 40% of adverse responses to synthetic cannabinoids. The most frequently reported being tachycardia (50%) and
hypertension (20%). To test our hypothesis, Sprague Dawley rats were fitted with chronic indwelling arterial and venous catheters to record blood pressure and heart rate. Conscious rats were given intravenous injections of vehicle (20% cyclodextrin) then incremental doses of the synthetic cannabinoid, WIN 55, 212-2 (25, 50, 100 ug/kg + 0.2 ml saline flush). We observed that injection of WIN resulted in rapid onset dose dependent increases in blood pressure and heart rate that peaked at approximately 18+/-6 mm Hg and 90+/-15 bpm. We also observed that interruption of autonomic function via ganglion blockade abolished the pressor effect of WIN. Lastly, we also used immunohistochemistry to assess the role of the PVN in the response to WIN. Collectively, the data are consistent with the view that synthetic cannabinoids, such as WIN, increase blood pressure and heart rate via autonomic mechanisms that may be controlled by the brain.

Key words: Blood Pressure, Heart Rate, Synthetic Cannabinoids, Neuronal Activation, Paraventricular Nucleus, Amygdala, Autonomic Function
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I. INTRODUCTION

Cardiovascular Disease Statistics

Cardiovascular diseases: including coronary heart disease, heart failure, stroke, and hypertension, make up the number one major cause of death in the United States, affecting 440, 460 males, and 418, 665 females per year (Virani et al., 2021). Cancer, accidents, chronic lower respiratory disease, Alzheimer’s disease, and diabetes all cause significantly fewer deaths in comparison (Figure 1). Between the ages of 20-39, 29.8% of males, and 17.2% of females have some form of cardiovascular disease. At the middle age bracket, 40-59 years old, over 50% of both males and females have some form of cardiovascular disease (Virani et al., 2021, pp. e340-e342). As of 2017, there are over 600 thousand deaths attributed to cardiovascular diseases (Virani et al., 2021).

Figure 1. Chart taken from: (Virani et al., 2021, p. e342)
Hypertension, or dysregulation of blood pressure, is an underlying causative factor that promotes all of these cardiovascular diseases. Hypertension affects 47.3% of adults in the United States. While approximately 11% of individuals die due to high blood pressure, dysregulation of blood pressure is a contributing factor to other diseases such as coronary artery disease, heart failure, kidney disease, and stroke that also have a substantial mortality risk. For example, coronary heart disease is the primary cause of death (42.1%) in the United States (Virani et al., 2021). About every 39 seconds, someone in the United States has a heart attack. Hypertension is highly prevalent. Globally, 1.13 billion people suffer from hypertension, 26% of the world’s population. About 75% of those individuals’ live in low-income countries (Organization, 2021). Less than 1 out of 5 people who are diagnosed with hypertension have it controlled, high systolic BP is the number one risk factor for years of life lost, globally in 2019 (Virani et

---


**Figure 2.** Chart taken from: (Virani et al., 2021, p. e340)
Since cardiovascular disease is so prevalent, we know that dysregulation of blood pressure is an important factor in these diseases, factors that increase blood pressure can have deleterious effects. We are interested in the cardiovascular response to synthetic cannabinoids.

**Synthetic Cannabinoids (Nomenclature/History)**

Synthetic Cannabinoids, commonly called “synthetic marijuana” are considered “designer drugs”, as they are created in a lab and act on the same receptors as THC in marijuana, however, they bind to those receptors more strongly than THC, produce much stronger and different effects, and do not show up on a standard drug test. Synthetic cannabinoids are part of a group of drugs called novel psychoactive substances (NPS). Non classical synthetic cannabinoids have no medical benefit at present and have a high potential for abuse. Consumers can buy synthetic cannabinoids in convenience stores, from individual drug dealers, or online as incense or natural herbal products. They are sold under a number of brand names, including K2, Spice, and others. Cannabinoids is a generic name that encompasses both naturally occurring cannabis (marijuana) delta 9 tetrahydrocannabinol, but also synthetic cannabinoids, which can be either classical, with a structure similar to THC such as dronabinol and nabilone, which are cannabis related, or the chemically derived “non-classical” synthetic cannabinoids which have a wide variety of chemical structures, as they are entirely derived chemically and synthesized in labs, and while they interact with the same systems in the body, they have very different effects.

The endocannabinoid system is involved in controlling many different processes in the body: cognitive processing, behavior, memory, appetite, pain, motor functioning,
immune functioning, gastrointestinal functionality, nervous system, and cardiovascular health (Le Boisselier, Alexandre, Lelong-Boulouard, & Debruyne, 2017). There are three identified cannabinoid receptor ligands. Endocannabinoids (anandamide), naturally occurring cannabinoids-phytocannabinoids (delta-9-tetrahydrocannabinol, THC) and then synthetic cannabinoids.

| Table 4. Clinical features and Maximum Poisoning Severity Scores (PSS) for the three most commonly used Synthetic Cannabinoid Receptor Agonist products, without reported co-use of other substances. |
|---|---|---|
| **Clinical features** | **Black Mamba** | **Pandora’s Box** | **Clockwork Orange** |
| Cardiovascular | 36 (40.9%) | 28 (43.1%) | 12 (44.4%) |
| Neurological | 19 (21.6%) | 12 (18.5%) | 12 (44.4%) |
| Psychiatric | 25 (28.4%) | 17 (26.2%) | 8 (29.6%) |
| Gastrointestinal | 7 (8.0%) | 9 (13.8%) | 2 (7.4%) |
| General | 5 (5.7%) | 2 (3.1%) | 6 (22.2%) |
| Lab findings | 7 (8.0%) | 4 (6.2%) | 1 (3.7%) |
| **Maximum PSS** | | | |
| None (0) | 7 (8.0%) | 7 (10.8%) | 1 (3.7%) |
| Minor (1) | 53 (60.2%) | 31 (47.7%) | 23 (85.2%) |
| Moderate (2) | 22 (25.0%) | 22 (33.8%) | 3 (11.1%) |
| Severe (3) | 6 (6.8%) | 5 (7.7%) | 0 (0%) |

Figure 3. *Table taken from: (Waugh et al., 2016)*

Figure 3 shows a variety of adverse effects to 3 different types of synthetic cannabinoids. One of the things I found striking was the fact that people take it for its psychological effects, but adverse psychological responses only account for about 30% of the effects, and the cardiovascular adverse responses are far more prevalent than any of the other effects.

The first synthetic cannabinoids were created for pharmacological research purposes to study the endocannabinoid system. Researchers found a way to separate the THC from the plant to use for therapeutic medicinal treatments. In the 1960s,
neuroscience research transitioned to searching for analgesic and anti-inflammatory properties of cannabinoid receptor agonists (Lindsay & White, 2012). A majority of SCs previously synthesized for research purposes were named after the scientist or corporation that was involved in their synthesis. Pfizer, in 1970, created their own non-classical cannabinoid, called the CP series, after Charles Pfizer to be utilized for recreational purposes. In 1995, JWH compounds contained in SCs are named after John W. Huffman, the Clemson University professor that synthesized it to study its pharmaceutical effects on the brain (Brents & Prather, 2014) Whereas the AM compounds are named after a renowned scientist known as Alexandros Makriyannis of Northeastern University. Furthermore, HU compounds are affiliated to Hebrew University, created by chemist, Raphael Mechoulam based in Jerusalem, basing HU’s compound on the structure of THC (Heath, Burroughs, Thompson, & Tecklenburg, 2012). WIN was developed by the research company, Sterling-Winthrop through their research on pravadoline by conformationally restraining aminoalkylindole analogs, however, they dropped their research when they discovered WIN was cannabimetic (Wiley, Marusich, Huffman, Balster, & Thomas, 2011). The classification of SCs for recreational use was intended to help advertise and market products containing SC. For instance, AKB-48 denotes the title of a South Korean girl rock band, while XLR-11 was associated with the first USA-created liquid fuel rocket used by aircrafts. Currently, a significant percentage of synthetic cannabinoids are given names that derive from their innate structural compounds, that is, their core, tail name, linker, and associated groups. However, using the term ‘synthetic marijuana’ to refer to products with synthetic cannabinoids is debatable and a mistake according to Lewis Nelson, a renowned
toxicologist in the United States (Ibrahim, Al-Saffar, & Wannenburg, 2014). Nelson argues that products that contain synthetic cannabinoids are highly variable and significantly more potent than marijuana, and as long as marijuana is considered a criminalized drug and not regulated, synthetic cannabinoids, like “spice” and “K2” will be rampantly available as ‘legal marijuana’ on the market, causing toxic exposures and clinical consequences (Lapoint et al., 2011; Takematsu et al., 2014). Thus, given that the term ‘synthetic’ does not denote any known plant, using the phrase synthetic cannabinoid seems suitable. Synthetic cannabinoids are known by many street names such as K2, Bombay Blue, and Black Mamba. Fanatics of products containing synthetic cannabinoids in Spanish nations like Chile, refer to these products as ‘cripy’. In convenience stores and smoke shops, products with synthetic cannabinoids are referred to as “herbal incense” or “herbal smoking blends,” with colorful packaging, often labelled as incense or potpourri often labeled with “not for human consumption, keep out of reach of children” (Zawilska & Wojcieszak, 2014).

**Epidemiology**

A good deal of epidemiological data indicates that synthetic cannabinoids have a history of substantial use and impact on health. From the findings, it was determined that the drug is highly abused in the U.S. Military and Navy, and for civilians, the abuse is common among adolescents. Synthetic cannabinoids are a diverse group of compounds designed to mimic cannabis. While not as frequent as cannabis, their use is still quite widespread. In 2011, 30 Airmen at an Air Force Base in Oklahoma, were discharged for spice use. In October of the same year, 64 Sailors were busted for spice use aboard the carrier, while, one month later, 28 more Sailors were caught on a different carrier. Spice
use led to the expulsion of 16 men from the Naval Academy in 2011, followed by 14 Coast Guard Academy cadets in January 2012. Between 2011-2012, over 150 personnel were apprehended for synthetic cannabinoid use in five separate incidents in different military branches which shows the extent of the problem among the disciplined forces (White, 2017).

Synthetic cannabinoid use is also highly prevalent in the general public. Kelley et al., 2013 conducted a field-based study in New York City nightclubs in 2012, finding that 8.2% of 1749 adults with a mean age of 26.4, 55% of those being male, reported using synthetic cannabinoids in the past year (Kelly et al., 2013). Whereas, in (Winstock et al., 2011), a 2011 study on synthetic cannabinoid prevalence in the UK, 12.6% of 2700 adults reported using a synthetic cannabinoid in the past year. According to NIH, in 2012, 11% of American high school seniors had used synthetic cannabinoids in the past year the majority being white males from urban locations (Gunderson, Haughey, Ait-Daoud, Joshi, & Hart, 2014). In this study, the researchers also found that the proportion of adolescent males who engaged in synthetic cannabinoid use was 70%. In 2011, 28, 531 emergency room visits, were linked to synthetic cannabinoid use, out of those, 78% were between the ages of 12-29. In a worldwide survey of nearly 15,000 participants, 17% reported synthetic cannabinoid use (Singh et al., 2018). Synthetic cannabinoid use is associated with serious consequences. In Mississippi from April through May of 2015 approximately 1200 emergency room visits, and 17 deaths were attributed synthetic cannabinoids (White, 2017). In 2015, the American Association of Poison Centers identified 3500 calls related to synthetic cannabinoid use, a dramatic 299% increase from just 6 months prior in 2014 (LT George Loeffler, 2012). The increase was immense and
reveals the problem related to the illegal and unprescribed use of the substance. In 2016, there were 130 emergency department visits due to synthetic cannabinoid use in New York City in a single week in July 2016 (White, 2017). According to a study done by Gloria Martz, between 2014 and 2016, 49.8% of 429 adolescents between the ages of 13-17 were admitted to a Children’s Recovery Center in Oklahoma for synthetic cannabinoid use. 22.4% of these children reported being daily SC users. Martz, in this same study, discussed that young individuals are at an increased risk for using synthetic cannabinoid products, as they are relatively inexpensive, easily available, known to produce higher subjective effects, and difficult to detect on drug tests (Martz, Tankersley, Mekala, & Motiwala, 2018). In summary, the current epidemiological data indicate that synthetic cannabinoids are used relatively extensively. There is also a substantial body of data showing that these substances can have a substantial impact on health.

Figure 4. Data derived from: (Roehler, Hoots, & Vivolo-Kantor, 2020)
Figure 4 shows the regional disparity as a trend over time on the proportion of ER visits related to Synthetic Cannabinoid exposure. Note the regional differences with a rather stable national trend (the green line) from 2016-2019. On the West (orange line), you can see the biggest decline in exposure, however, in the Midwest (the black line) there is a recent upwards trajectory in exposure.

Effects of Synthetic Cannabinoids in Humans

While synthetic cannabinoids interact with the same endogenous system as cannabis, their effects are quite different and poorly understood. As seen in Figure 3, synthetic cannabinoids adversely affect many physiological processes including gastrointestinal (7.4-13.8% of cases), psychiatric (26.2-29.6%), neurological (18.5-44.4%) and the most commonly presenting affect is cardiovascular (40.9-44.4%). Serious adverse cardiovascular effects have presented following both acute and chronic synthetic cannabinoid use within case reports and clinical studies, resulting in stroke, arrhythmias, cardiomyopathy, myocardial infarction, and cardiac arrest (Pacher, Steffens, Haskó, Schindler, & Kunos, 2018). In addition to psychological effects that trigger their use, in humans these substances may cause substantial cardiovascular morbidity including, increased heart rate and blood pressure which may contribute to deadly effects such as heart attacks (White, 2017). It seems that the synthetic cannabinoids may activate the autonomic nervous system, however, how this happens is not yet understood. Unfortunately, there remains a lot of controversy surrounding how synthetic cannabinoids affect the cardiovascular system.
Figure 5 shows data taken from multiple case studies, regarding the distribution of acute cardiovascular adverse events in patients using synthetic cannabinoids. The most predominant effects are related to increases in heart rate, blood pressure and cardiac arrhythmia. Patients have also presented with angina, which is indicative of disturbances in cardiac electrical activity and coronary ischemia, respectively.

**Cardiovascular Concerns of Excessive Uses of Synthetic Cannabinoids**

Researchers have suspected the excessive use of products containing synthetic cannabinoids for over five decades have resulted in adverse cardiovascular effects. However, the last decade has experienced a steady increase in reported frequencies of cardiovascular-related complications. A recent systematic review has concluded that
there is a positive correlation between excessive use of products containing synthetic cannabinoids and cardiovascular complications among patients in many hospitals across the United States (Hermanns-Clausen et al., 2016). To put this into perspective, the United Kingdom National Poisons Information Service has recorded that between 40-45 percent of all synthetic cannabinoid-linked incidents are of a cardiovascular nature among youths and individuals in their early adult ages between 2006 and 2010 (Waugh et al., 2016). Moreover, the prevalence of cardiovascular-related difficulties grew from 1.1% during 2006, to about 3.6% during 2010, whereas the death rate linked to those complications reached 25% by the end of 2010 (Heath et al., 2012). Synthetic Cannabinoid users who display these complications are often young males and, importantly, have no underlying or genetic link for cardiovascular conditions. Common cardiovascular difficulties of products containing synthetic cannabinoids range from mild to severe depending on the type of complication. A variety of cardiovascular related complications have been reported.

\textit{Cerebrovascular Disease}

Excessive use of products with synthetic cannabinoids leads to the early development of cerebrovascular disease among individuals in their early 20s, and patients undergoing cancer treatment procedures. Patients with cardiovascular conditions showcase symptoms such as neurological impairment and ischemic stroke as the most common adverse impact as a result of using products containing synthetic cannabinoids (Singh et al., 2018). Furthermore, a study conducted among a segment of an Australian population demonstrated that individuals aged between 18 and 44 years suffered from
ischemic events due to excessive use of products containing synthetic cannabinoids (Jouanjus et al., 2017; Wolff & Jouanjus, 2017).

Chronic use and smoking of synthetic cannabinoids trigger cerebrovascular events that impact an individual’s cardiovascular condition. Some of the mechanisms that are linked to probable cerebrovascular dangers such as direct vasculo-toxic impacts, modifications in hemodynamics, and atrial fibrillation (Lindsay & White, 2012; White, 2017). Also, temporary exposure to smoke produced by synthetic cannabinoid can trigger endothelial dysfunction among patients (Singh et al., 2018; Von Der Haar et al., 2016). A population review in US-based hospitals reveals that people with habitual smoking of products containing synthetic cannabinoids experience a three-fold increase in the possibility of developing intense cerebrovascular events (Hermanns-Clausen et al., 2016). Also, a case study series by the American Heart Association demonstrated that individuals with ischemic stroke are in danger of suffering a persistent stroke when they start re-using products containing synthetic cannabinoids (DeFilippis et al., 2020; Khan, Hanif, & Wilson, 2018; Wolff & Jouanjus, 2017). The male population in the United States is more at risk to suffer from cerebrovascular disease than women and the fact that males more commonly are the ones who smoke synthetic cannabinoids is concerning.

**Arrhythmias**

Incessant usage of products with high concentrations of synthetic cannabinoids leads to severe cases of arrhythmias and a wide variation of electrical impacts that deteriorates an individual’s cardiovascular condition. These conditions include atrial fibrillation, asystole, and supraventricular tachycardia that intensifies an individual’s CVD situation (Al Fawaz et al., 2019). Additionally, high dosages of synthetic
cannabinoids caused increases in catecholamines, and the stimulation of cardiac beta-adrenergic receptors may heighten the likelihood of a patient developing arrhythmias (Ozturk, Yetkin, & Ozturk, 2019). Atrial fibrillation is the most common form of arrhythmias among patients who use products containing synthetic cannabinoids (Pacher et al., 2018; White, 2017).

The incessant use of synthetic cannabinoids triggers huge fluctuations in cardiac rhythms. The rhythm variations include sinus tachycardia, ectopic atrial, and ventricular fibrillation that often leads to sudden cardiac death among patients who are consistent users. Cardiac tachyarrhythmias are due to excessive use of synthetic cannabinoids causing the introduction of a hyperadrenergic state (Ozturk et al., 2018; Ozturk et al., 2019; White, 2017). Further, a recent electrophysiology review to investigate the effects of excessive use of synthetic cannabinoid compounds reveals the prevalence of inducible and untimely ventricular contractions. Similarly, myocardial ischemia that occurs due to microvascular spasm in response to synthetic cannabinoid use may lead to severe dizziness, syncope, cardiac arrest, and eventually an abrupt cardiac death (Cohen & Weinstein, 2018).

Cannabis Arteritis

Cannabis vasculopathy is indistinguishable from thrombo-angiitis obliterans, however, it presents at an earlier age in those who consume both synthetic cannabinoids and tobacco (Ibrahim et al., 2014). Additionally, subacute and advanced ischemia for upper and lower limits impacted by arteritis may result in severe difficulties such as tissue necrosis with deteriorating effects when an individual uses synthetic cannabinoids (Ibrahim et al., 2014). Additional vascular difficulties of nonstop use of synthetic
cannabinoid products are migratory thrombophlebitis, renal artery segmentation, and vasculopathy concerning the occlusion of the primary retinal veins (Singh et al., 2018).

**Clinical Implications**

The two German investigators who smoked “Spice Diamond” experienced the acute effects due to smoking which included change in opinions and thinking, and dry mouth (White, 2017). According to their study, the use of synthetic cannabinoids causes tachycardia, impairment of mood and perception, anxiety, loss of concentration, paranoia, and then post use, extreme fatigue. The most common adverse events associated with the use of synthetic cannabinoids include agitation, drowsiness, vomiting, and other extreme effects which easily lead to death. Males who are over the age of 30 years were more likely to die and experience serious symptoms as compared to the female counterparts (Zaurova et al., 2016). The most common cardiovascular symptom associated with the use of synthetic cannabinoids include tachycardia with a heart rate of 90-170 beats every minute. In addition, at least a third of the patients exhibited hypertension (Zaurova et al., 2016). Hypokalemia, muscle pain, and acute psychosis were also some of the major symptoms that developed and last for several days. The typical psychotic and neurological effects include the drowsiness, paranoia, and euphoria which developed with continuous and use of synthetic cannabinoids. A study conducted between patients with exposure to synthetic cannabinoids and other patients who had smoked traditional marijuana revealed modified and altered mental status and cardiotoxicity of the individuals who had used the synthetic cannabinoids (Zaurova et al., 2016).
**Effects of Synthetic Cannabinoids in Rats**

Short term administration of these agents to anesthetized animals result in an overall decrease in blood pressure (Malinowska, Baranowska-Kuczko, & Schlicker, 2012; Malinowska, Toczek, Pedzinska-Betiuk, & Schlicker, 2019; O'Sullivan, 2015). In contrast, research in conscious animals is more limited, but these agents appear to cause an increase in blood pressure (Ho & Gardiner, 2009; S.M. Gardiner, 2001; Schindler, Gramling, Justinova, Thorndike, & Baumann, 2017). Schindler (Schindler et al., 2017) saw increases in blood pressure with a series of synthetic cannabinoids. Gardiner (S.M. Gardiner, 2001) responses show dose dependent increases in blood pressure. However, both of these studies showed a decrease in heart rate. (Niederhoffer & Szabo, 1999) reported a decrease in blood pressure with WIN 55, 212-2 in conscious rabbits. Therefore, there remains considerable controversy regarding the effects of synthetic cannabinoids in cardiovascular function in animals, which limits understanding of the mechanisms involved. Several pharmacologic effects are associated with synthetic cannabinoids which was determined to induce anxiety in animals. For instance, in rats, it contributes to higher serum corticosterone concentrations that directly refer to the aspect of being responsive to stress (White, 2017). (Niederhoffer & Szabo, 1999) however, in their study they found no evidence for vasodilation in response to WIN 55, 212-2.

**Evidence for PVN in Cardiovascular Control**

The paraventricular nucleus of the hypothalamus (PVN) amongst many other functions, is known to regulate the autonomic nervous system by controlling the sympathetic outflow to the heart and the blood vessels. Due to its critical role in regulating sympathetic tone, dysregulation in sympathetic activity directly correlates to
dysregulation of the heart, and in some cases, heart failure. Pre-autonomic neurons of the PVN become disinhibited and lead to increased sympathetic activity via glutamate and angiotensin II (Pyner, 2014). The PVN plays a critical role in regulating blood volume via the mechanoreceptors in the heart. An increase in blood volume stimulates increases in heart rate, renal vasodilation via vasopressin neurotransmission, increases in urine output, and sodium loss (Dampney, Michelini, Li, & Pan, 2018). It is also known that the PVN is critical in chronic stress responses, psychological stress, such as conditioned fear, as well as continuous increases in sympathetic nerve activity (Dampney et al., 2018). It is known that in spontaneously hypertensive rats, the PVN pre-sympathetic neuronal excitement and sympathetic outflow is significantly increased in comparison to normotensive rats, such as Sprague Dawley rats. PVN pre-sympathetic neurons are critical to increases in sympathetic vasomotor activation that causes increases in blood pressure and hypertension (Dampney et al., 2018). Some work suggests that cannabinoid receptors are expressed in the PVN (Grzeda et al., 2015; Ozturk et al., 2019). Thus, the PVN may be involved in the cardiovascular responses to synthetic cannabinoids.

**cFos as a Neuronal Marker**

cFos is a gene that is activated through sensory stimulation, as it encodes for the Fos transcription factor. The Fos protein is produced in the cytoplasm, then travels to the nucleus where it combines with other proteins to create heterodimeric transcription complexes in order to regulate transcription of other genes (Kovacs, 2008). Neuronal Fos signal activation occurs where the animal is exposed to a novel stimulus for a 90-minute period of time. Immunostaining, which is the application of antibodies that work against
the desired protein, in this case, cFos protein as a neuronal marker, can be observed (Chung, 2015).

**Summary of Rationale**

The use of synthetic cannabinoids is prevalent and increasing in some countries. Epidemiological data indicate that use of synthetic cannabinoids is associated with significant cardiovascular mortality. Data in animals is mixed with some data showing decreases in blood pressure and heart rate whereas, other data shows increase in blood pressure and heart rate. A major difference occurs between anesthetized and conscious animals, suggesting involvement of the brain. CB receptors are extensively distributed in the brain and are known to be found in the PVN.

**Statement of Hypothesis**

We hypothesize that the PVN is a site in the brain that synthetic cannabinoids act on to cause increased blood pressure by activating the sympathetic autonomic nervous system, thereby, wiping out the response by inhibiting neurotransmission. Looking at the big picture, this project aims to identify the role of the PVN in the abuse of synthetic cannabinoids and how they are associated with cardiovascular adverse effects, so if we understand their mechanisms of action, we can figure out effective treatments.

**Specific Aims**

1) Determine the blood pressure and heart rate responses to a synthetic cannabinoid, WIN 55, 212-2, in conscious rats.

2) Determine the role of the autonomic nervous system in these responses.

3) Determine if the hypothalamic paraventricular nucleus is involved in the response to synthetic cannabinoids.
II. MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (300-400 grams) from ENVIGO or the Animal Resource Center (ARC) 60-90 days old were used. The animals were housed in the ARC two per cage, in a room with many other rats. The room is set on a specific light/dark cycle, where the lights turn on at 6 am and turn off at 6 pm. The protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) and observed NIH guidelines.

Surgical Interventions

Male Sprague Dawley rats (300-400 grams) were randomly selected and placed in an anesthesia chamber connect to a gas anesthetic (isoflurane) vaporizer. Isoflurane (5%) with an oxygen flow rate of 3 L/min was used to induce anesthesia. Once the animal had lost the righting reflex, the isoflurane was adjusted to 2-3% to maintain anesthesia. The rat was shaved at the surgical locations, the femoral area and the nape of the neck. Each surgical area was wiped with iodine scrub (x2) and 70% isopropyl alcohol ending with a wipe of regular iodine to disinfect the surgical sites. The rat was then transferred to a water jacket heating pad (set at 45 degrees C), taped down in the dorsal recumbent position, artificial tears were placed on the eyes, and the rat placed back in the nose cone with the isoflurane anesthesia this time at the 2.5 level. Anesthesia was adjusted based on need as judged by the respiratory rate or reaction to pinch. The rat’s tail was labeled with indelible marker for permanent identification. The surgical area surrounding the rat was
wiped down with 70% isopropyl alcohol. A surgical pack that had been autoclaved at a sterilization temperature of 270 degrees for 20 minutes, with a 15-minute drying time was placed next to the surgical area. The rats were covered with a piece of Press and Seal wrap (Glad Wrap) to serve as a sterile surgical drape. The surface was scrubbed with 70% isopropyl alcohol. Sterile gloves and a surgical mask were put on, and the surgical pack was opened.

The rats were treated with Buprenorphine SR (1.2 mg/mL/kg) to provide analgesia. A fourth of an inch incision was made through the skin at the femoral area vertically towards the midline using scissors. Then, blunt dissection using forceps was used to separate tissues until the femoral artery and vein were revealed. The vessels were isolated from each other using blunt dissection with forceps, and a 3-0 black silk non-absorbable suture was placed around each vessel at both the proximal and distal ends. During this process, the area was infiltrated with 2% lidocaine to provide local anesthesia and relax the vessels for catheter insertion. The sutures were first used to occlude the flow of the vessels during catheter insertion. Then a small cut was made on the vessels using micro-Vanna spring scissors to introduce the catheters into the vessels.

Polyethylene Tubing (PE-50) connected to a syringe containing heparinized saline (25 U/ml) for the venous catheter tubing. The arterial catheter consisted of Tygon microbore tubing (0.020”) glued together with a 1” long piece of micro-renathane MRE-025 for the intravascular portion. The catheter was glued together days prior to the surgery using E6000 to bind it together. The micro-renathane provided a non-thrombotic surface to prolong catheter patency during chronic implantation. The venous and arterial catheters were soaked for an hour in iodine and then for an hour in 70% isopropyl alcohol and
flushed with heparin saline prior to implantation. The catheters were then introduced into the vessels about 1”, tied in place, then tunneled subcutaneously to the back of the neck using a trochar. The femoral area was sutured closed with 4-0 violet braided, polyglycolic acid, absorbable suture followed by 2-4 stables and vet bond. The area at the back of the neck was sutured around the catheters with 3-0 black silk non absorbable suture, wrapping the catheters around 6 times, and sealed with vet bond to help hold the catheters during chronic implantation. The catheters were trimmed to about ¾” sticking out between the ears, flushed with heparin saline, and plugged. For plugs, we used a blunted and crimped 22 gage hypodermic needle about ¼” long, injected with E6000 glue which was allowed to dry a few days prior to surgery to bond and seal. The wounds were covered with a topical antibiotic ointment (bacitracin). The rats were returned to their home cage. After the rats regained consciousness and were mobile, they were returned to the animal quarters to recover.

Protocol

The animals were then allowed 3-5 days of recovery, then trained to the recording area for 2-3 days. The animals were checked on daily, to assess for general condition, mobility, leg functioning, and weight. On the training days, the rats were brought to the recording room so they could acclimate to the environment. On the training days, the rat’s arterial and venous catheters were flushed with heparin saline to ensure there were no clots. On the experimental days, the arterial line was connected to a pressure transducer connected to the BioPac recording system where arterial pressure and heart rate were recorded continuously.
The rationale for the doses of WIN were taken from the literature (S.M. Gardiner, 2001). The cyclodextrin vehicle is made up from a 20% weight to volume ratio with sterile water and then vortexed until into solution. Initially, we attempted 50, 100, and 300 µg/kg doses, but the 300 µg/kg had severe adverse behavioral effects, so we switched to doses of 25, 50, and 100 µg/kg. The WIN was made up by weighing out between 400-700 micrograms, and then mixed with it 20% Cyclodextrin (vehicle) to make the stock solution of 100 µg/kg. To go into solution, the 100 µg/kg dose of WIN was put in the sonicator for about 20 minutes, then placed in a beaker of water on a hot plate at level 8 for 10 minutes, vortexed, then placed back on the sonicator for 20 minutes. This procedure was repeated until the drug went into solution. To make the subsequent doses, we used serial dilutions. The 50 µg/kg was made from diluting part of the stock solution with the vehicle by a one-to-one ratio, then the 25 µg/kg was made from diluting part of the 50 µg/kg with the vehicle by a one-to-one ratio.

A baseline recording (about 30 minutes to an hour) was obtained, until the rats exhibited a calm, stable blood pressure and heart rate. The rats then received an intravenous injection of 0.2 mL 20% cyclodextrin vehicle followed by 0.2 mL saline flush. Responses to the vehicle injection were recorded for approximately 20 minutes. After this, the rats received injections of 0.2 mL of a synthetic cannabinoid, WIN 55, 212-2, at the doses of 25, 50, and 100 µg/kg, followed by a 0.2 mL saline flush monitoring for between 30 minutes to an hour in-between each dose. Blood pressure and heart rate were monitored for approximately 20 minutes after each injection. In all cases, blood pressure and heart rate were allowed to return to baseline prior to the next injection.
**Ganglion Block Procedure**

Some of the rats were subjected to ganglionic blockade. After getting a successful response from the 3 doses of WIN and allowing blood pressure and heart rate to return to baseline, the rats received an intravenous injection of a ganglion blocker. Ganglionic blockade was used to assess the role of the autonomic nervous system in the responses to WIN injection. Ganglionic blockade was achieved by intravenous injection of chlorisondamine at a dose of 10 mg/kg/0.1ml. The chlorisondamine was made by weighing out an amount of drug based on the animal’s weight and sufficient to make up about 0.3-0.4 milliliters of solution (extra to account for amounts lost in the needle hub and line). Chlorisondamine iodide is relatively insoluble in aqueous solutions. It was dissolved by adding the drug powder to the appropriate amount of sterile water in a sterile test tube. The solution was then gently heated on a water bath in 1-minute increments with vortexing at each step until the drug went into solution. Once in solution, the chlorisondamine solution was loaded into a 1 ml syringe to deliver the drug to the rat. 0.1 mL of solution was delivered intravenously followed by a 0.2 ml saline flush. After this injection, the rats were monitored until blood pressure and heart rate became stable (roughly 10 minutes) then given an injection of 0.2 mL 100 microgram per kilogram WIN 55, 212-2 followed by a 0.2 mL saline flush. The rats were then monitored for 30 minutes to observe the effect of WIN after ganglion blockade.

**Brain Collection Procedure**

Immunohistochemistry was used to assess the brain involvement in the responses to WIN. The following procedure was used to collect the brain for this purpose. The animal was injected with either 0.2 mL WIN (100 µg/kg) or 20% cyclodextrin vehicle
followed by a 0.2 mL saline flush through the venous catheter then monitored for 90 minutes. The rats were then anesthetized deeply with urethane chloralose (800 mg/kg: 80 mg/kg). After waiting for 15 minutes, deep anesthesia was confirmed by tail pinch. Then, the animal was placed in a collection tray in the fume hood. A cut was made at the xiphoid process, snipping up on both sides of the diaphragm until the heart was exposed. The right atrium was then snipped to release blood, then 40-50 mL of heparin saline was injected into the left ventricle to flush the vascular system of blood. This was followed by 40-50 mL of 4% paraformaldehyde for perfusion fixation of the brain. A big scissor was then used to separate the head from the body, the skin on the top of the head was peeled back to reveal the skull then a rongeur is used to carefully chip away the skull like an eggshell to collect the brain. The brain was then placed in a solution of 4% paraformaldehyde for 24 hours for post fixation. After which the brain was transferred for long-term storage to a 20% sucrose solution to cryoprotect the brain in preparation for cryosectioning.

**Cryosectioning Procedure**

The cryostat was set to -20 degrees and to cut sections at 30 microns. The frontal lobe and the occipital lobe of the brain were then cut off using a razor blade to block the brain for mounting on the sectioning pedestals. The brain was then mounted onto the pedestal using Tissue-Tek O.C.T compound and then placed into a bucket of crushed dry ice for a few minutes to ensure rapid, complete and uniform freezing. Then, the mounted brain was placed into the chuck of the cryostat. 30-micron sections were taken, and the chuck adjusted until an even section was obtained. Sections were discarded until the optic chiasm and 3rd ventricle became visible. The sections were then collected, three per
slide, from approximately bregma -1.30 to -2.30 mm using anatomical markers (e.g., optic chiasm) as depicted in the atlas of Paxinos and Watson. The sections are mounted to the slides by carefully placing them on the slide and then placing a warm finger underneath so that it would heat up and bind to the slide. The section to slide arrangement are as follows: the first slide: sections 1, 4, and 7, second slide: 2, 5, and 8. third slide: 3, 6, and 9; and so forth. This staggered order on successive slides was used to allow close proximity of sections destined for immunohistochemical and histochemical staining.

Immunohistochemistry Procedure

First, a hydrophobic barrier with a Vector laboratories ImmEdge™ Pen was applied around each section on each slide. Then, the sections were subjected to a heat-induced antigen retrieval protocol as follows. 100 mL of citrate buffer with a pH of 6 was boiled for five minutes in a beaker. Then, the tissue slides were placed in a six-well plastic slide box and the citrate buffer was poured over the top of the slides, and then boiled in the microwave again for seven minutes. After boiling, the plastic slide box was immersed in an ice bath for one hour to cool the sections.

After one hour in the ice bath, the following IHC protocol was performed. The slides were washed 5 times each with 1% phosphate buffered saline containing 1% (v/v) Tween detergent (PBS-T). The 1% PBS-T is made up of a phosphate buffered saline tablet (Sigma P4417) in 200 mL of ultra-pure water and 2 mL Tween 20. Then, the slides were placed back into the plastic slide box for application of antibody solutions. The primary antibodies were made in a solution of 10% goat serum in 1% PBS-T. The 10% goat serum in 1% PBS-T is made up of 1 mL Goat Serum in 9 mL 1% PBS-T. The primary antibody used was an Abcam rabbit polyclonal to cFos (ab190289) (1mg/ml).
For application to the sections, a 1:100 dilution was used by taking 1-part Abcam primaries to 99 parts 10% goat serum in 1% PBS-T (10 µL Abcam and 990 µL 10% Goat Serum in 1% PBST). For staining, 10 µL of the cFos primary antibody solution was placed on the first section and the third section of each slide. The middle section on each slide was used as a primary antibody negative control section. This section was treated with a negative control of 10 µL 10% goat serum in 1% PBS-T which did not contain the primary antibody. Then, the plastic slide box was placed in the fridge on the rocker at 4 degrees Celsius for 24-hours. After 24 to 48 hours, the slides were thoroughly washed five times with 1% PBS-T prior to application of the secondary antibody. The secondary antibodies were applied to each of the three sections on the slides. The secondary antibodies are made up from a donkey or goat anti-rabbit highly cross-absorbed secondary antibody, Alexa Fluor Pluss 488 (2 mg/mL) Invitrogen (donkey: A32766, goat: A32731). 10 µL of the anti-donkey or anti-goat secondary antibody was diluted in 1990 µL of 10% goat serum in PBST. 10 µLs was then applied to each section on the slide. The slides were then left in a covered box for two hours at room temperature for incubation and to protect from light since the fluorescent antibodies are subject to photobleaching. After two hours incubating in the secondary antibodies, the slides are thoroughly washed five times with 1% PBS-T and then a coverslip mounting medium containing 4’,6-diamidino-2-phenylindole, DAPI (to visualize nuclei) was placed on each of the sections on the slide, and then a dust-free coverslip was placed over the top. The slides were stored in the covered opaque box in the fridge at 4 degrees Celsius for 40 minutes. After 40 minutes, the sections were then used for fluorescent microscopy.
**Cresyl Violet Staining Procedure**

Cresyl violet stains the DNA and RNA nucleic acids in the brain in order to detect the shapes of the structures in the brain. Slides adjacent to the ones we ran for the immunohistochemistry procedure were then taken for cresyl violet staining, to confirm the location of the PVN. The cresyl violet stock solution is made up of 0.2 grams cresyl violet-acetate with 150 mL distilled water, then mixed with a stir bar for 20 minutes. The buffer solution (pH 3.5) was made from 282 mL of 0.1 M acetic acid (6 mL concentrated acetic acid per 1000 mL distilled water) with 18 mL of 0.1 M sodium acetate (13.6 grams in 1000 mL distilled water). Then, 30 mL of cresyl violet stock solution with 300 mL of buffer were mixed for 30 minutes. First, the slides were placed in Xylene for 5 minutes, then 95% isopropyl alcohol for 3 minutes, then 70% isopropyl alcohol for 3 minutes, then deionized distilled water for 3 minutes, then cresyl violet for 8 minutes, distilled water for 3 minutes, 70% isopropyl alcohol for 3 minutes, 95% alcohol for 1 minute, then one dip of 100% isopropyl alcohol, then HemoD for 5 minutes, then Citrisol for up to 24 hours. Then, the slides were cover slipped with a histological mounting medium and left overnight to dry before observing under the microscope.

**Fluorescent Microscopy**

A Leica DMLB microscope with Leica Application Suite X software was used for the fluorescent microscopy procedure. An emission wavelength of 488 was used to visualize cFos under the microscope and the DAPI channel was used to get emission at wavelength of 461 fluorescence for DAPI. Images were taken for the WIN injected brain’s as well as the vehicle injected brains at 10x, 20x, and 40x of both 488 and DAPI for the PVN area with and without primary antibodies, the Amygdala area with and
without primary antibodies. At least 24 images per slide were collected. The cFos staining with the DAPI staining at each magnification level for each location were then overlayed to identify if the perceived cFos staining was within the nuclei. Then, the cresyl violet slides were observed through light/bright field under the microscope at 5x magnification and images were taken at both the PVN and Amygdala for each slide.

III. DATA ANALYSIS

Blood Pressure and Heart Rate Data

The blood pressure and heart rate data were analyzed from each tracing by measuring the baseline blood pressure and heart rate before each injection, then the blood pressure and heart rate for one-minute durations every five minutes post injection to obtain the time course of each response. The peak heart rate and blood pressure to each of the doses of WIN injection were also collected for a one-minute duration. Once acquiring these data were compiled into Excel spreadsheets and transferred to an analysis package (GraphPad Prism) for analysis. Data was checked for outliers using Grubb’s test. One blood pressure data point was removed since it tested out to be an outlier. Data that did not meet criteria for parametric analysis were log transformed prior to analysis.

Image J Analysis of Immunofluorescence Images

TIFF images of the overlay for cFos and DAPI staining were imported into NIH Image J. These color images were then separated into their red (nonexistent), green (cFos) and blue (DAPI) component channels. Background subtraction was performed on each channel. The threshold feature of Image J was then used to despeckle the images. An area encompassing the region of interest (PVN or Amygdala) was defined using the
polygon tool on Image J. The bounded area was then analyzed using the analyze particle function from the analysis menu. This produced an output that included the number of particles (counts). Matched areas were used to analyze the green (cFos) and blue (DAPI) channels. The particle counts from each analysis was entered into an Excel spreadsheet. cFos staining was expressed as a proportion of overall cell number in the selected area as reflected by the DAPI particle counts (% cFos staining = (cFos particle count/DAPI particle count) x 100). Staining was evaluated on the sections with and without primary antibody, and in rats treated with WIN or with vehicle.
IV. RESULTS

Effect of WIN 55, 212-2 on blood pressure and heart rate in conscious rats.

Raw tracings for blood pressure and heart rate are shown in figure 6. Briefly, cyclodextrin vehicle did not appear to affect blood pressure or heart rate whereas WIN injection increased both blood pressure and heart rate. Summary data are presented in figures 7 and 8. Baseline blood pressure and heart rate averaged approximately 122± 4 mm Hg and 356± 11 bpm prior to the injection of the cyclodextrin vehicle. Injection of cyclodextrin vehicle caused changes in blood pressure 0.2± 1 mm Hg and heart rate -1± 2 bpm that were not significantly different from baseline. In contrast, intravenous injection
of WIN produced markedly different effects. These data are illustrated in figures 6 (MAP) and 7 (HR). At the lowest dose of 25 µg/kg, blood pressure increased by 7±2 mm Hg while heart rate increased by 30±7 bpm. At the middle dose of 50 µg/kg, these responses were 16±3 mm Hg and 58±13 bpm. At our highest dose of 100 µg/kg, we observed an increase of 18±2 mm Hg in blood pressure, and 74±10 bpm in heart rate. These responses were significantly greater than that caused by vehicle injection. Therefore, intravenous injection of WIN was associated with dose dependent increases in blood pressure and heart rate.

![Figure 7. This figure shows the changes in mean arterial pressure obtained in conscious rats (n=10-11) injected with vehicle (0) or incremental doses of a synthetic cannabinoid, WIN 55, 212-2 at doses of 25, 50 and 100 µg/kg. ANOVA followed by Dunnett’s Test. * Indicates significant difference from vehicle control p<0.05](image-url)
We also measured the MAP and HR responses for 20 minutes after injection to establish a time course. The data are shown in figures 9 (MAP) and 10 (HR). As shown in figure 9, the blood pressure response reached its highest point within the first 5 minutes after injection and then declined thereafter. However, blood pressure remained significantly elevated compared to vehicle injection up to the 15-minute mark. At the lowest dose, blood pressure was no longer significantly higher than vehicle injection at 20 minutes post injection. As might be expected, higher doses had longer durations and the blood pressure responses to WIN remained significantly above those for vehicle even after 20 minutes.

**Figure 8.** This figure shows the changes in heart rate obtained in conscious rats (n=10-11) injected with vehicle (0) or incremental doses of a synthetic cannabinoid, WIN 55,212-2 at doses of 25, 50 and 100 µg/kg. ANOVA followed by Dunnett’s Test. * Indicates significant difference from vehicle control p<0.05
A somewhat similar pattern was observed in terms of heart rate. WIN injection produced very rapid onset increases in heart rate that appeared to peak at around 5 minutes post injection. Somewhat different from blood pressure however, heart rate did not rapidly decrease over time and were in fact maintained for up to 20 minutes (figure 10).

Figure 9. This figure shows the time course of the changes in blood pressure obtained in conscious rats (n=10) injected with vehicle or incremental doses of a synthetic cannabinoid, WIN 55, 212-2 at doses of 25, 50 and 100 ug/kg. Repeated measures ANOVA followed by Student Newman Keul test. * p<0.05 vehicle vs 25 ug/kg, Ф p<0.05 vehicle vs 50 ug/kg, Т p<0.05 vehicle vs 100 ug/kg
Effect of Ganglion Blockade

The cardiovascular responses to synthetic cannabinoids may involve an autonomic nervous system component. We used pharmacological ganglion blockade to assess the role of the autonomic nervous system. Ganglion blockers work by blocking the transmission at the sympathetic and parasympathetic ganglia in the autonomic nervous system. Administration of the ganglion blocking agent, chlorisondamine, reduced both blood pressure (120±5 to 80±4 mm Hg) and heart rate (381±12 to 345±17 bpm). Figure 10 shows compares the MAP and HR responses to WIN 100 µg/kg obtained before and after ganglionic. This data shows that Synthetic Cannabinoid, WIN, acts by
increasing sympathetic outflow. Pre-ganglionic blockade, we observed a change in mean arterial blood pressure of 17±2 and change in heart rate of 63±9. Post ganglionic blockade, we observed a MAP response of 1±2 mm Hg and a HR response of -13±5 bpm. Therefore, ganglionic blockade abolished the response to WIN.

**Figure 11.** This figure shows the effect of autonomic blockade on blood pressure and heart rate obtained in conscious rats (n=6) injected with synthetic cannabinoid, WIN 55,212-2 at a dose 100 µg/kg. Paired t-test. * Indicates significant difference from vehicle control p<0.05

**Immunohistochemistry**

We used cFos immunohistochemistry as an approach to detect neuronal activation in response to drug injection. Each immunohistochemistry run included a section in which no primary antibody was included. This section served as an antibody negative control as illustrated in figure 12. Very little punctate green (cFos) staining was observed in these sections indicating little to no non-specific binding of the fluorescent secondary antibody.
Two animals received vehicle injection prior to brain collection and served as vehicle controls for immunohistochemistry. The results from these two rats were variable as illustrated in figure 13. In one case there was some positive cFos staining observed as depicted in figure 12. This staining was relatively sparsely distributed but appeared to be localized primarily along the lower ventricular margin of the PVN. In the other vehicle injected rat, no staining was observed in the PVN.

**Figure 12.** This figure shows fluorescent immunohistochemistry images for cFos (green) and DAPI staining in a section where the primary antibody to cFos was omitted. These sections served as antibody negative controls. From the top left shows 488 emissions, then DAPI staining (top right), overlay of 488 and DAPI low power (bottom left) and high power (bottom middle). The lower right shows a cresyl violet image to identify location. There is virtually no green punctate staining indicating that there was very little nonspecific binding of the secondary antibody. Scale bars are shown in each figure. 3rd V = third ventricle, PVN = paraventricular nucleus
WIN injection was associated with an increase in cFos staining. An example of this staining is shown in figure 14. Punctate green staining was observed in the region of the PVN in discrete cells. DAPI overlay was consistent with the idea that this staining occurred in the nucleus, consistent with cFos staining. The cresyl violet (Nissl stain) image shows that this section was taken in the early-mid PVN region.
Summary data for PVN cFos staining is shown in figure 15. These data are expressed as the proportion of cFos positive cells to DAPI positive cell counts. Virtually no staining consistent with cFos staining was observed in the antibody negative control sections (0.6±0.4%). Vehicle injection was associated with a small but observable increase in the proportion of cells exhibiting cFos staining in the PVN region (3±1%). WIN injection also elicited an increase in the proportion of cells staining positive for cFos in the PVN region (9±2%). The WIN induced increase in cFos staining was significantly greater than that observed for either antibody negative control or vehicle control.
Although not part of our overall hypothesis, we also observed increases in cFos staining a region ventral and lateral to the PVN. This area corresponds to an area described as the medial amygdaloid nucleus in the atlas of Paxinos and Watson (figure 16).
Figure 16. Image of the coronal section from the atlas of Paxinos and Watson shown a coronal section through the brain region where we detected positive cFos staining follow injection of WIN 55, 212-2. Arrow shows location of medial amygdaloid nucleus.

Figure 17 shows images for staining observed in an antibody negative control section in the region of the MeAD. Very little punctate green (cFos) staining was observed in these sections indicating little to no non-specific binding of the fluorescent secondary antibody.
As observed with the PVN, vehicle injection appeared to increase positive cFos staining in the MeAD region as well as shown in figure 18. cFos staining was relatively sparsely distributed through this region. Nevertheless, high power examining did show nice overlay of the green and blue staining consistent with positive cFos staining.

**Figure 17.** This figure shows fluorescent immunohistochemistry images for cFos (green) and DAPI (blue) staining in a section where the primary antibody to cFos was omitted. These sections served as antibody negative controls. From the top left shows 488 emissions, then DAPI staining (top right), overlay of 488 and DAPI low power (bottom left) and high power (bottom middle). The lower right shows a cresyl violet image to identify location. There is virtually no green punctate staining indicating that there was very little non-specific binding of the secondary antibody. Scale bars are shown in each figure. MeAD = Medial Amygdaloid Nucleus
WIN injection was also associated with an increase in positive cFos staining in the MeAD region. An example of this staining is shown in figure 19. Punctate green staining was observed in discrete cells. DAPI overlay was consistent with the idea that this staining occurred in the nucleus, consistent with cFos staining. Visually this staining appeared to be denser than that produced by vehicle injection. The cresyl violet (Nissl stain) image shows that this section was taken in the Medial Amygdaloid Nucleus.

Summary data for Amygdala cFos staining is shown in figure 20. These data are expressed as the proportion of cFos positive cells to DAPI positive cell counts.
Virtually no staining consistent with cFos staining was observed in the antibody negative control sections

![Composite immunohistochemistry images of Amygdala observed in a rat that received WIN 55, 212-2 injection.](image)

**Figure 19.** Composite immunohistochemistry images of Amygdala observed in a rat that received WIN 55, 212-2 injection. Magnification at 10x using Leica DMLB microscope with Leica Application Suite X software. Top left: cFos staining observed at emission wavelength of 488. Note the punctate green staining absent in the antibody negative control sections in figure 17. Top right: DAPI staining observed at emission wavelength of 461. Bottom left: Overlay of cFos and DAPI at 10x magnification. Bottom middle is overlay at 20x. Scale bars are shown in the figures. Bottom right: Cresyl Violet Staining observed through light field at 5x.

(0.36±0.17%). Vehicle injection was associated with a small but observable increase in the proportion of cells exhibiting cFos staining in the Amygdala region (4.5±1.8%). WIN injection also elicited an increase in the proportion of cells staining positive for cFos in the amygdala region (22±5%). The WIN induced increase in cFos staining was significantly greater than that observed for either antibody negative control.
Figure 20. This figure shows the counts of cFos staining in percent DAPI at the Amygdala with no primary (n=5), animals treated with vehicle only (n=2), and animals treated with WIN 55, 212-2 (n=5). ANOVA followed by Student Newman Keul test. * Indicates significant difference from vehicle control p<0.05.
V. DISCUSSION

In our study of the hemodynamic responses to WIN 55, 212-2, we saw dose dependent increases in blood pressure and heart rate. At our highest dose of WIN 55, 212-2, 100 µg/kg, we observed an increase of 18±2 mm Hg in blood pressure, and 74 ±10 bpm in heart rate. (Schindler et al., 2017) used several other synthetic cannabinoids: JWH-018 AM2201, XLR-11, CP55, 940. They saw dose dependent increases in blood pressure with peak changes of about 15 mm Hg. Our findings using WIN 55, 212-2 are consistent with this, as we saw changes in blood pressure of about 18 mm Hg. A pressor response appears to be a general feature of synthetic cannabinoids, as all of them increase blood pressure in conscious rats. As an example, (Schindler et al., 2017) found that CP55, 940, JWH-018, and AM2201, and XLR-11 produced a hypertensive effect. The literature is consistent with our findings on synthetic cannabinoids in blood pressure. In contrast, (Schindler et al., 2017) found no change in heart rate, where we observed significant dose dependent increases in heart rate. It is difficult to reconcile these opposing findings. However, (Schindler et al., 2017) used subcutaneous injection, where we used direct intravenous injection, which may have allowed greater access of the drugs to the brain.

Also, in contrast to our findings, (Niederhoffer & Szabo, 1999) reported that intravenous injection of WIN 55, 212-2 acts to decrease blood pressure and heart rate in conscious rabbits. They ascribed this to peripheral sympathetic inhibition. These differences are difficult to explain but may be due to the difference in species used. In addition, (Malinowska et al., 2012) summarized the effects of various synthetic cannabinoids on blood pressure and heart rate as seen in the literature, which overwhelmingly suggests that there is a difference in response that is seen upon
administration of a synthetic cannabinoid in conscious animals versus anesthetized animals. In conscious animals, synthetic cannabinoids are widely reported to produce increases in blood pressure and heart rate, whereas in anesthetized animals, decreases are seen. As an example, (Kristy D. Lake, 1997) reported dose dependent decreases in mean arterial pressure and heart rate, but their work used urethane chloralose anesthetized rats. (Malinowska et al., 2012) confirmed the marked distinction between conscious and anesthetized responses. As referenced in figure 5, this is important, since humans use the drug while they are conscious, and the human data shows tachycardia and hypertension. The data for conscious responses is more relevant to what is seen in humans. Our data is consistent with the responses seen in humans.

The hypertensive effects of synthetic cannabinoids are an opposing response than what seen in cannabis. Although there have been some conflicting reports, the bulk of the evidence suggests that THC seems to lower blood pressure and produce bradycardia in both conscious and anesthetized animals, as well as in humans (Sultan, Millar, O'Sullivan, & England, 2018). This reinforces the view that while they presumably act on the same system, synthetic cannabinoids produce markedly different responses than synthetic cannabinoids.

(López-Dyck et al., 2017; Spiller et al., 2019) studied the expression of CB1 and CB2 receptors in the vasculature using two synthetic cannabinoids, ACPA, and JWH-133. They looked at what the effects of these synthetic cannabinoids did on each receptor in terms of vascular tone. They found that if you activate CB1 or CB2 receptors, you get vascular dilation, which lowers blood pressure and heart rate (López-Dyck et al., 2017). However, we saw increases in blood pressure and heart rate, our findings suggest that the
increased blood pressure and heart rate are occurring due to a mechanism within the brain.

There are cannabinoid receptors located all throughout the different regions of the brain. (Spiller et al., 2019) found that CB1 and CB2 receptors are expressed both in the vasculature, as well as in the reward and aversion centers of the brain, including the ventral tegmental area and the nucleus accumbens. The CB1 receptors in the PVN have been shown to affect cardiovascular responses, as Grzeda (Grzeda et al., 2015) saw that upon microinjection of a CB1 receptor antagonist into the PVN, then injection of CP55, 940 into the PVN, stimulation of CB2 receptors in the PVN caused increases in blood pressure and heart rate. In addition, lesions of the PVN abolished their responses to intravenous injection of CP55, 940.

Prior work has shown that autonomic blockade attenuates the responses to synthetic cannabinoids, (S.M. Gardiner, 2001) and (Schindler et al., 2017). We injected chlorisondamine iodide prior to injection of our highest dose of WIN, 100 µg/kg to block autonomic functioning to determine if the sympathetic nervous system is involved in the mechanism of increased blood pressure and heart rate seen when injecting WIN 55, 212-2. Our findings show that injection of ganglionic blocker prior to injection of WIN 55, 212-2 completely blocks the effect of WIN. The literature supports our findings that the autonomic nervous system mediates the cardiovascular effects of WIN 55, 212-2. Gardiner used pentolinium for a ganglion blocker and found that it antagonized the pressor effect of WIN (S.M. Gardiner, 2001). (Schindler et al., 2017) found that pretreatment of hexamethonium as a ganglion blocker, antagonizes the increased blood pressure and heart rate responses seen using two other synthetic cannabinoids. Our work
is consistent with that found in the literature. Under our conditions, with our animals, using WIN 55, 212-2, we have determined that it appears the increases in blood pressure and heart rate caused by WIN is mediated through the brain via the autonomic nervous system. Possibly, the reason others have reported seeing vasodilator effects, is because of the study done by (Niederhoffer & Szabo, 1999) that shows that it actually blocks the release of norepinephrine, but maybe that is overcome by the central nervous system, so when we block the nerves at the ganglia, there is no norepinephrine to block to prevent release, so that is why we did not see that effect in our responses.

The current literature in combination with our findings suggest that synthetic cannabinoids cause a sympathetic pressor effect via the brain, when administered within conscious animals. When injected prior to WIN 55, 212-2; the chlorisondamine iodide (10 mg/kg/0.1 mL) blocks the effect of WIN 55, 212-2 on the rest of the system, suggesting that the autonomic nervous system is involved. The PVN is involved in autonomic control (Dampney et al., 2018; Pyner, 2014). Injection of synthetic cannabinoids into the PVN increases blood pressure and heart rate (Grzeda et al., 2015).

We used cFos immunohistochemistry, to assess brain involvement, focusing on the PVN. In our study, even though we did see cFos staining within the vehicle, specifically within the PVN and amygdala (figure 18). WIN produced a much stronger increase in cFos staining (figure 15, figure 20) than the vehicle in both the PVN and Amygdala. Any effect of the vehicle seen in the PVN, and Amygdala are likely due to handling or stress. The PVN is involved in autonomic control, blood pressure and heart rate responses (Dampney et al., 2018), and has CB receptors (Grzeda et al., 2015), so it is logical to conclude that the PVN is part of the circuitry involved in the blood pressure...
and heart rate response, as we saw. Stimulation of the amygdala causes many physiological responses including negative emotions, anxiety, stress, increases in sympathetic nervous system activation, and increases in blood pressure and heart rate (Kraynak, Marsland, & Gianaros, 2018). Some reports have suggested that CB receptors are found in the amygdala (Katona, 2001). So, it seems reasonable to suggest that the amygdala is involved in the responses that we saw. Since cFos is a marker of neuronal activation, our results suggest that the PVN and the Amygdala are both involved in the mechanism of the hypertensive and tachycardic cardiovascular responses we saw in response to WIN 55, 212-2.

VI. CONCLUSION

Our data is consistent with the view that synthetic cannabinoids increase blood pressure and heart rate via the autonomic nervous system to adversely affect the cardiovascular system. The immunohistochemistry showed cFos activation within the PVN and the Amygdala, which suggests that the brain is involved in the cardiovascular effect of synthetic cannabinoid, WIN 55, 212-2. In the human data, increases in heart rate are the most frequent response seen, followed by increases in blood pressure being second, as seen in figure 5. Cardiovascular adverse effects are the most common response to synthetic cannabinoids, as seen in figure 4. Human causes of death upon consumption of synthetic cannabinoids are as a result of the severe cardiovascular responses. More research to explore the underlying mechanisms may lead to better treatment options for the adverse effects of synthetic cannabinoids.

Further research is needed to prove the linkage between these processes. Going forward, we would like to investigate the causal link between the cardiovascular
responses to WIN and PVN. We would like to block the amygdala as well as the PVN to see if the cardiovascular responses go away. Blocking the CB1 and then the CB2 receptors in the PVN to see if it changes response. Another option would be to block the CB1 and CB2 receptors in the amygdala to see if that changes the response.


VIII. APPENDIX


**IPS/MSPS Scientific Sessions Outstanding Undergraduate Oral Presentation** 2020


**U Discover Scholar** May 26, 2020-August 3, 2020

Grant awarded to fund research project evaluating impact of synthetic cannabinoids on cardiovascular health. PI: Dr. Doug Martin, Sanford School of Medicine’s Basic Biomedical Sciences


**SPURA Scholar** May 20, 2019-August 16, 2019

Undergraduate research in addiction fellowship grant awarded to fund research project evaluating mechanisms of cardiovascular responses to synthetic cannabinoids. PI: Dr. Doug Martin, Sanford School of Medicine’s Basic Biomedical Sciences