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Characterization of cardiovascular function in adult offspring following prenatal exposure to methamphetamine

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CHARACTERIZATION OF CARDIOVASCULAR FUNCTION IN ADULT OFFSPRING FOLLOWING PRENATAL EXPOSURE TO METHAMPHETAMINE

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APPROVAL OF THESIS

We, the faculty supervising the work of Hasitha Chavva, affirm that the thesis, *Characterization of Cardiovascular Function in Adult Offspring Following Prenatal Exposure to Methamphetamine*, meets the high academic standards for original scholarship and creative work established by the Master of Science in Pharmaceutical Sciences and the Marshall University School of Pharmacy. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABBREVIATIONS

Ach – Acetylcholine

Ang-II – Angiotensin-II

ANOVA – Analysis of variance

cDNA – Complimentary DNA

DA – Dopamine

DAT – Dopamine transporter

EDP – End diastolic pressure

eNOS – Endothelial nitric oxide synthase

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

I/R – Ischemia-reperfusion

KCl – Potassium chloride

L-NAME – N(gamma)-nitro-L-arginine methyl ester

MAO – Monoamine oxidase

Meth - Methamphetamine

mM – Millimolar

mRNA – messenger RNA

NE – Norepinephrine

NET – Norepinephrine transporter

NO – Nitric oxide

PE – Phenylephrine

PKC-ε – Protein kinase C-epsilon

PVAT – Perivascular adipose tissue

PVCF – Perivascular adipose tissue derived contractile factors

PVRF – Perivascular adipose tissue derived relaxation factors

SDS-PAGE – Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis

S.E.M. – Standard error of the mean

SNP – sodium nitroprusside

5-HT – Serotonin

SERT – Serotonin transporter

VMAT-2 – Vesicular monoamine transporter-2

+dP/dT – Positive derivative of pressure over time

-dP/dT – Negative derivative of pressure over time

µg – Micrograms

µL – Microliter

ABSTRACT

Methamphetamine (meth) is a synthetic stimulant, and its abuse is a significant public health concern in the United States and all over the world. Methamphetamine is a highly addictive drug, and its abuse is widespread among women of child-bearing age. The consequences of methamphetamine abuse are not only of relevance to pregnant women, but also their unborn children, as amphetamine type substances can cross the placental barrier and effect the fetus during gestation. Most previous studies investigating the impact of prenatal methamphetamine exposure on the offspring have focused on neurological and behavioral effects. The goal of this study was to investigate the impact of prenatal methamphetamine exposure on the cardiovascular system of adult offspring. Using a rat model, the primary finding of this work was that prenatal exposure to methamphetamine produces long-lasting and sex-dependent effects in the cardiovascular system of adult offspring. Specifically, prenatal exposure to methamphetamine hypersensitizes the female heart to ischemic injury regardless of whether exposure to methamphetamine occurs during the first or second half of the gestational period. Although this effect of prenatal methamphetamine persists in 2-month-old offspring, it subsides before 1 year of abstinence. This work also demonstrated that prenatal exposure to methamphetamine leads to perivascular adipose tissue dysfunction, disruption of NO signaling, and potentiation of angiotensin II-induced contraction of the aorta in a sex-dependent manner. These data demonstrate that prenatal exposure to methamphetamine produces sex-dependent effects in the heart and vasculature of offspring that persist into adulthood.

CHAPTER 1

INTRODUCTION

Alcohol and substance abuse is a significant public health concern. Substances such as cocaine, amphetamines, hallucinogens, and cannabis have psychomimetic properties, whose use or abuse give rise to psychotic symptoms (Fiorentini et al., 2011). Substance use disorders are one of the most common psychotic disorders found in the United States. According to the 2019 National Survey on Drug Use and Health, 60.1 % of the population aged 12 or older used alcohol (50.8 %), tobacco (21.1 %), or illicit drugs (13 %) within the past month in the United States (Substance Abuse and Mental Health Services Administration [SAMHSA], 2020).

One of the most widespread drugs of illicit use is methamphetamine. Methamphetamine is a highly addictive, powerful stimulant that affects the central nervous system and cardiovascular system. It is known by a variety of names including ice, blue, meth, and crystal. Methamphetamine is available legally only through a nonrefillable prescription and has been classified as a Schedule II stimulant by the U.S. Drug Enforcement Administration. Therapeutic use of methamphetamine is limited to attention deficit hyperactivity disorder (ADHD) and exogenous obesity, and it is available only through a prescription (National Institute on Drug Abuse [NIDA], 2021a). However, its illicit misuse is far more common than prescribed doses (NIDA, 2021a). Methamphetamine was used by approximately 1.6 million people (0.6% of the population) in 2016 and the average age of methamphetamine users was 23.3 years as reported by the 2017 National Survey on Drug Use and Health (NSDUH) (NIDA, 2021b). The National Survey on Drug Use and Health states that there was an average of 510 new users of methamphetamine each day in people aged 12 or older in 2019 (70 new users/ day in people aged 12 to 17; 170 new users in people aged 18 to 25; 260 new users in people aged 26 or older) (SAMHSA, 2020). Drug addiction is a chronic, relapsing

disorder in which obsessive drug-seeking and drug-taking behavior persists despite serious negative consequences. The major reasons for not receiving substance use treatment included: not ready to stop using (39.9 %), do not know where to go for treatment (23.8 %), and lack of health coverage or inability to afford the cost of treatment (20.9 %) (SAMHSA, 2020).

Individuals exposed to risk factors (hypoxia, malnutrition, toxins) during the fetal and perinatal period are at risk of developing diseases later as adults (Mathew & Ayyar, 2012). The Fetal Origins of Adult Diseases (FOAD) hypothesis states that events occurring during early development can have a profound impact on one's risk for development of disease in the future adult life (Calkins & Devaskar, 2011). This hypothesis was proposed by Barker and his associates. The "Barker hypothesis" is also sometimes called the "developmental origins of adult disease hypothesis" or "fetal origins of adult disease hypothesis". Barker's hypothesis suggested that an adverse uterine environment results in poor fetal and infant growth and is followed by a high risk of developing disease during adulthood (Barker, 2004). The fetal origins hypothesis proposes that diseases in the adult life occur as a result of the fetal adaptations to an adverse uterine environment and that these adaptations may be cardiovascular, metabolic, or endocrine. There is an increased risk of death from cardiovascular and chronic lung disease in men and women who had a lower birth weight (Osmond et al., 1993). Lower birth weight leads to several adverse outcomes in later life which include increased risk factors for later disease, insulin resistance, stress responses, and reduced glucose tolerance, lung function, increased clinical disease (type 2 diabetes, coronary heart disease, chronic renal disease and chronic lung disease) and increased all-cause and cardiovascular mortality (Phillips, 2007; Risnes et al., 2011; Shaheen, 1997; Victora et al., 2008; Whincup et al., 2008).

The use of tobacco, alcohol, and some illicit drugs or misuse of prescription drugs by pregnant women can have severe health consequences in the offspring. The risk of still births is doubled and sometimes tripled when pregnant women take prescription pain relievers (opioids) or other illicit drugs (National Institute of Child Health and Human Development [NICHD], 2013). Some substances (alcohol, barbiturates, benzodiazepines, and caffeine) can cause neonatal abstinence syndrome (NAS), in which the newborn experiences withdrawal symptoms shortly after delivery (Hudak & Tan, 2012). Long-term effects which can be fatal to the baby when pregnant women consume drugs include birth defects, and sudden infant death syndrome (SIDS) (MedlinePlus, n.d.).

Substance use and misuse by pregnant women has been increasing globally. In contrast to the primary methamphetamine users (17% of female drug abusers used only methamphetamine), 38% of them used it during pregnancy (Marwick, 2000). Use of methamphetamine continues to grow and there are very few studies focusing on its use during pregnancy and its effects on offspring. When a pregnant woman takes methamphetamine, it crosses the placenta and reaches the fetus through the umbilical circulation and impacts the developing offspring (Tamayo R. A. C., 2015). Prenatal methamphetamine exposure affects fetal growth, birth weight, being small for gestational age, eye disorders, developmental delays and sensorimotor functions (Acuff-Smith et al., 1992; Acuff-Smith et al., 1996; Little et al., 1988; Nguyen et al., 2010; Slamberová et al., 2006).

Methamphetamine is a stimulant drug, which when smoked or injected reaches high concentrations in the lung, placenta, kidney, intestine, liver, brain and heart (Burchfield et al., 1991). Methamphetamine administered by intranasal or intravenous routes has an elimination half-life of 11 hours in humans (Schifano et al., 2007). The clearance of methamphetamine from the

organs is fastest in heart and lungs, followed by kidneys, spleen, pancreas, brain and liver (Volkow et al., 2010). Due to the longer elimination half-life of methamphetamine in the fetus, the plasma drug concentration is higher in fetus compared to the mother (Burchfield et al., 1991).

CNS stimulants have their chemical structure like monoamine neurotransmitters and are indirectly acting sympathomimetics. Cocaine and methamphetamine differ in their mechanisms of action but produce similar physiological effects. Cocaine inhibits reuptake of epinephrine, norepinephrine, and dopamine from sympathetic and dopaminergic neurons, whereas methamphetamine causes the release of these catecholamines from sympathetic and dopaminergic neurons. Methamphetamine exerts its actions on mainly three molecular targets: (1) plasma membrane transporters, (2) vesicular monoamine transporter (VMAT), and (3) monoamine oxidase (MAO) (Ferrucci et al., 2019; Sulzer et al., 2005). Normally, neurotransmitters (dopamine (DA), norepinephrine (NE), serotonin (SER)) released into the synapse are transported back into the nerve ending by plasma membrane transporters (Dopamine transporter, Norepinephrine transporter, Serotonin transporter) and back into the synaptic vesicle by vesicular monoamine transporter-2 (VMAT-2) **[Fig. 1.1 A]**. However, methamphetamine causes opposite changes, which causes translocation of dopamine from synaptic vesicle to neuronal cytoplasm by vesicular monoamine transporter 2 and reverse transport of dopamine from cytoplasm into the synapse via the dopamine transporter (Ferrucci et al., 2019; Kish, 2008). The third molecular target is monoamine oxidase (MAO), which is involved in the metabolism of neurotransmitters. Methamphetamine impairs this enzyme MAO and leads to the availability of free neurotransmitters into the nerve axoplasm and then into the synapse (Ferrucci et al., 2019). Therefore, methamphetamine acts by blocking the reuptake of catecholamines, inducing the

release or catecholamines, and increasing the concentration of catecholamines in the synaptic cleft **[Fig. 1.1 B]**.

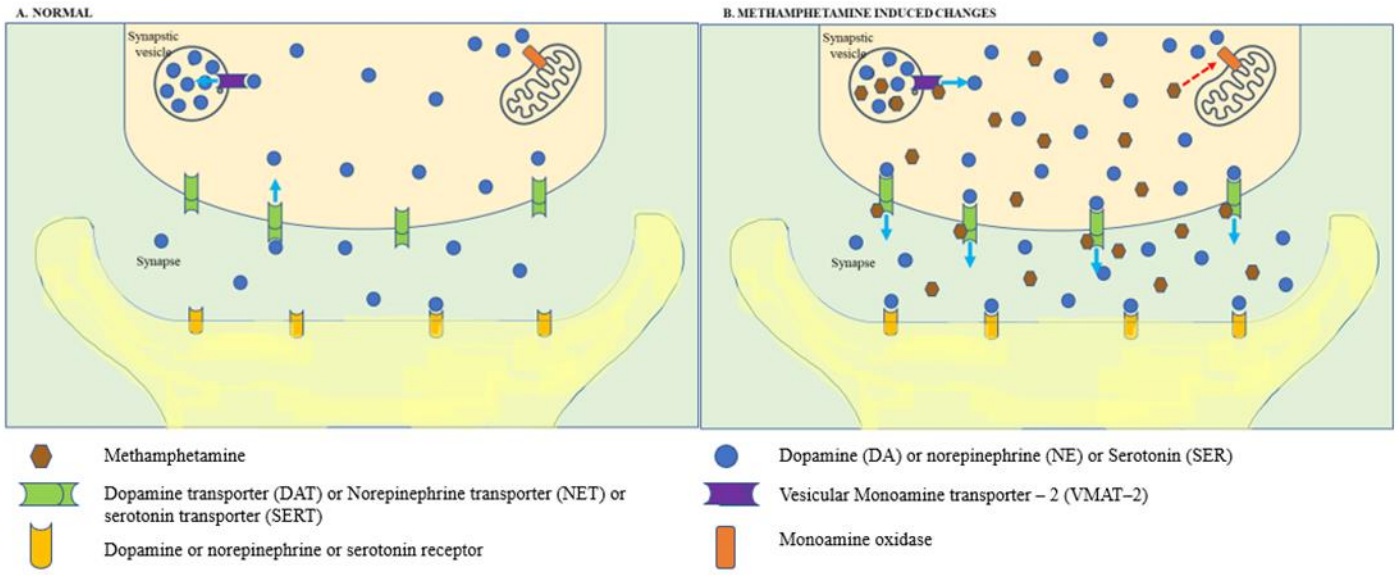

Figure 1.1. Mechanism of action of methamphetamine.

Methamphetamine exerts its actions on: (1) plasma membrane transporters, (2) vesicular monoamine transporter (VMAT), and (3) monoamine oxidase (MAO). Neurotransmitters (dopamine (DA), norepinephrine (NE), serotonin (SER)) released into the synapse are transported back into the nerve ending by plasma membrane transporters (DAT, NET, SERT) and back into the synaptic vesicle by vesicular monoamine transporter-2 (VMAT-2). However, methamphetamine causes opposite changes, which causes translocation of dopamine from synaptic vesicle to neuronal cytoplasm by vesicular monoamine transporter 2 and reverse transport of dopamine from cytoplasm into the synapse via the dopamine transporter. Methamphetamine impairs the enzyme MAO and leads to the availability of free neurotransmitters into the nerve axoplasm and then into the synapse. Therefore, methamphetamine acts by blocking the reuptake of catecholamines, inducing the release or catecholamines, and increasing the concentration of catecholamines in the synaptic cleft.

Cardiovascular disease is the world's leading cause of death, and accounts for nearly half of all noncommunicable diseases (Laslett et al., 2012). Neurologic, obstetric, gastrointestinal, renal, and endocrine issues are serious side effects of methamphetamine abuse, with long-term damage and cardiovascular disease being the most common complaint among methamphetamine users (Derlet & Horowitz, 1995). Methamphetamine abuse leads to an increased risk of adverse cardiovascular consequences which include ischemic stroke, arrhythmias, pulmonary hypertension, cardiomyopathy and myocardial infarction (Chen, 2007; Haning & Goebert, 2007; Huang et al., 2016; Neeki et al., 2016).

Earlier studies focused on the impact of prenatal exposure to methamphetamine on developmental, behavioral, and neuronal outcomes in the offspring. The offspring born to pregnant women exposed to methamphetamine were small for gestational age and exhibited decreased growth, body length and head circumference (Little et al., 1988; Nguyen et al., 2010; Smith et al., 2003). Both adult and prenatal methamphetamine exposure leads to impairment of cognitive function and behavior (Dong et al., 2018; Macúchová et al., 2014). There were several effects in the developmental stages leading to cognitive dysfunction in six- to seven-year-old children on prenatal exposure to methamphetamine (Kwiatkowski et al., 2018). In addition, prenatal exposure to methamphetamine was associated with neurobehavioral patterns of increased physiological stress, increased CNS stress, decreased arousal and increased lethargy in the neonatal period (Smith et al., 2008).

Parallel to methamphetamine, most studies of prenatal cocaine exposure have also focused on neurological and behavioral outcomes. Prenatal cocaine exposure led to lower visual and auditory orienting skills, poor motor abilities, decreased interactive behavior, and more abnormal reflexes (Chasnoff et al., 1985; Chasnoff et al., 1989). Bae et al., demonstrated that prenatal

exposure to cocaine increases myocardial sensitivity to ischemic injury in adult male offspring (Bae et al., 2005). Prenatal cocaine exposure also led to hypertrophy and increased apoptosis in neonatal cardiomyocytes of rat (Bae & Zhang, 2005), methylation of the protein kinase C-ε (PKC-ε) promoter, and decreased the expression of the cardioprotective protein PKC-ε (Zhang et al., 2007).

Prenatal cocaine not only affects the cardiac muscle, but also alters the adult vascular function. Prenatal cocaine exposure had no effect on basal blood pressure in adult rats, but increased norepinephrine-induced blood pressure (Xiao, Huang, et al., 2009). This is consistent with potentiated norepinephrine-induced vasoconstriction, and increased myofilament sensitivity to calcium in mesenteric arteries (Xiao, Huang, et al., 2009). In addition, there was sex-dependent attenuation of endothelium-dependent relaxation and a suppressed baroreflex in adult male rats following prenatal exposure to cocaine. These data suggest that fetal exposure to cocaine alters both cardiac function and vascular function in adult offspring in a sex-dependent manner.

Previous studies regarding the consequences of fetal exposure to methamphetamine focused on behavior, locomotor, or CNS effects. There were very few studies of the impact of prenatal methamphetamine on the adult cardiovascular function. Considering the high prevalence of cardiovascular disease and the increasing use of methamphetamine around the world, it is important to understand the impact of prenatal exposure to methamphetamine on the adult cardiovascular system. Thus, the goal of this work was to determine how prenatal exposure to methamphetamine impacts the adult cardiovascular system. This work addresses the following specific aims.

Specific aims:

1. Aim 1 was to identify the gestational period (first half vs second half of pregnancy) of risk of myocardial ischemic injury in adult offspring on exposure to methamphetamine in their prenatal period. **Hypothesis:** Methamphetamine increases risk of myocardial ischemic injury in the adult offspring if exposed either in the first half or second half of gestational period.

In the primary phase of gestation period, the development of the embryo takes place which includes placentogenesis and organogenesis, though in the later half only fetal and placental growth occurs (Blum et al., 2017). However, typically, a rat heart develops from embryonic day (ED 9-15) to complete heart development by ED16 (Marcela et al., 2012). Thus, the development of a rat heart takes place partly in first half and partly in the second half of the gestation period. It can be implied from this that the heart is prone to injury if the intrauterine environment is altered in the first or second half of gestation period. Thus, we hypothesize that methamphetamine exposure either in the first half or second half of pregnancy worsens cardiac ischemic injury in the adult rats.

2. Aim 2 was to determine if sensitization to myocardial ischemic injury following prenatal exposure to methamphetamine persists into geriatric phase of life. **Hypothesis:** Prenatal exposure to methamphetamine causes increased sensitivity to myocardial ischemic injury after they are abstinent for the first one year of their life.

Prenatal exposure to methamphetamine causes myocardial hypersensitivity to I/R injury in 2-month-old young adult rat offspring (Rorabaugh et al., 2016). Fetal stress (maternal undernutrition, hypoxia, toxins) increases the vulnerability to cardiovascular diseases in adult life (Rodríguez-Rodríguez et al., 2017). Prenatal hypoxia or exposure to nicotine led to cardiac alterations and cardiovascular dysfunction in aged adult offspring, although they were being

abstinent after birth (Aljunaidy et al., 2018; Xiao et al., 2016). Thus, we hypothesize that prenatal methamphetamine would also increase the myocardial sensitivity to I/R injury in aged adult offspring even after being abstinent after birth.

3. Aim 3 was to determine if prenatal exposure to methamphetamine alters the vascular function in adult offspring. **Hypothesis:** Prenatal exposure to methamphetamine alters the blood pressure and vascular function in adult offspring.

Previous studies demonstrated that prenatal exposure to cocaine leads to endothelial dysfunction in mesenteric arteries of adult male (but not female) rats (Xiao, Huang, et al., 2009). In addition to this, these animals had decreased sensitivity of the baroreceptor reflex, potentiation of norepinephrine-stimulated increase in blood pressure, and dysfunctional regulation of myogenic tone in coronary arteries (Xiao, Yang, et al., 2009). On prenatal exposure to nicotine, similar effects are observed (Rorabaugh, 2021) and also altered perivascular adipose tissue function in adult rats (Gao et al., 2005). Cocaine, nicotine, and methamphetamine belong to CNS stimulants and have different mechanisms of action. However, cocaine and methamphetamine both act on central and peripheral sympathetic neurons to increase sympathetic stimulation to heart, blood vessels, and other innervated tissues. Thus, we hypothesize that prenatal methamphetamine leads to altered vascular function in adult offspring in a sex-dependent manner.

CHAPTER 2

METHAMPHETAMINE EXPOSURE DURING EITHER THE FIRST OR SECOND HALF OF GESTATION WORSENS CARDIAC ISCHEMIC INJURY IN ADULT FEMALE OFFSPRING.

2.1 Introduction

Drug abuse by pregnant women is a very serious problem today. Pregnancy and infancy are periods of sensitivity to environment factors that influence health and disease later in adult life. Dr. David Barker proposed the concept of fetal origins of adult disease (FOAD) which hypothesizes that events occurring during early development of the fetus have an intense impact on one's risk for development of future adult disease (Barker, 1990). This hypothesis proposes that fetal nutrition, stress and toxins induce long-term changes in the fetus leading to decreased size at birth, lower birth weight and increasing the risk factors for future adult diseases including coronary heart disease, diabetes, hypertension, chronic lung and kidney disease (de Boo & Harding, 2006; Fall, 2013). Barker's hypothesis proposed that environment exposures causing poor fetal and infant growth predicts a high risk of ischemic heart disease (Barker et al., 1989).

The gestational timing of exposure to CNS stimulants has various consequences in the resulting adult offspring. Prenatal exposure to cocaine during embryonic days E10 - E20 had increased susceptibility to convulsant-induced seizures in both adult male and female rats (Snyder-Keller & Keller, 2001). Pregnant mice that were injected with caffeine during gestational days 6.5-9.5 had a significant decrease in DNA methylation (Buscariollo et al., 2014). The biological process by which methyl group is added to DNA molecule is known as DNA methylation and is an epigenetic alteration that leads to cancer, atherosclerosis, nervous disorders, and cardiovascular disorders. Many of the differential methylated areas were located in genes associated with cardiac

hypertrophy and cardiomyopathy. These changes did not occur in adult mice that were exposed to caffeine during gestational days 10.5-13.5 indicating that caffeine-induced cardiac effects occur during specific time frame during the gestational period.

Methamphetamine abuse by pregnant women is common. In contrast to the primary methamphetamine users (17% of female drug abusers used only methamphetamine), 38% of them used it during pregnancy (Marwick, 2000). Among pregnant women who abuse methamphetamine, 84.3% abuse it during first trimester, 56% use it till second trimester and 42.4% of these women continue it till the third trimester (Della Grotta et al., 2010). In-utero exposure to methamphetamine leads to decreased attention span, reduced working-memory capability, behavioral dysregulation, and impairment of spatial memory in the offspring (Kiblawi et al., 2013; Piper et al., 2011; Roussotte et al., 2011; Twomey et al., 2013). Behavioral and neuroanatomical outcomes of prenatal methamphetamine exposure critically depend on the timing and duration of exposure. Adult male (but not female) rats that had been exposed to methamphetamine during gestational day 1-11 had increased social play behavior. This effect was not seen either in males or females when exposed to methamphetamine during gestational day 12-22 (Malinová-Ševčíková et al., 2014; Ševčíková et al., 2020). Methamphetamine exposure during embryonic day 1-11 or embryonic day 12-22 and during postnatal period 1-11 affects the spatial learning in adult male rats (Hrebíčková et al., 2016). When pregnant rats were administered methamphetamine during gestational day 10 to 20, there were morphological alterations in fetal brains, including microgyria, ectopia, and hemorrhage (Cui et al., 2006). Adult male and female rats that were exposed to methamphetamine during gestational day 13-20 resulted in selective functional alterations of brain 5-HT systems (Cabrera et al., 1993). Most of the studies performed with respect to the timing of gestational exposure were related to locomotion, social behavior, and CNS function.

Prenatal exposure to methamphetamine throughout the whole gestation period increases the myocardial sensitivity to I/R injury in adult female rat offspring (Rorabaugh et al., 2016). However, we do not know whether this effect occurs when the adult offspring is exposed to methamphetamine in the first or second half of gestation period. Thus, the purpose of this study is to determine whether methamphetamine exposure during the first or second half of gestation period sensitizes the heart to ischemic injury.

2.2 Methods

Animals: Female Sprague-Dawley rats (8 weeks of age) were used for breeding. The presence of a vaginal plug was considered as gestational day 0. The rats were housed in standard cages with free access to food and water and on 12/12hr light/dark cycle (lights on at 0600). Pregnant rats were divided into four groups **[Fig. 2.1]**. *Group 1* received daily saline injections starting on gestational day 1 and continuing until the pups were born. *Group 2* received daily methamphetamine (5mg/kg/day) injections starting on gestational day 1 and continuing until the pups were born. *Group 3* received daily methamphetamine (5 mg/kg) injections on gestational days 1-11 and received saline injections on gestational days 12-22. *Group 4* received daily saline injections on gestational days 1-11 and daily methamphetamine (5 mg/kg) injections on gestational days 12-22. Subcutaneous injections were administered once per day (at 0800) starting at gestational day 1 and continuing until the pups were born. The pups were weaned on postnatal day 28 and housed two to three per cage. All rats were housed 2 animals per cage after they reached 100 grams body weight. Only female offspring were used for the study as only adult female offspring was hypersensitive to myocardial ischemic injury on prenatal exposure to methamphetamine (Rorabaugh et al., 2016). The pups were weighed at weaning and at 8 weeks

age. All procedures were approved by the Institutional Animal Care and Use Committee of Marshall University (IACUC protocol no. 719).

Langendorff isolated heart experiments: The hearts were rapidly removed after anesthetizing the rats with sodium pentobarbital (100mg/kg ip) and mounted on a Langendorff isolated heart apparatus as previously described (Rorabaugh et al., 2016). Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 0.5 Na_2EDTA, 11 glucose, and 2.5 $CaCl_2$, pH 7.4) was perfused at a constant pressure of 80 mm Hg through an aortic cannula. The contractile function of the left ventricle was measured using an intraventricular balloon connected to a pressure transducer and inflated to an end diastolic pressure of 4mm Hg. Powerlab 4SP data acquisition system (AD Instruments, Colorado Springs, CO) was used for recording the data.

Throughout the experiment, hearts were submerged in Krebs solution to maintain the temperature of the heart at 37.5 ± 0.5 ºC and temperature was continuously monitored using a thermocouple placed on the surface of the heart. Hearts were equilibrated for 25 min prior to the onset of 30 min of ischemia and 2 hours of reperfusion. If the developed pressure was <100 mm Hg, coronary flow rate was >25ml/min, or if there were persistent arrhythmias, hearts were excluded after the 25 min equilibration period. Data was continuously recorded immediately prior to ischemia, preischemic contractile function was measured and postischemic recovery of contractile function was measured following 5, 10, 20, 30, 40, 50, 60 and 120 min of reperfusion. Hearts were reperfused for a total of 2 hours prior to triphenyl tetrazolium chloride staining.

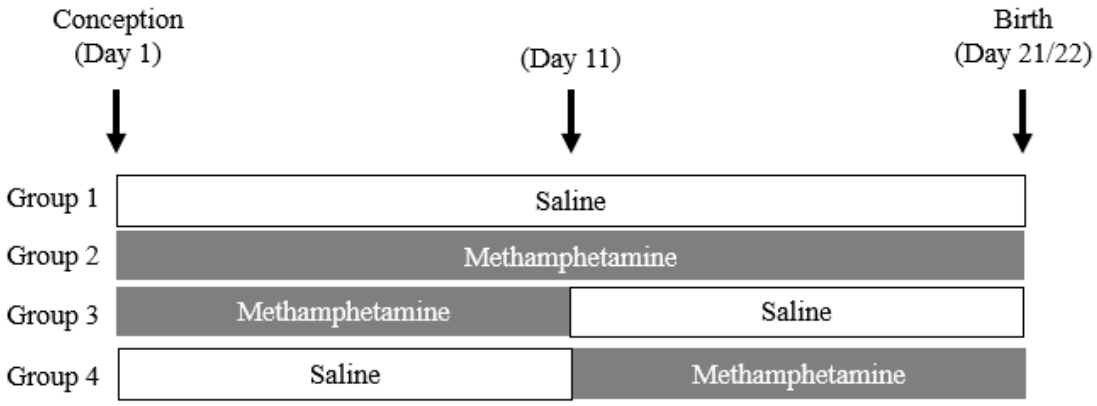

Figure 2.1. This figure represents the four treatment groups.

The animals in group 1 injected with saline and group 2 with methamphetamine (5mg/kg) throughout the period of gestation. The animals in group 3 were injected with methamphetamine during the first half of gestation and with saline during the second half of the gestational period. The animals in group 4 were injected with saline in the first half of gestation and with methamphetamine in the second half of gestation period.

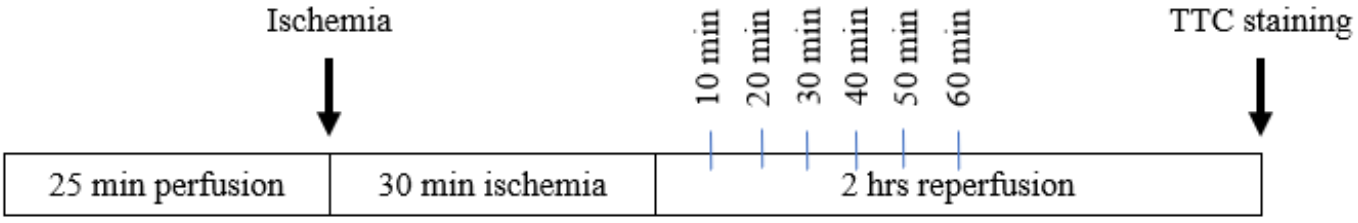

Figure 2.2. Workflow of Langendorff isolated heart experiment.

The hearts were initially perfused for 25 min, followed by subjecting them to ischemia for 30 min and finally reperfusing them for 2 hours.

Measurement of infarct size: Hearts were perfused with 1% triphenyl tetrazolium chloride (TTC) at a rate of 7.5ml/min for 8 min and then submerged in 1% triphenyl tetrazolium chloride for 12 min at 37 ºC. Hearts were subsequently frozen at -80 ºC, sliced into ~1-mm sections, soaked in 10% neutral buffered formalin, and then photographed with a Nikon SMZ 800 microscope equipped with a Nikon DS-Fi1 digital camera. Image J software was used for measuring the infarcted surface area and total surface area of each slice. Infarct size was expressed as percentage of area at risk of the entire ventricular myocardium.

Statistical analysis: One-way ANOVA was used for analyzing infarct sizes and Tukey's posthoc analysis was used for comparing the infarct sizes between the groups. Repeated measures two-way ANOVA was used for analyzing parameters of cardiac function (developed pressure, +dP/dT, -dP/dT, heartrate, end diastolic pressure and coronary flow rate) and Tukey's posthoc analysis with time (preischemic vs postischemic recovery) and drug treatment (methamphetamine vs saline) as factors. All data is represented as mean ± SEM.

2.3 Results

Prenatal exposure to methamphetamine significantly decreased body weight at weaning. Body weight at the time of weaning was significantly decreased in pups that had been prenatally treated with methamphetamine **[Fig. 2.3 A]**. One-way ANOVA indicated a significant effect of methamphetamine on body weight at weaning ($p < 0.0001$). Tukey's posthoc analysis indicated a significantly lower body weight ($p < 0.0001$) in pups from group 2 (methamphetamine) (80 ± 1.2 grams), group 3 (methamphetamine + saline) (81 ± 2.3 grams), and group 4 (saline + methamphetamine) (70 ± 2.5 grams) compared to pups from group 1 (saline) (93 ± 1.3 grams). Also, pups from group 4 (saline + methamphetamine) weighed less ($p < 0.05$) compared to group 2 (methamphetamine); and pups from group 3 (methamphetamine + saline) weighed more ($p < 0.05$) compared to group 4 **[Fig. 2.3 A].** These changes in body weight were not seen when the rats reached 2-months of age **[Fig. 2.3 B]**.

Methamphetamine exposure during the first half or second half of the gestation period increases the infarct size and decreases the postischemic recovery of contractile function in adult female offspring. The impact of prenatal methamphetamine on the ischemic heart was measured after the female offspring attained 2 months age. The hearts were mounted on a Langendorff heart apparatus and subjected to 30 min of ischemia and 2 hours of reperfusion. One-way ANOVA indicated significant effect of methamphetamine on infarct size in adult female hearts [$F = 5\ (3, 39)$, $p < 0.05$] **[Fig. 2.4]**. Tukey's posthoc analysis indicated significantly ($p < 0.05$) increased infarct size in Group 3 (methamphetamine + saline) and Group 4 (saline + methamphetamine) compared to Group 1 (saline only throughout gestation). Two-way ANOVA indicated no significant effect of methamphetamine on preischemic parameters of contractile function including developed pressure **[Fig. 2.5 A]**, end diastolic pressure **[Fig. 2.5 B]**, +dP/dT

[Fig. 2.5 C], -dP/dT **[Fig. 2.5 D]**, heart rate **[Fig. 2.5 E]** and coronary flowrate **[Fig. 2.5 F]**. However, methamphetamine significantly increased postischemic recovery of developed pressure [$F = 4.5$ (1, 17), $p < 0.05$] **[Fig. 2.5 A]**, decreased postischemic recovery of -dP/dT [$F = 5.5$ (1, 17), $p < 0.05$] **[Fig. 2.5 D]** and there was a significant interaction between time and drug treatment for heart rate [$F = 2.7$ (8, 136), $p < 0.01$] **[Fig. 2.5 E].** Furthermore, two-way ANOVA indicated a significant effect of time (preischemic vs postischemic recovery) for developed pressure, EDP, +dP/dT, -dP/dT, heartrate and coronary flowrate **[Fig. 2.5 A-F]**

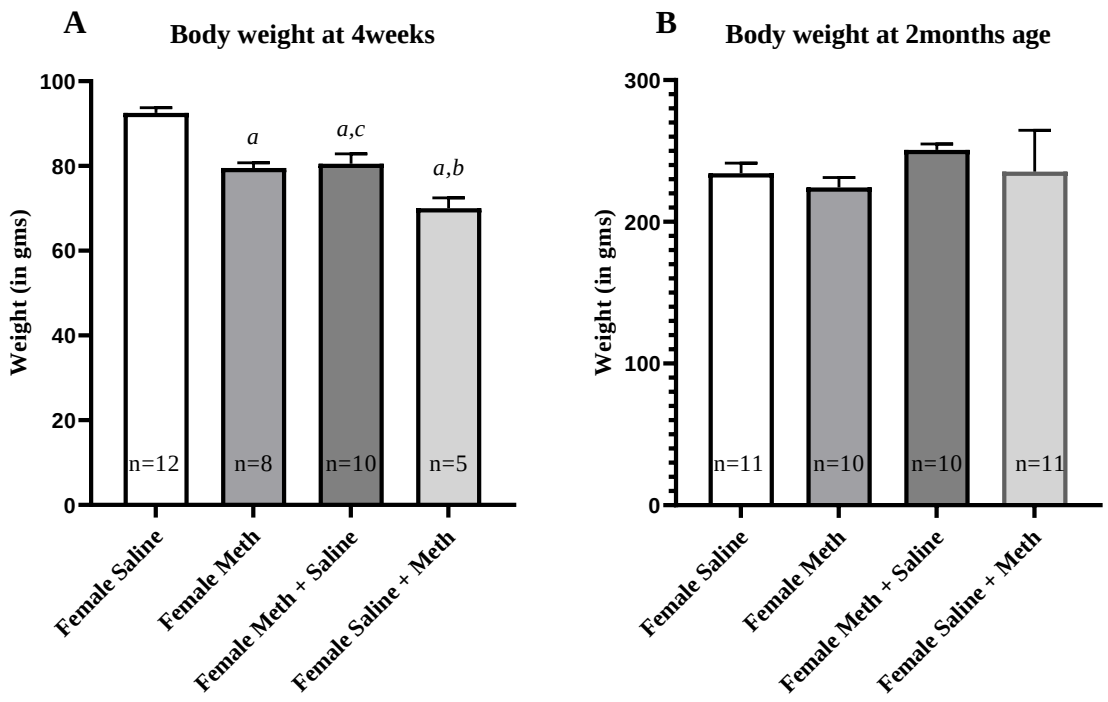

Figure 2.3. Effect of prenatal methamphetamine on body weight at weaning and 2-months age.

Prenatal exposure to methamphetamine had a significant impact on body weight of offspring at weaning (A). Ordinary one-way ANOVA indicated a significant effect of methamphetamine on body weight at weaning ($p < 0.0001$). [a] indicates $p < 0.001$ compared to saline female. [b] indicates $p < 0.05$ compared to methamphetamine female. [c] indicates $p < 0.05$ compared to saline + methamphetamine female. Prenatal exposure to methamphetamine had no effect on body weight of offspring at 2-months age (B).

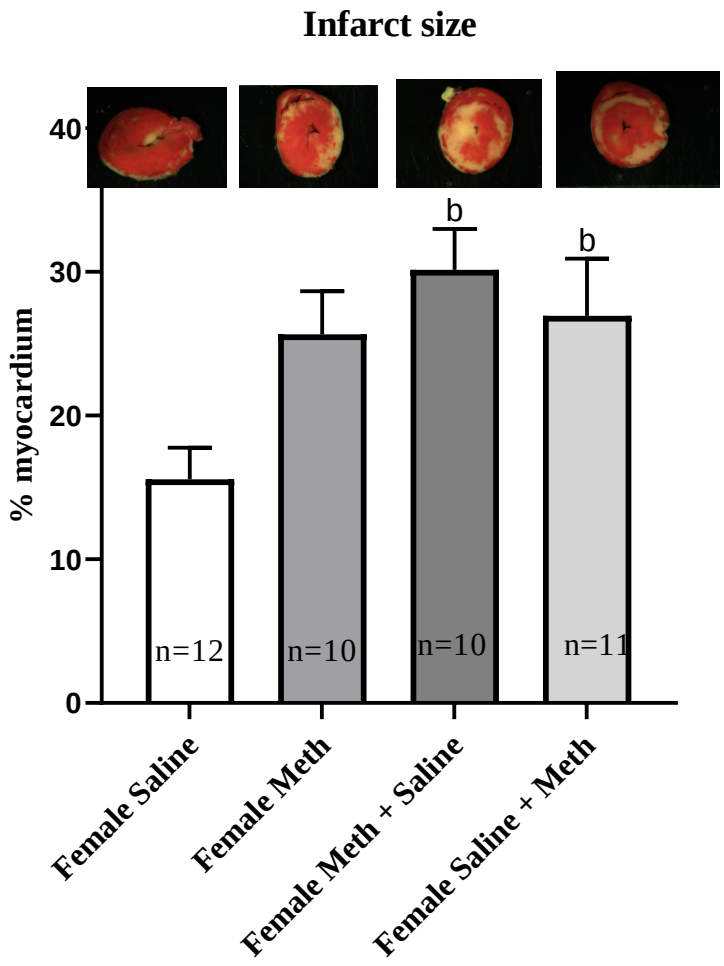

Figure 2.4. Impact of prenatal methamphetamine exposure on infarct size in adult female hearts.

Hearts were subjected to 30 min of ischemia and 2 hours of reperfusion on a Langendorff apparatus. Hearts were stained with triphenyl tetrazolium chloride and infarct sizes were measured. Ordinary one-way ANOVA indicated a significant effect of methamphetamine on infarct size [$F = 5$ (3,39), $p < 0.05$]. [b] indicates $p < 0.05$ compared to saline only group compared by Tukey's posthoc analysis.

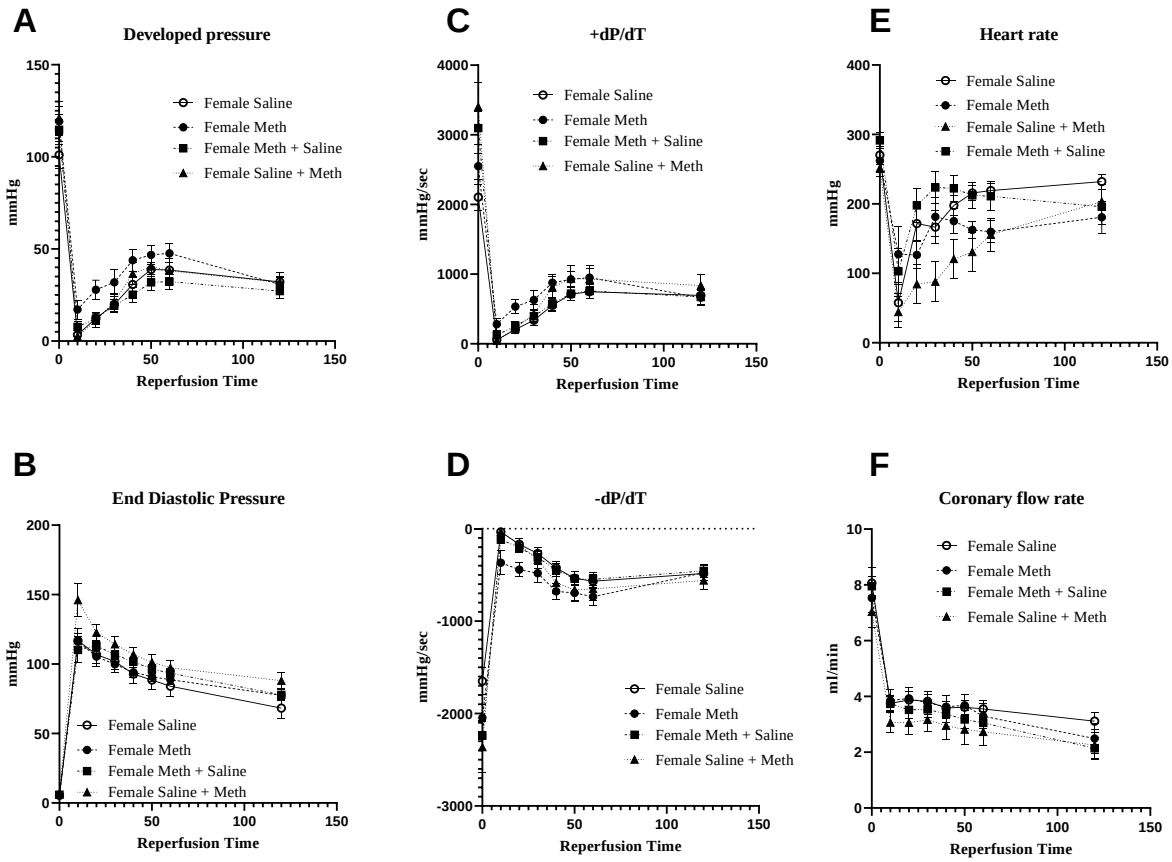

Figure 2.5. Parameters of preischemic contractile function and postischemic recovery of contractile function in female hearts prenatally exposed to methamphetamine or saline or methamphetamine + saline or saline + methamphetamine.

Preischemic contractile function was measured following a 25 min equilibration period immediately prior to the onset of ischemia. Two-way ANOVA indicated no significant effect of methamphetamine on parameters of preischemic contractile function including developed pressure (A), end diastolic pressure (B), +dP/dT (C), -dP/dT (D), heart rate (E) and coronary flowrate (F). Postischemic recovery was measured following 10, 20, 30, 40, 50, 60 and 120 min of reperfusion. Methamphetamine significantly increased postischemic recovery of developed pressure [$F = 4.5$ (1, 17), $p < 0.05$] (A), decreased postischemic recovery of -dP/dT [$F = 5.5$ (1, 17), $p < 0.05$] (D) and significant interaction between time and drug treatment for heart rate [$F = 2.7$ (8, 136), $p < 0.01$] (E). Two way ANOVA indicated a significant effect of time (preischemic vs postischemic recovery) for developed pressure [$F = 13$ (3, 57), $p < 0.0001$] (A), end diastolic pressure [$F = 269$ (2, 32), $p < 0.0001$] (B), +dP/dT [$F = 13$ (3, 49), $p < 0.0001$] (C), -dP/dT [$F = 13$ (3, 51), $p < 0.0001$] (D), heart rate [$F = 15$ (3,55), $p < 0.0001$] (E) and coronary flow rate [$F = 140$ (3, 39), $p < 0.0001$] (F). All data are represented as mean ± S.E.M of 10-12 rats.

2.4 Discussion

The primary finding of this study was that methamphetamine worsened cardiac ischemic injury in adult female offspring if exposed during either the first half or second half of gestation period. There is no difference due to time of exposure to methamphetamine during gestation period in myocardial sensitivity to ischemic injury. Earlier studies indicated that methamphetamine exposure throughout the gestational period worsens cardiac I/R injury in a sex-dependent manner in adult offspring {Rorabaugh, 2016 #198} (Bae et al., 2005; Lawrence et al., 2008; Rorabaugh et al., 2016). However, we did not know whether methamphetamine exposure during the first or second half of the gestational period makes the adult offspring prone to cardiac I/R injury. The current study indicates that exposure to methamphetamine throughout either the first or second half of gestation hypersensitizes the adult female heart to ischemia.

When the pups were weighed at weaning, pups from Group 2 (methamphetamine), Group 3 (methamphetamine + saline) and Group 4 (saline + methamphetamine) had significantly lower body weights compared to those from dams treated with saline throughout gestation (Group 1). Also, pups from Group 4 (saline + methamphetamine dams) weighed less compared to those from Group 2 (methamphetamine) and Group 3 (methamphetamine + saline) dams. The beginning of gestation is characterized by cell differentiation and organogenesis while the end of gestation is characterized by growth (Blum et al., 2017). This could be the reason behind pups from Group 4 (saline + methamphetamine treated dams) weighing less than those from Group 3 (methamphetamine + saline treated dams).

Previous studies have documented that methamphetamine abuse during specific stages of gestation in pregnant mothers has negative effects on fetal growth, infant behavior, and development of the frontal lobe of the fetus, leading to impaired social functioning in adulthood

(Behnke, 2013; Jablonski et al., 2016; Kolb et al., 2012). The period of brain development in the rat (ED 1-22) corresponds to the first (ED 1-11) and the second (ED 12-22) human trimester equivalents, during which organogenesis, neurulation and histogenesis occur in brain (Fentress, 1988; Kelley & Berridge, 2002). The structures in the fetal brain develop at different periods which effect the social and non-social behavior when exposed to methamphetamine during the prenatal or neonatal periods. Each period of brain development may be more critical for effects on locomotion, behavior, and memory as exposure during these periods lead to a range of anatomical abnormalities in the brain (Acuff-Smith et al., 1992; Melo et al., 2006; Melo et al., 2008). Therefore, prenatal exposure to methamphetamine in different developmental stages of the fetal brain produce effects that are specific to a particular region in the brain. A rat heart typically develops from embryonic day 9 -15 and the complete heart develops by the end of this period (Marcela et al., 2012). So, methamphetamine exposure during either the first or second half of gestation period might lead to changes in the heart. Therefore, we can conclude that methamphetamine worsens cardiac ischemic injury in adult female offspring either exposed in the first half or second half or the whole period of gestation.

The purpose of this study was to identify the gestational time of exposure to methamphetamine that makes the offspring more sensitive to ischemic injury as an adult. According to the results obtained from the current study, it can be inferred that methamphetamine increases the myocardial sensitivity to I/R injury irrespective of exposure in the first or second half of pregnancy. If a woman finds that she is pregnant and decides to stop using methamphetamine around mid-term to save her child from being sensitive to cardiac ischemic injury, the child is still affected. In fact, the offspring is nominally more sensitive to ischemic injury if exposed during the first half of gestation period rather than being exposed for the whole period of gestation (however,

this difference was not significant). The reason behind this is unknown but may be related to the embryo becoming less sensitive or tolerant to methamphetamine over time. However, the offspring still has increased myocardial sensitivity to ischemic injury irrespective of the exposure to methamphetamine in the first half or second half of the gestation period.

CHAPTER 3

PRENATAL METHAMPHETAMINE INDUCED MYOCARDIAL HYPERSENSITIVITY TO ISCHEMIA-REPERFUSION INJURY DOES NOT PERSIST FOLLOWING 1 YEAR OF ABSTINENCE.

3.1 Introduction

The intrauterine environment plays a key role in the development of healthy offspring. During pregnancy, variation in the quality or quantity of nutrients consumed or other factors that create an adverse uterine environment (stress, hypoxia, drug exposure) can exert permanent and powerful effects on the developing fetus (Barker, 1999). The fetal origins of adult disease (FOAD) hypothesis proposes that events occurring during early development of the fetus (fetal stress, malnutrition and toxins) have a powerful impact on the development of diseases in later life (Barker, 1990; de Boo & Harding, 2006). Fetal malnutrition and low birth weight are known to be associated with adult-onset of cardiovascular diseases (Barker, 1999; Rocchini, 1994).

Prenatal exposure to central nervous stimulants (CNS) can have long-lasting effects on adult offspring. Substance (alcohol, tobacco, illicit drugs) abuse during pregnancy can have a negative impact on the developing fetus affecting cognition, anxiety and susceptibility to drug addiction in adulthood (Cantacorps et al., 2020; Dong et al., 2018; Salas-Ramirez et al., 2010; Sobrian et al., 2003). Earlier studies on prenatal CNS stimulant exposure have focused on behavioral and neurological outcomes. However, recent studies on prenatal CNS stimulant (cocaine, nicotine, methamphetamine, caffeine) exposure indicated that these stimulants can lead to diabetes, obesity, vascular dysfunction and increased susceptibility to cardiac ischemic injury in adult offspring (Bae et al., 2005; Fan et al., 2016; Korchynska et al., 2020; Rorabaugh et al.,

2016; Xiao et al., 2011; Xiao, Yang, et al., 2009). Prenatal exposure to CNS stimulants can have both short-term and long-term effects on the developing offspring (Rorabaugh, 2021).

Adult and prenatal methamphetamine exposure to methamphetamine increased myocardial hypersensitivity to ischemia-reperfusion injury in female rat hearts at the age of 2 months. Similar effects are seen in 2-month-old adult male rat offspring following prenatal exposure to cocaine (Bae et al., 2005) or nicotine (Lawrence et al., 2008). However, it is unclear whether these changes are permanent or how long they persist in adult life. Cardiovascular disease occurs primarily in older people. However, up to this point, experimental data have been limited to studies conducted in young adult animals.

There have been no studies performed to determine whether these effects persist into the geriatric phase of life when an individual is more likely to experience a heart attack. Thus, the goal of this study was to determine whether prenatal exposure to methamphetamine increases myocardial sensitivity to I/R injury in adult female rat following 1 year of postnatal abstinence from the drug.

3.2 Methods

Animals: Female Sprague-Dawley rats (8 weeks of age) were used for breeding. The day on which a vaginal plug was identified was considered as gestational day 0. The rats were housed in standard cages with free access to food and water and on 12/12hr light/dark cycle (lights on at 0600). Pregnant rats were administered saline or methamphetamine (5mg/kg/day) by subcutaneous injection once per day (at 0800) starting at gestational day 1 and continuing until the pups were born. At 28 days of age, pups were weaned and housed two to three per cage and only female offspring were used for the study. All rats were housed 2 animals per cage after they reached 100 grams body weight. The pups were weighed at weaning, at six months of age, and at the age of one year before performing Langendorff isolated heart experiments. All procedures were approved by the Institutional Animal Care and Use Committee of Marshall University (IACUC protocol no. 719).

Refer to section 2.2 for additional methods.

Statistical Analysis: Body weight and infarct size were analyzed by t-test. Repeated measures two-way ANOVA was used for analyzing parameters of cardiac function (developed pressure, +dP/dT, -dP/dT, heart rate, end diastolic pressure, and coronary flow rate) and Tukey's posthoc analysis with time (preischemic vs postischemic recovery) and drug treatment (methamphetamine vs saline) as factors. All data is represented as mean ± SEM.

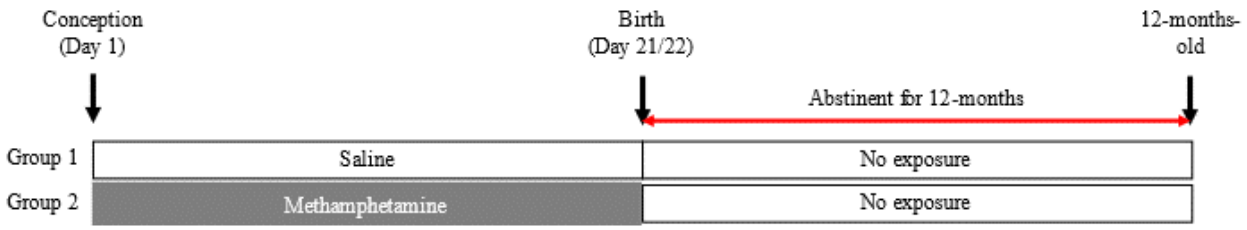

Figure 3.1. This figure represents the treatment groups and the timeline for experiments.

3.3 Results

Prenatal methamphetamine has no effect on body weight at the age of 6-months and at one-year. Prenatally methamphetamine exposure had no effect on body weight at 6 months **[Fig. 3.2 A]** or 12 months **[Fig. 3.2 B]** of age.

Prenatal methamphetamine has no effect on infarct size in 1 year old adult female offspring. The impact of prenatal methamphetamine on the ischemic heart was measured following prenatal exposure to methamphetamine (GD 1-22) and 1 year of subsequent abstinence. Hearts were subjected to 30 min of ischemia and 2 hours of reperfusion on a Langendorff isolated heart apparatus. Unpaired t-test indicated no significant effect of methamphetamine on infarct size ($p = 0.73$) after 1 year of abstinence **[Fig. 3.3]**. Two-way ANOVA indicated a significant effect of time (preischemic vs postischemic recovery) for developed pressure [$F = 94$ (8,117), $p < 0.0001$] **[Fig. 3.4 A]**, EDP [$F = 52$ (8,117), $p < 0.0001$] **[Fig. 3.4 B]**, +dP/dT [$F = 100$ (8,117), $p < 0.001$] **[Fig. 3.4 C]**, -dP/dT [$F = 94$ (8,117), $p < 0.0001$] **[Fig. 3.4 D]**, heartrate [$F = 12$ (8,117), $p < 0.0001$] **[Fig. 3.4 E]** and coronary flow rate [$F = 56$ (8,117), $p < 0.0001$] **[Fig. 3.4 F].** However, two-way ANOVA indicated no significant effect of prenatal methamphetamine on parameters of preischemic contractile function and postischemic recovery of contractile function in adult rat hearts following 1 year of abstinence **[Fig. 3.4]**.

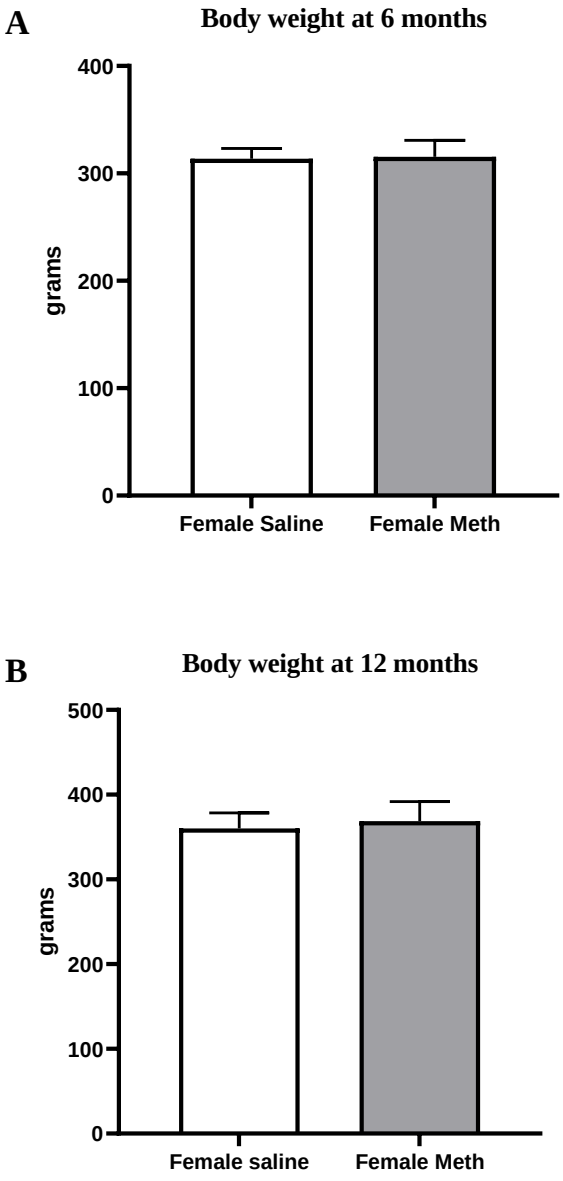

Figure 3.2. Prenatal methamphetamine had no effect on body weight of adult offspring at 6-months and 12-months age.

Prenatally methamphetamine exposed female rats did not differ in their body weight when they reached 6-months (A) and 12-months age (B). All data is represented as mean ± S.E.M of hearts from 7-8 rats.

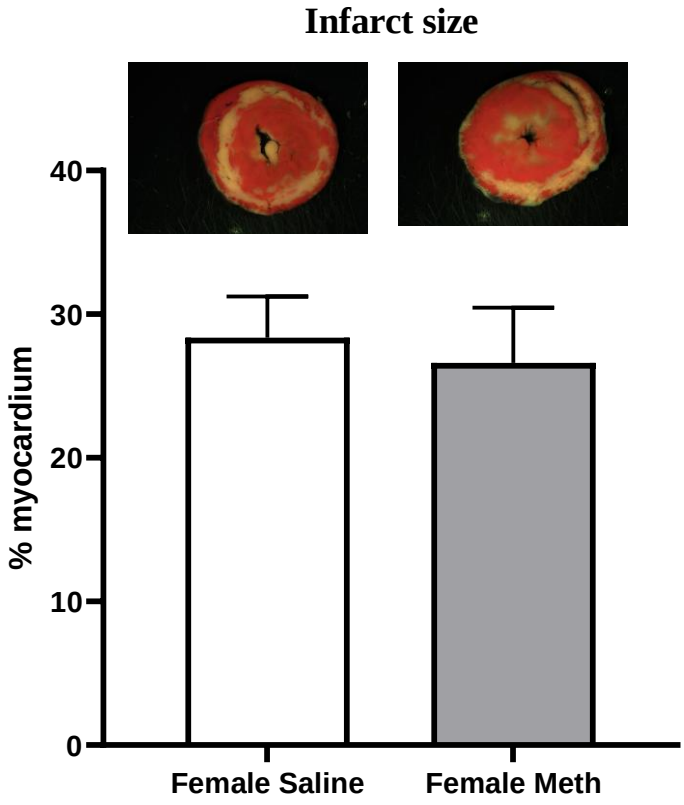

Figure 3.3. Infarct sizes in 1-year-old female hearts following prenatal exposure to saline or methamphetamine.

Unpaired t-test indicated there is no significant effect ($p = 0.726$) of prenatal methamphetamine on female hearts following 1 year of abstinence. All data is represented as mean ± S.E.M of hearts from 7-8 rats.

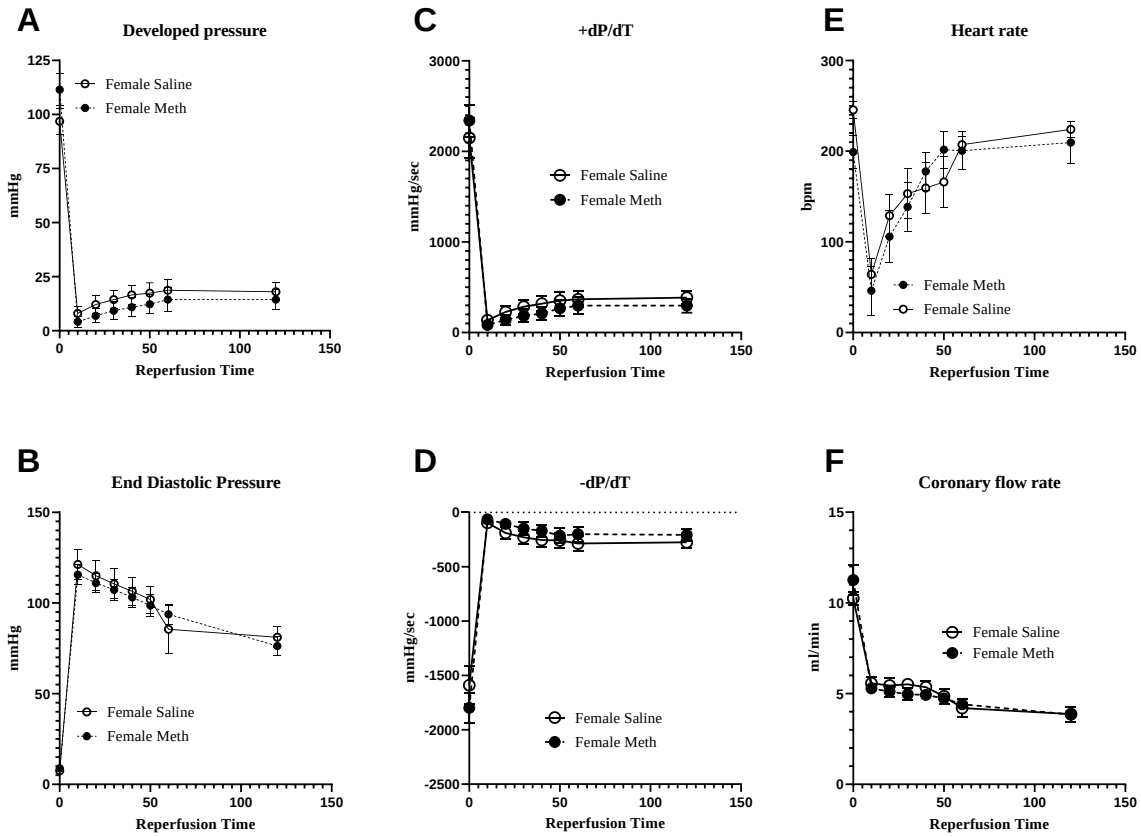

Figure 3.4. Parameters of preischemic contractile function and postischemic recovery of contractile function in female hearts treated prenatally with methamphetamine or saline and abstinent for 1 year.

Preischemic contractile function was measured following a 25-minute equilibration period immediately prior to the onset of ischemia. Postischemic recovery was measured following 10, 20, 20, 40, 50, 60 and 120 min of reperfusion. Two way ANOVA indicated a significant effect of time (preischemic vs postischemic recovery) for developed pressure [$F = 94$ (8,117), $p < 0.0001$] (A), end diastolic pressure [$F = 52$ (8,117), $p < 0.0001$] (B), +dP/dT [$F = 100$ (8,117), $p < 0.0001$] (C), -dP/dT [$F = 93$ (8,117), $p < 0.0001$] (D), heart rate [$F = 12$ (8,117), $p < 0.0001$] (E), coronary flow rate [$F = 56$ (8,117), $p < 0.0001$] (F). Two-way ANOVA indicated no significant effect of prenatal methamphetamine on parameters of preischemic contractile function and postischemic recovery of contractile function in adult rat hearts following 1 year of abstinence. All data is represented as mean ± S.E.M of hearts from 7-8 rats.

3.4 Discussion

Prenatal exposure to methamphetamine increases myocardial sensitivity to I/R injury in adult 2-month-old female hearts despite the fact that they had been abstinent from methamphetamine since birth (Rorabaugh et al., 2016). In addition, these animals exhibited changes in the expression of PKC-ε and the phosphorylation of Akt, proteins that have well established roles in protecting the heart from ischemic injury (Rorabaugh et al., 2016). This suggests that prenatal methamphetamine induces long-lasting changes in the heart that persist following at least 2 months of postnatal abstinence from the drug. The age of rodents can be comparable to that of human age and each month duration in a rat's age is comparable to three human years (Sengupta, 2013). The age of rats used in this study group is one year, which is approximately 36-year-old human. However, the current data indicate that this effect does not persist into the geriatric phase of life when heart attacks are most likely to occur.

Fetal stress (hypoxia, maternal undernutrition, toxins) increases the susceptibility to cardiovascular diseases (ventricular hypertrophy, hypertension, ischemic heart disease) in adult life (Rodríguez-Rodríguez et al., 2017; Vieira-Rocha et al., 2019; Xiao et al., 2016). Prenatal hypoxia increased oxidative stress and cardiac diastolic dysfunction in 7-month-old adult male and female rats. Also, prenatal hypoxia demonstrated an age related decrease in vascular sensitivity in 13-month-old adult male rat offspring (Aljunaidy et al., 2018). Maternal undernutrition is one of the major risk factors for heart disease in adult offspring. Pregnant female rats that received only half of the normal feed intake affected the health of 22-month-old offspring of either sex. The adult offspring had cardiac alterations structurally (larger left ventricular mass, ventricular hypertrophy) and functionally (hypertension, lower ejection fraction) (Rodríguez-Rodríguez et al., 2017). Fetal nicotine exposure increased sensitivity to I/R injury and decreased post-ischemic recovery of

contractile function in 8-month-old adult male offspring (Xiao et al., 2016). Fetal stress (hypoxia, malnutrition, toxins, illicit drugs) can lead to long-lasting effects that persist into adulthood.

However, we found no studies on prenatal exposure to methamphetamine and the effects on cardiovascular function in aged adults. Prenatal exposure to methamphetamine did not have any effect on body weight when they reached one year age, which suggests that there is no effect of methamphetamine on growth of an individual in the long-term. Therefore, the current study investigates the effects in aged rats to determine if prenatal exposure to methamphetamine alters myocardial sensitivity to I/R injury after being abstinent in the first year of their life. Unlike in the 2-month-old rat offspring that were prenatally exposed to methamphetamine that had increased sensitivity to I/R injury, one year old adult offspring that were prenatally exposed to methamphetamine had no effect on the infarct size after subjecting to I/R injury. Our findings indicated that myocardial hypersensitivity to I/R injury on prenatal ischemic injury decreases as time progresses and there is no effect of methamphetamine after the rats are abstinent for the first one year of their postnatal period. This suggests that sensitization to myocardial ischemic injury following prenatal exposure to methamphetamine decreases as time progresses and a pregnant woman who is addicted to methamphetamine can still save her child from cardiac damage later in the life by not exposing the child directly to methamphetamine for the whole lifetime.

CHAPTER 4

SEX-DEPENDENT CHANGES IN VASCULAR FUNCTION OF ADULT RATS FOLLOWING PRENATAL EXPOSURE TO METHAMPHETAMINE

4.1 Introduction

Previous studies in our lab have focused on the impact of methamphetamine on myocardial ischemic injury. Methamphetamine exposure during either the early adult period or during gestation causes females (but not males) to become hypersensitive to cardiac ischemic injury (Rorabaugh et al., 2016; Rorabaugh et al., 2017). However, there have been no studies on vascular function in adult offspring following prenatal exposure to methamphetamine. The present study tested the hypothesis that prenatal exposure to methamphetamine results in vascular dysfunction in adult offspring. We also investigated the impact of prenatal methamphetamine on the function of perivascular adipose tissue (PVAT)

PVAT surrounds most of the vasculature, provides structural support to the blood vessel, regulates vascular homeostasis and thermogenicity, and has become recognized as an important regulator of vascular function (Liang et al., 2020). PVAT is structurally composed of adipocytes, fibroblasts, and T-lymphocytes and macrophages. Depending on the location, cell types and precursor cells of PVAT are different, but having similarities with both brown and white adipose tissue (Gil-Ortega et al., 2015). PVAT of large and small vessels differs due to the presence of an anatomical barrier in large blood vessels composed of collagen and elastic fibers and fibroblasts. This anatomical barrier is not present in small vessels and PVAT is an intrinsic component of the vessel wall in small vessels (Gil-Ortega et al., 2015; Ramirez et al., 2017).

PVAT is known to regulate vascular tone of blood vessels (large and small vessels) by releasing biologically active molecules like adipocytokines, chemokines, and growth factors, all

of which are broadly classified into PVAT-derived relaxing factors (PVRF) and PVAT-derived contractile factors (PVCF). PVCF like angiotensin II (Ang-II), reactive oxygen species (ROS), and other unidentified factors can induce blood vessel constriction, whereas PVRF like adiponectin, nitric oxide (NO), hydrogen sulfide (H_2S), prostacyclin, angiotensin 1-7, and methyl palmitate promote vasodilation by targeting medial and endothelial layers of blood vessels (Xia & Li, 2017). Thus, PVAT plays a key role in regulation of vascular tone and blood pressure. PVAT becomes dysfunctional in age-related vascular diseases and various pathophysiological conditions like obesity, type 2 diabetes, vascular injury and aging (Queiroz & Sena, 2020; Xia & Li, 2017). Dysfunctional PVAT may indirectly increase the risk of vascular events, hypertension, and other cardiovascular diseases. Thus, the current study investigated the impact of prenatal methamphetamine on adult vascular function. In light of the importance of PVAT in cardiovascular disease, we specifically investigated the potential impact of prenatal methamphetamine on vessels in the presence and absence of PVAT.

4.2 Methods

Animals: Female Sprague-Dawley rats (8weeks of age) were used for breeding. The rats were housed in standard cages with free access to food and water and on 12/12hr light/dark cycle (lights on at 0600). The presence of a vaginal plug was considered as gestational day 0. Pregnant rats were administered saline or methamphetamine (5mg/kg/day) by subcutaneous injection once per day (at 0800) starting at gestational day 1 and continuing until the pups were born. At 28 days of age, pups were weaned and housed two animals per cage. All procedures were approved by the Institutional Animal Care and Use Committee of Marshall University (IACUC protocol no. 719).

Blood pressures: Blood pressure was measured when the offspring reached 5 months age using CODA® High Throughput System (Kent Scientific Corporation, Torrington, CT). Each

session was set to have a total of 20 cycles with the first five being acclimation cycles. The blood pressure was measured for five consecutive days. The first three days were for acclimatization to the apparatus. Blood pressure measurements on days 4 and 5 were averaged to represent the blood pressure of each animal. Blood pressure was measured between 9am to 12 pm, at a room temperature between 32-35°C.

Preparation of the aorta: Following anesthesia with pentobarbital sodium (100 mg/kg), aortas were collected and cut into 2-mm long rings. Two aortic rings were used from each rat. One aortic ring had perivascular adipose tissue intact, and the other ring had perivascular adipose tissue removed. The segments were mounted on a Radnoti 4-unit tissue bath system (Radnoti LLC, Monrovia, CA) in chambers filled with 10 ml of Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 0.5 Na_2EDTA, 11 glucose, and 2.5 $CaCl_2$, pH 7.4) bubbled with 95% O_2 and 5% CO_2 at 37°C. The tension was set to 0.5g and gradually increased until a resting tension of 3g was obtained. The tissue was then equilibrated for one hour. Krebs solution was changed every 15 min during the equilibration phase.

Concentration response curves: Viability of the aortic tissue was verified by stimulating with 60 mM potassium chloride (KCl). 200 µL of the 30 mM KCl stock solution was added to the 10 ml chamber to make the final KCl concentration 60 mM KCl. Two 10 min KCl responses were recorded and the second KCl response was used to normalize the subsequent agonist-induced contractile responses. Contractile responses to phenylephrine (PE, 10^{-11} – 10^{-5} mol/L), angiotensin-II (Ang-II, 10^{-11} – 10^{-6} mol/L) and serotonin (5-HT, 10^{-11} – 10^{-4} mol/L) were measured. Relaxation responses to acetylcholine (Ach, 10^{-11} – 10^{-5} mol/L) and sodium nitroprusside (SNP, 10^{-11} – 10^{-5} mol/L) were measured following precontraction with 10 µM phenylephrine. These experiments were conducted in two different sets of rats. In the first set, the aortic rings were treated with 60

mM KCl, followed by 3 washes prior to the phenylephrine-induced concentration response curve. Relaxation responses to Ach were then measured in the precontracted tissue, followed by serotonin-induced concentration response curve. In the second set of rats, the aortic rings were initially treated with 60 mM KCl, followed by 3 washes. Ang-II concentration responses were then measured. This was followed by 3 washes (each for 5 minutes). The tissue was then precontracted with 10 µM PE prior to measuring SNP-induced relaxation. The tissues were washed between each treatment. The contractile responses caused by PE, Ang-II, and serotonin were normalized to the response induced by 60 mM KCl. Relaxation responses caused by Ach or SNP were normalized to precontraction induced by 10 µM PE. The signals were recorded using a PowerLab data acquisition system and LabChart 8 software (AD instruments, Colorado Springs, CO).

Determining the involvement of eNOS (endothelial nitric oxide synthase) activity using synthetic eNOS inhibitor L-NAME: The same experiments were performed as described above except that 100 µM L-NAME was added to the tissue bath 20 min prior to starting each concentration response curve.

Measurement of PVAT assisted relaxation: Aortic rings were mounted on the tissue bath with the passive tension set to 0 grams. Then, a cumulative passive tension of 0.25, 0.5, 1, 2, 4 and 6 grams was applied to the aortal rings. After each adjustment of tension, the tissue was allowed to relax for 30 min and the tension was recorded. The tissue was then treated with PE (10 µM), allowed to reach a plateau and the response was recorded, followed by 30-minute washout period.

Western blots: PVAT from the aorta was removed and homogenized with a Polytron in 500 µL solubilization buffer [50mM Tris, pH 7.4, 1 mM EDTA, 1% sodium dodecyl sulfate, phosphatase inhibitor cocktail 2 (catalog no. P5726, Sigma), phosphatase inhibitor cocktail 3 (catalog no. P0044, Sigma), and protease inhibitor cocktail (catalog no. P8340, Sigma)] using an

electric tissue homogenizer. The homogenate was boiled for 5 min and then centrifuged at 14,000 rpm for 10 min. The BCA assay was used to measure the protein concentration of each sample. Thirty µg of protein were loaded on to a 10% polyacrylamide gel. Western blotting was used to measure the expression of eNOS and GAPDH. Antibodies for eNOS (Catalog no. 9572) and GAPDH (Catalog no. 5174) were obtained from Cell Signaling Technology (Danvers, MA). The band densities were measured and quantified using ImageJ software and eNOS expression was normalized to GAPDH.

Quantitative polymerase chain reaction (qPCR): RNA was extracted from the aortas (devoid of PVAT) and the abundance of transcripts encoding angiotensin-II receptors [Angiotensin receptor subtype 1a (AT1aR), angiotensin receptor subtype 1b (AT1bR) and angiotensin receptor type 2 (AT2R)] was determined by quantitative PCR. Total RNA was isolated using Trizol (Thermo Fisher, Waltham, MA) as per the manufacturer's instructions, resuspended in 20 µL nuclease free water and the total RNA concentrations were determined using Nanovue spectrophotometer (GE Healthcare, USA). cDNA was synthesized using 323 ng of total RNA with Superscript™ VILO™ cDNA synthesis kit (Thermofisher, CA). For each receptor of interest, 2 µL of cDNA was used. Bio-Rad CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc. Hercules CA) was used for quantifying the gene expression of angiotensin II receptor (catalog no. 4331182) by TaqMan™ single gene expression assays (Thermofisher Scientific, Foster City, CA). The following qPCR protocol was used: 95°C for 30 sec (enzyme activation), followed by 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 30sec (annealing/extension), finally a melt curve at 65°C - 95°C. Alien RNA (catalog no. 300600) obtained from Agilent Technologies (La Jolla, CA) was used as a reference and for normalization of the curves. PCR was performed in duplicate, and the threshold cycle numbers were averaged.

Measurement of vasomotor responses in third order mesenteric arteries. Third order mesenteric arteries (~250 μm in diameter) were isolated from 6-month-old male rats and placed in calcium-free physiological saline solution (PSS) (in mM: 130 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 5.5 glucose, 0.026 EDTA; pH 7.4). Mesenteric rings (with PVAT either attached or removed) were cut to a length of 1.8 mm and mounted in a prewarmed (37° C) 4-channel wire myograph (DMT 620M, Danish Myo Technology, Denmark), chamber filled with PSS containing 40 mM $CaCl_2$ that was continuously aerated with 95 % O_2 / 5 % CO_2. Contractile function was continuously recorded by a Power Lab 4/30 data acquisition system (AD Instruments, Colorado, US) using Lab Chart Pro v8.1.19 (AD Instruments, Colorado, US) software. Mounted vessels were normalized by using the micrometer to progressively stretch the tissue until it reached a wall tension equivalent to 100 mmHg of pressure (del Campo & Ferrer, 2015; Wenceslau et al., 2021). The vessels were then prepared for experimentation using a "wake-up" protocol in which the tissue was contracted with physiological saline containing high potassium (KPSS) (in mM: 74.7 NaCl, 60 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.0 $NaHCO_3$, 5.5 glucose, 0.026 EDTA, 1.6 $CaCl_2$) and then washed three times. The tissue was then contracted with 10 μM norepinephrine and washed three times. Functionality of the tissue was tested by precontracting the vessels with 1 μM norepinephrine followed by relaxation of the precontracted tissue with 10 μM acetylcholine. Vessels that failed to contract to 1 μM norepinephrine or to relax in response to 10 μM acetylcholine were not used for further experiments. The vessels were then contracted with KPSS for 10 min followed by three washes. Once the KPSS was washed out by PSS, contractile responses to cumulative concentrations (10pM – 10μM) of phenylephrine were measured. The tissue was washed three times and then contracted with 10μM phenylephrine prior to measuring relaxation with cumulative concentrations of acetylcholine. The tissue was then washed three more times

before precontracting the tissue with 10μM phenylephrine and measuring relaxation in response to cumulative concentrations (10pM – 10μM) of sodium nitroprusside. The phenylephrine-induced contractile response was normalized to the response following 10 min of contraction with KPSS. Relaxation responses to acetylcholine and nitroprusside were normalized to precontraction tension induced by 10μM phenylephrine.

Statistical analyses: Gestational weight gain was analyzed by two-way ANOVA (time and treatment as factors). Concentration response curves were analyzed by non-linear regression. pEC_{50} values and the magnitude of agonist-induced contractile or relaxation responses were analyzed by two-way ANOVA with treatment (prenatal saline vs prenatal methamphetamine) and the presence or absence of PVAT as factors and Tukey's posthoc analysis. Contractile response curves for male and female vessels were analyzed separately. Blood pressure was analyzed by two-way ANOVA (sex and treatment) and Tukey's posthoc analysis. P values ≤ 0.05 were considered statistically significant. GraphPad prism 8 (San Diego, CA) software was used for all the analyses.

4.3 Results

Methamphetamine had no effect on gestational weight. Female rats had similar weights on gestational day 1 that were treated with saline (288 ± 3; n = 3) or methamphetamine (290 ± 14; n = 4). Two-way ANOVA indicated a significant effect of gestational day [$F = 140$ (2,11), $p < 0.0001$] on gestational weight **[Fig. 4.1]**. However, there was no effect of methamphetamine on gestational weight. The litter size was similar in saline (13 ± 0.3) and methamphetamine treated dams (11 ± 1).

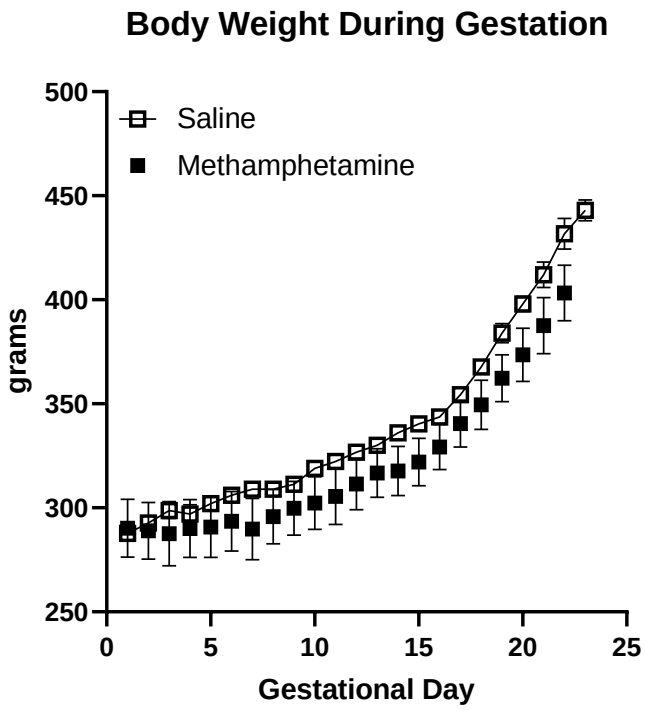

Figure 4.1. Gestational weight in dams treated with saline or methamphetamine.

Weight of pregnant rats was recorded while administering them with saline or methamphetamine. Two-way ANOVA indicated a significant effect of gestational day [$F = 140$ (2,11), $p < 0.0001$]. All data is represented as mean ± S.E.M of hearts from 3-4 rats.

Prenatal exposure to methamphetamine impairs acetylcholine-induced relaxation of adult male aorta. Acetylcholine induced a concentration dependent relaxation of precontracted aortas. However, acetylcholine-induced relaxation was impaired in male aortas only when PVAT was intact **[Fig. 4.2A]**. Prenatal methamphetamine had no effect on acetylcholine-induced relaxation when PVAT was removed. Two-way ANOVA indicated a significant effect of methamphetamine on pIC50 [$F = 11$ (1,21), $p < 0.0005$] and maximal response [$F = 8$ (1,21), $p = 0.01$] for acetylcholine-induced relaxation in male aortas **[Table 1]**. L-NAME completely blocked acetylcholine-induced relaxation in male aortas **[Fig. 4.2 E] [Table 1]**. In contrast, prenatal exposure to methamphetamine had no impact on acetylcholine-induced relaxation in female aortas in the presence or absence of PVAT **[Fig. 4.2 B] [Table 1]**.

When nitroprusside-induced relaxation was measured in male aortas, two-way ANOVA indicated a significant effect of PVAT on pIC50 [$F = 24$ (1,22), $p < 0.0001$] and maximal relaxation response [$F = 22$ (1,22), $p < 0.0001$] in male aortas **[Fig. 4.2 C]**. There was also a significant effect of PVAT on the pIC50 of nitroprusside-induced relaxation in female aortas [$F = 18$ (1,19), $p < 0.0005$] **[Fig. 4.2 D]**. Although PVAT had a significant effect on pIC50 of nitroprusside induced relaxation in both male and female aortas, prenatal exposure to methamphetamine had no impact on either pIC50 or maximal relaxation response induced by nitroprusside in aortas of either sex **[Table 1 and 2].**

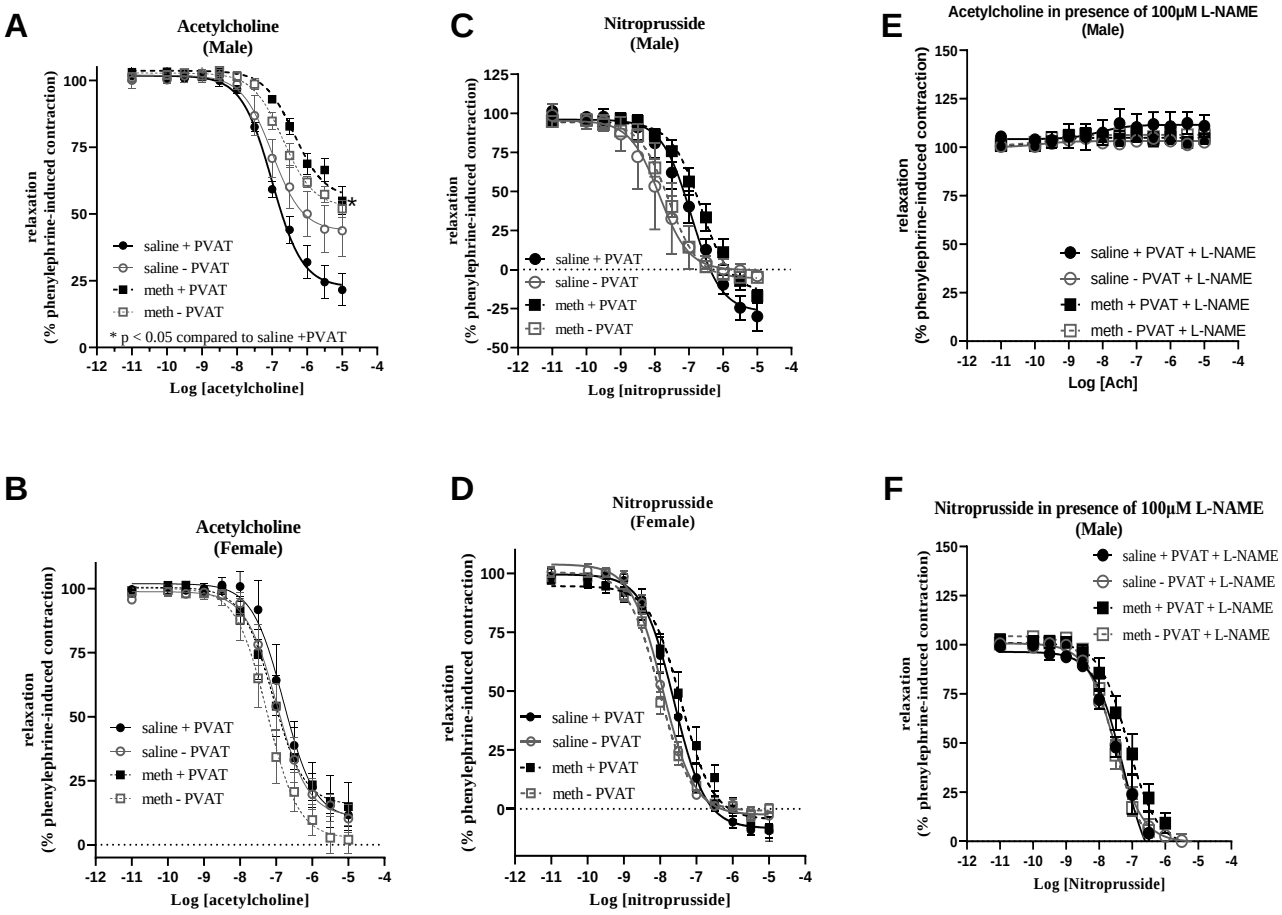

Figure 4.2. Prenatal methamphetamine causes sex dependent dysfunction of perivascular adipose tissue (PVAT) of the adult aorta.

Two-way ANOVA indicated a significant effect of prenatal methamphetamine [F = 8 (1,21), p = 0.01] on acetylcholine-induced relaxation in male (A) but not female (B) aortas. Prenatal methamphetamine had no effect on nitroprusside-induced relaxation (C, D). The response to acetylcholine was abolished by L-NAME (E). All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=4-6).

Potentiation of angiotensin-II induced contractile responses in the aorta following prenatal exposure to methamphetamine in a sex dependent manner. Contractile responses to Ang-II, phenylephrine, and serotonin were measured in aortas from male and female rats that were prenatally exposed to saline or methamphetamine. Ang-II induced contractile responses were potentiated in aortas of male rats following prenatal exposure to methamphetamine irrespective of the presence or absence of PVAT **[Fig. 4.3 A] [Table 1]**. This potentiation was blocked by L-NAME **[Fig. 4.3 B] [Table 1]**. In contrast to male aortas, Ang-II induced responses were not affected by prenatal methamphetamine in female aortas **[Fig. 4.3 C] [Table 2]**. The number of mRNA transcripts encoding AT_{1A}, AT_{1B}, and AT_2 receptor subtypes in aorta were not affected by prenatal methamphetamine **[Fig. 4.3 D].**

Prenatal exposure to methamphetamine had no effect on phenylephrine-induced contractions in either male or female aortas **[Fig. 4.4 A-B] [Table 1 and 2]**. Two-way ANOVA indicated a significant effect of methamphetamine on pEC50 of serotonin-induced contractile responses in male aortas [$F = 5\ (1,18)$, $p < 0.05$] **[Fig. 4.4 C] [Table 1]**, but Tukey's posthoc analysis indicated no differences between individual groups. Serotonin-induced contractions were not affected in female aortas **[Fig. 4.4 D] [Table 2**].

Prenatal methamphetamine has no effect on eNOS expression in the PVAT. eNOS expression was assessed in PVAT of aortas from male rats. Prenatal methamphetamine had no effect on eNOS expression **[Fig. 4.5]**

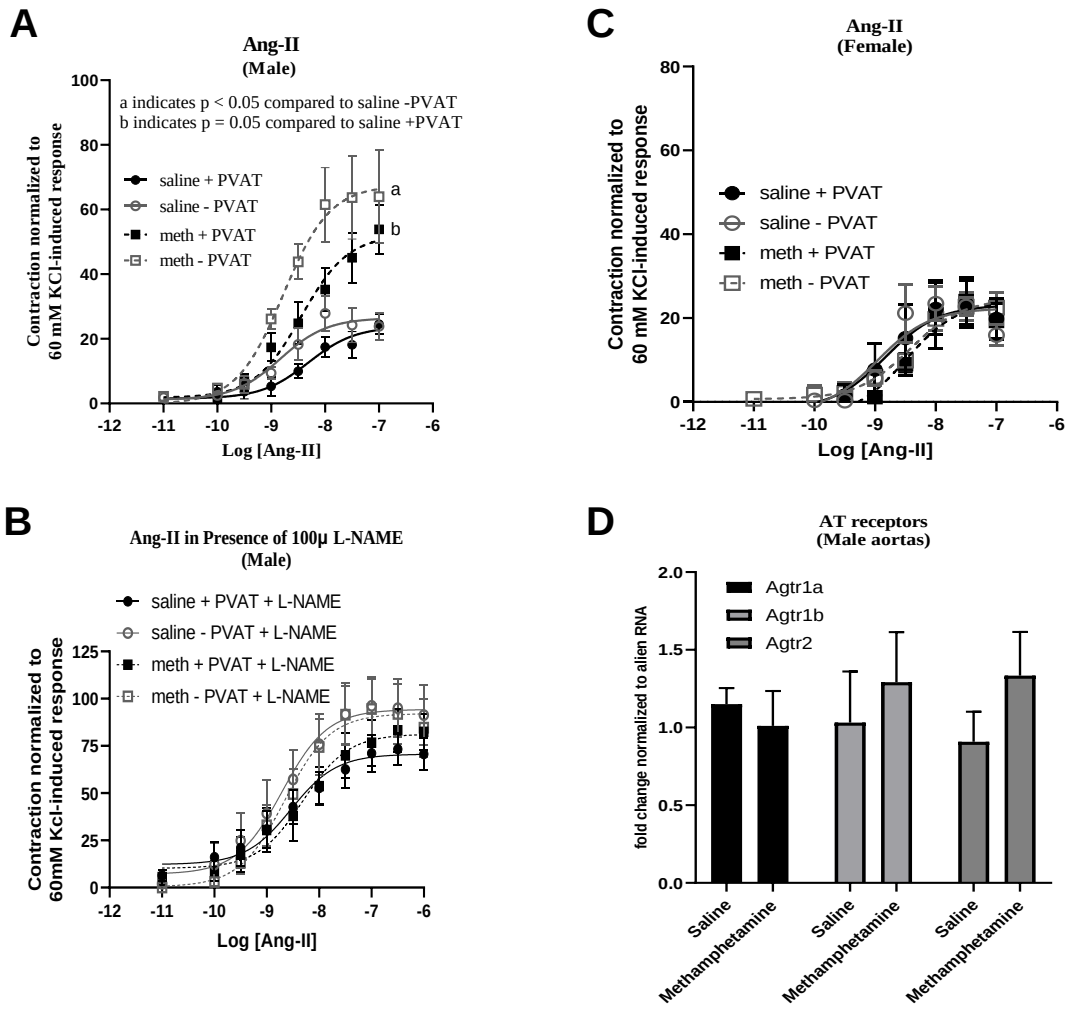

Figure 4.3. Prenatal exposure to methamphetamine potentiates Ang-II induced contraction of aortas in male offspring.

Aortas from adult male offspring that were prenatally exposed to methamphetamine had significantly [F = 15 (1,25), p < 0.001] potentiated Ang-II induced contractions irrespective of the presence / absence of PVAT (A). There was no effect of prenatal methamphetamine on Ang-II induced responses in male aortas in presence of 100µM L-NAME (B). Angiotensin-II induced contractile responses were unaffected in female aortas that were prenatally exposed to methamphetamine (C). Prenatal methamphetamine did not induce changes in transcripts encoding AT1 and AT2 receptors (D). All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=4-6).

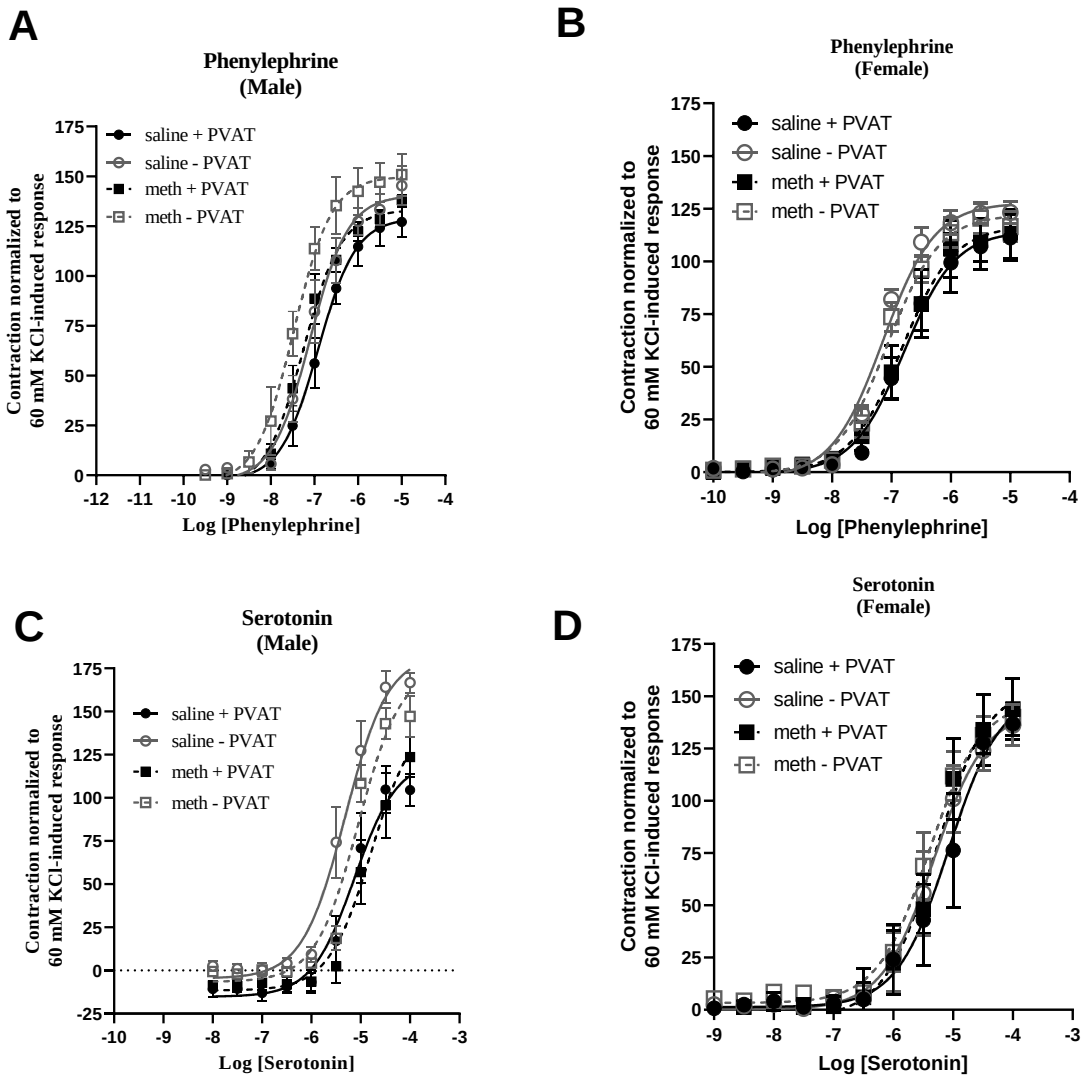

Figure 4.4. Prenatal methamphetamine had no effect on phenylephrine or serotonin-induced contraction in aortas of female rats.

Phenylephrine-induced contraction was not altered by prenatal methamphetamine either in male (A) or female (B) aortas. Prenatal methamphetamine had significantly decreased serotonin-induced contraction in male (C) but not female (D) aortas. Two-way ANOVA indicated a significant effect of methamphetamine [$F = 5$ (1,18), $p < 0.05$] on pEC50 for serotonin-induced contraction. All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=4-6).

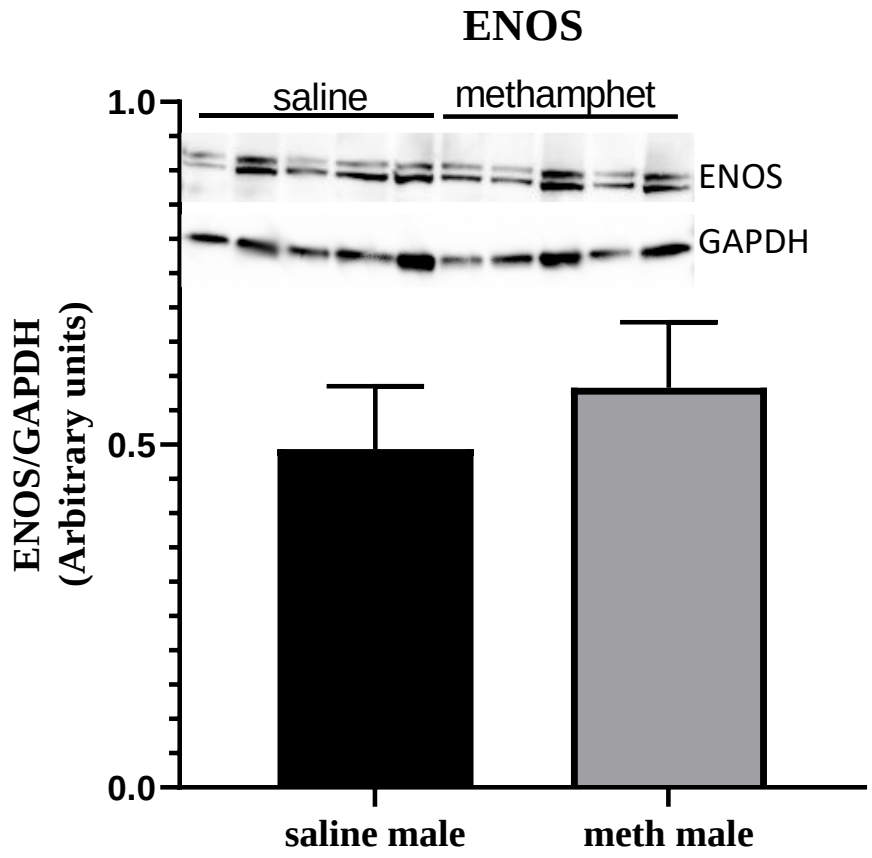

Figure 4.5. Prenatal methamphetamine had no effect on the expression of eNOS in PVAT of aortas of male offspring.

Data is represented as mean ± S.E.M of n (=5) for each group.

Prenatal methamphetamine has no effect on PVAT assisted relaxation. Aortas from male and female rats were stretched to cumulative tensions of 0.25, 0.5, 1, 2, 4 and 6 grams and stress induced relaxation was measured. Prenatal methamphetamine has no significant effect on PVAT assisted relaxation in either male or female aortas. However, there was a significant effect of PVAT on stretch induced relaxation in both male [$F = 29\ (4,72)$, $p < 0.001$] **[Fig. 4.6 A] [Table 1]** and female [$F\ 15.5\ (4,72)$, $p < 0.0001$] **[Fig. 4.6 B]** aortas. Also, there is significant effect of tension in male [$F = 1174\ (5,72)$, $p < 0.0001$] and female [$F = 1110\ (5,72)$, $p < 0.0001$] aortas and there is a significant interaction between tension and PVAT in male [$F = 29\ (4,72)$, $p < 0.0001$] and female [$F = 3\ (20,72)$, $p < 0.0001$] aortas.

Prenatal methamphetamine has no effect on vascular responses in mesenteric arteries. Prenatal exposure to methamphetamine had no effect on acetylcholine- or nitroprusside-induced relaxation in these vessels regardless of the presence or absence of PVAT **[Fig. 4.7 A-B] [Table 3]**.

Prenatal exposure to methamphetamine had no effect on blood pressure. The basal systolic or diastolic blood pressures were not affected by prenatal exposure to methamphetamine in either male or female rats **[Fig. 4.8 A-B]**.

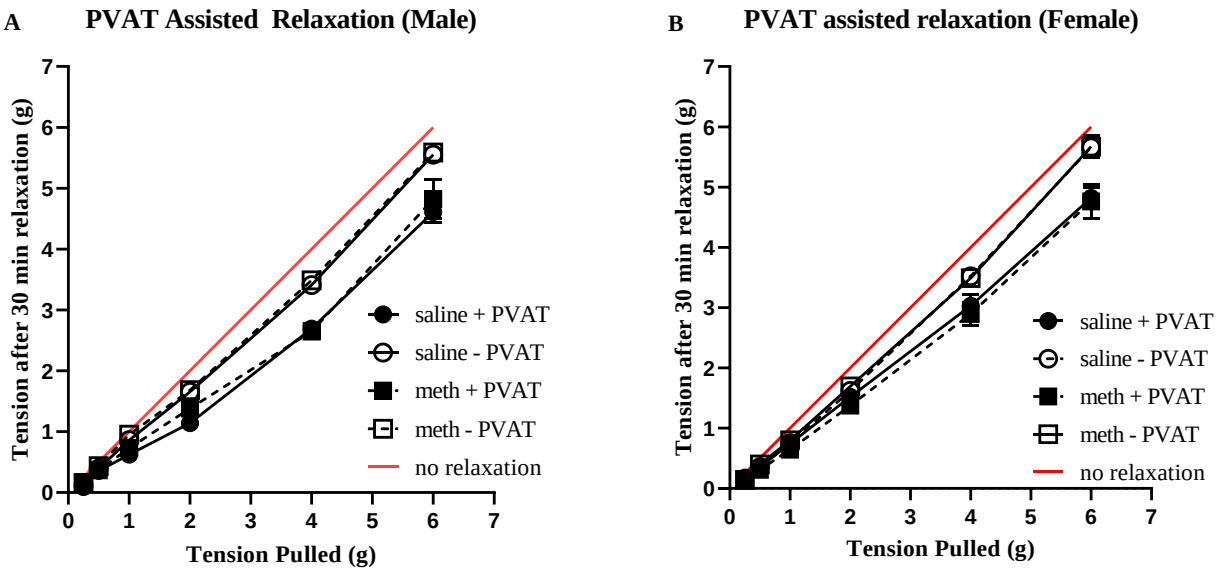

Figure 4.6. Prenatal methamphetamine had no impact on PVAT-assisted relaxation in aortas of adult offspring in either males or females.

All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=3).

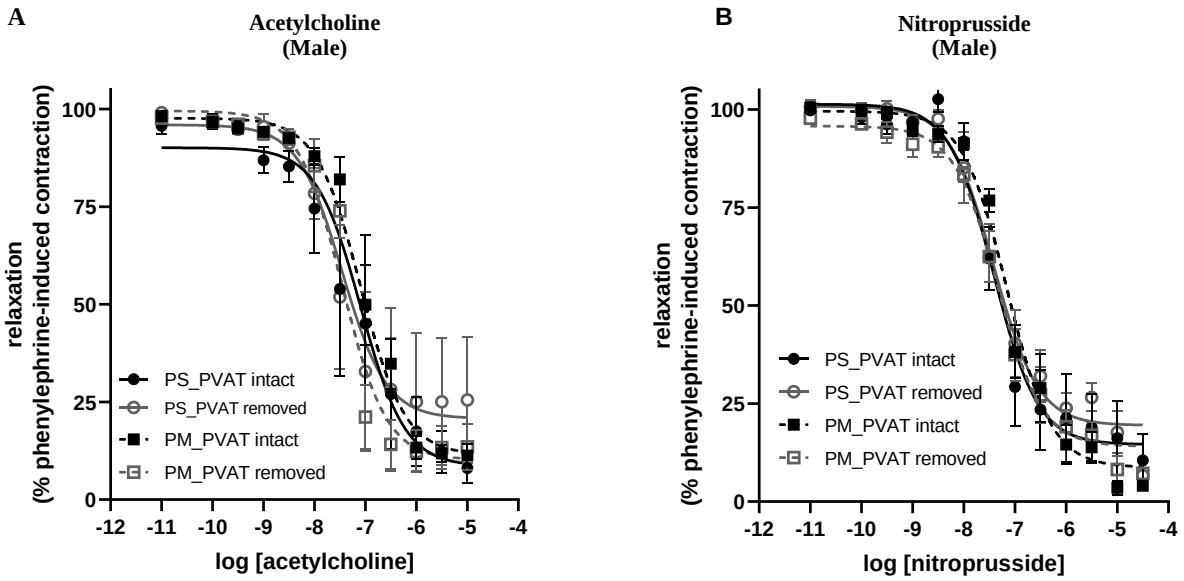

Figure 4.7. Prenatal methamphetamine had no effect on Acetylcholine (A) or nitroprusside-induced (B) relaxation in resistance mesenteric arteries of adult males.

All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=5).

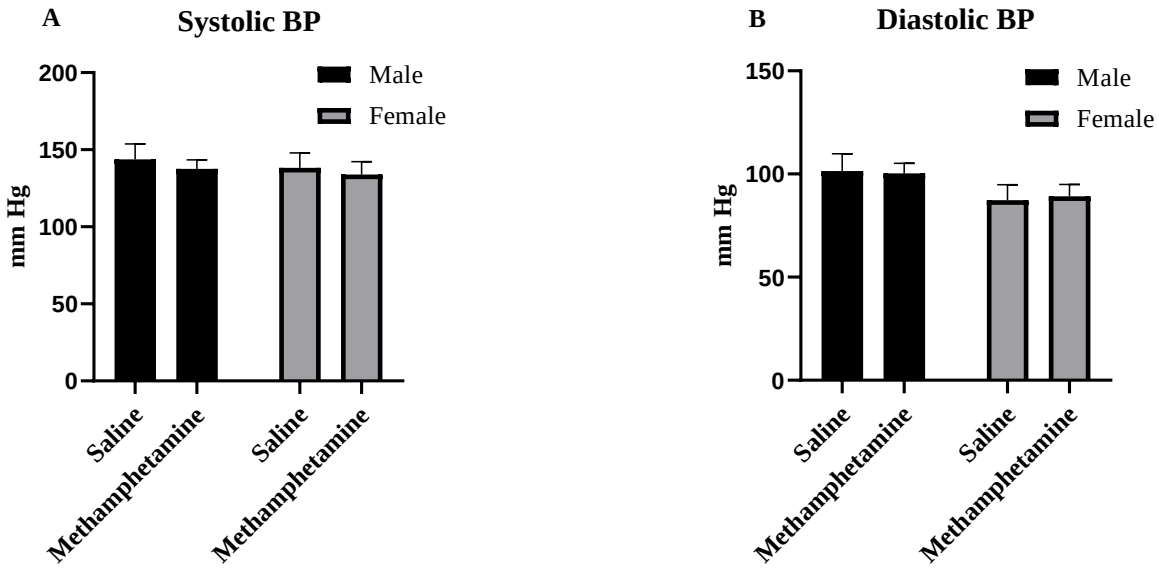

Figure 4.8. Prenatal methamphetamine had no effect on basal blood pressure.

Systolic and diastolic blood pressures are shown in (A) and (B) respectively in both male and female offspring. There was no effect of methamphetamine on baseline blood pressures in both male and female offspring. All data is represented as mean ± S.E.M of hearts from separate animals from each group n (=6).

Table 1. Contractile and relaxation responses in aortas of adult male offspring following prenatal exposure to saline or methamphetamine.

		pEC50		Maximal Response	
Male	**+/- PVAT**	**Saline**	**Meth**	**Saline**	**Meth**
PE	**+PVAT**	-6.9 ± 0.1	-7.2 ± 0.1	130 ± 7	143 ± 7
	-PVAT	-7.2 ± 0.2	-7.4 ± 0.04	135 ± 2	150 ± 10
Ang II	**+PVAT**	-8.4 ± 0.2	-8.3 ± 0.2	24 ± 4	58.6 ± 9[a]
	-PVAT	-8.8 ± 0.1	-9.0 ± 0.2	27 ± 5	62 ± 14[a, b]
Ang II + L-NAME	**+PVAT**	-8.8 ± 0.3	-8.4 ± 0.2	70 ± 8	81 ± 10
	- PVAT	-8.7 ± 0.2	-8.6 ± 0.1	95 ± 15	97 ± 16
Serotonin	**+PVAT**	-5.1 ± 0.1	-4.7 ± 0.2[b]	128 ± 11	149 ± 10
	-PVAT	-5.3 ± 0.1	-5.0 ± 0.1	189 ± 9[a]	180 ± 11[a]
Ach	**+PVAT**	-7.0 ± 0.1	-6.3 ± 0.1[a]	22 ± 6	55 ± 5[a]
	-PVAT	-6.8 ± 0.2	-6.9 ± 0.1	39 ± 10	53 ± 3
Ach + L-NAME	**+PVAT**	-7.7 ± 0.5	-8.0 ± 1.2	112 ± 6.5	105 ± 3
	-PVAT	-7.3 ± 2	-8.2 ± 1	10 ± 1	108 ± 3
Nitroprusside	**+PVAT**	-7.1 ± 0.2	-6.7 ± 0.2[b]	-21 ± 5	-17 ± 6[b]
	-PVAT	-7.9 ± 0.2[a]	-7.6 ± 0.1[c]	-3 ± 2[a]	3
Nitroprusside + L-NAME	**+PVAT**	-7.3 ± 0.1	-7.1 ± 0.2	-21 ± 13	-3 ± 2
	-PVAT	-7.5 ± 0.1	-7.5 ± 0.1	-1 ± 2	-6 ± 2

Data represent the mean ± SEM of n (4-6) separate animals for each group.
Two-way ANOVA indicated a significant effect of PVAT [F = 8 (1,25), $p < 0.01$] on pEC50 for Ang-II. Two Way ANOVA indicated a significant effect of methamphetamine [F= 15 (1, 25), p =

0.001] on maximal Ang-II induced contraction. Two-way ANOVA indicated a significant effect of methamphetamine [$F = 5$ (1, 18), $p < 0.05$] on pEC50 for serotonin. Two Way ANOVA indicated a significant effect of PVAT [$F= 20.5$ (1, 18), $p < 0.0005$] on maximal serotonin-induced contraction. Two-way ANOVA indicated a significant effect of methamphetamine [$F = 11$ (1, 21), $p < 0.005$] and a significant interaction between PVAT and methamphetamine [$F = 5.3$ (1, 21), $p > 0.05$] on pEC50 for Ach. Two-way ANOVA indicated a significant effect of methamphetamine [$F = 8$ (1,21), $p = 0.01$] on maximal Ach-induced relaxation. Two-way ANOVA indicated a significant effect of PVAT [$F = 24$(1,22), $p < 0.0001$] on pIC50 for SNP. Two-way ANOVA indicated a significant effect of PVAT [$F = 15$(1,22), $p < 0.005$] on maximal SNP induced relaxation.

[a] $p < 0.05$ compared to saline +PVAT.

[b] $p < 0.05$ compared to saline -PVAT.

[c] $p < 0.05$ compared to Methamphetamine +PVAT.

Table 2. Contractile and relaxation responses in aortas of adult female offspring following prenatal exposure to saline or methamphetamine.

		pEC50		Maximal Response	
Female	**+/-PVAT**	**Saline**	**Meth**	**Saline**	**Meth**
PE	+PVAT	-6.7 ± 0.2	-6.8 ± 0.1	118 ± 10	118 ± 11
	-PVAT	-7.1 ± 0.1[a]	-7.0 ± 0.1	128 ± 6	123 ± 6
Ang II	+PVAT	-8.6 ± 0.2	-8.4 ± 0.1	25 ± 6	25 ± 6
	-PVAT	-8.9 ± 0.1	-8.4 ± 0.1	23 ± 3	25 ± 4
Serotonin	+PVAT	-5.1 ± 0.3	-5.4 ± 0.2	163 ± 18	153 ± 26
	-PVAT	-5.3 ± 0.2	-5.4 ± 0.2	147 ± 8	152 ± 5
Ach	+PVAT	-6.8 ± 0.2	-7.1 ± 0.2	8 ± 4	13 ± 9
	-PVAT	-6.9 ± 0.2	-7.3 ± 0.2	9 ± 5	0.6 ± 6
Nitroprusside	+PVAT	-7.7 ± 0.1	-7.5 ± 0.2[b]	-9 ± 2	-4 ± 4
	-PVAT	-7.9 ± 0.1	-8.1 ± 0.01[c]	-3 ± 2	-1 ± 2

Data represent the mean ± SEM of n (=6) animals for each group.
Two-way ANOVA indicated a significant effect of PVAT [$F = 10.82(1,20)$, $p < 0.005$] on pEC50 for phenylephrine. Two-way ANOVA indicated a significant effect of methamphetamine [$F = 5.59(1,20)$, $p < 0.05$] on pEC50 for Ang-II. Two-way ANOVA indicated a significant effect of PVAT [$F = 18(1,19)$, $p < 0.0005$] on pIC50 for SNP.
[a] $p < 0.05$ compared to saline +PVAT.
[b] $p < 0.05$ compared to saline -PVAT.
[c] $p < 0.05$ compared to Methamphetamine +PVAT.

Table 3. Relaxation responses in resistance mesenteric arteries of adult male offspring following prenatal exposure to saline or methamphetamine.

		pIC50		Maximal Response	
Male	**+/- PVAT**	**Saline**	**Meth**	**Saline**	**Meth**
Ach	**+PVAT**	-7.22 ± 0.44	-6.97 ± 0.16	5 ± 4	7 ± 3
	-PVAT	-7.30 ± 0.43	-7.33 ± 0.07	21 ± 17	8 ± 6
Nitroprus side	**+PVAT**	-7.40 ± 0.12	-7.13 ± 0.1	14 ± 10	9 ± 5
	-PVAT	-7.36 ± 0.15	-7.31 ± 0.1	21 ± 4	15 ± 4

Data represent the mean ± SEM of n (=4-5) animals for each group.
Two-way ANOVA indicated no significant of PVAT or methamphetamine on pIC50 or maximal response for both acetylcholine and nitroprusside-induced relaxation in resistance mesenteric arteries of adult male offspring.

4.4 Discussion

The primary finding of this study was that prenatal exposure to methamphetamine alters vascular function in a sex-dependent manner. Previous work in our lab demonstrated that prenatal exposure to methamphetamine sex dependently sensitizes the adult heart to ischemic injury (Rorabaugh et al., 2016). However, we are not aware of any studies focusing on the impact of prenatal exposure to methamphetamine on vascular function in the adult offspring. This is the first study to show that prenatal methamphetamine exposure alters vasomotor function in the aorta of adult offspring. Our findings also show that prenatal exposure to methamphetamine leads to perivascular adipose tissue dysfunction and disruption of NO signaling in the aorta.

Prenatal exposure to cns stimulants alters cardiac function and vascular function (Rorabaugh, 2021). Vascular function was altered in adult offspring that had been exposed to cocaine prenatally. Prenatal cocaine had no effect on basal blood pressure, but had increased norepinephrine-induced blood pressure in adult rats (Xiao, Huang, et al., 2009). Norepinephrine-induced vasoconstriction was also potentiated in mesenteric resistance arteries, and myofilament sensitivity to calcium was increased in these vessels, following prenatal exposure to cocaine. Prenatal exposure to cocaine also resulted in attenuation of endothelium dependent relaxation in a sex-dependent manner. These data suggests that prenatal cocaine induces changes in both endothelium and vascular smooth muscle which alter the vascular function and increase the risk of developing hypertension.

Prenatal exposure to nicotine had no effect on basal blood pressure (Xiao et al., 2008) like cocaine (Xiao, Huang, et al., 2009) in adult rats. Prenatal exposure to nicotine increased norepinephrine and angiotensin II-induced blood pressure in adult rats (Xiao et al., 2015; Xiao et al., 2008) and enhanced vasoconstriction of mesenteric resistance arteries in adult rats (Xiao et al.,

2008). These effects seen with prenatal nicotine are associated with increased expression of angiotensin II receptors in vascular smooth muscle, increased thickening of arterial wall media, enhanced sensitivity of vascular smooth muscle to calcium, and disruption of perivascular adipose-dependent mechanisms that regulate vascular tone (Gao et al., 2008; Xiao et al., 2008). Some studies demonstrated that prenatal exposure to nicotine significantly increased basal blood pressure in adult rats (Gao et al., 2008; Pausová et al., 2003) and mice (Fox et al., 2012). These effects seen on blood pressure following prenatal exposure to nicotine were sex dependent. However, there were mixed effects of nicotine on basal blood pressure. In either case, prenatal nicotine can have long-term effects on the vasculature that may increase the risk of developing hypertension. Our finding that prenatal exposure to methamphetamine disrupts vascular function in the adult offspring is consistent with these previous studies.

Prenatal exposure to methamphetamine had no effect on baseline blood pressure in either male or female adult offspring. This was consistent with the studies performed on prenatal exposure to cocaine and nicotine (Xiao, Huang, et al., 2009; Xiao et al., 2008). Whether prenatal exposure to methamphetamine has an impact on angiotensin II or norepinephrine-induced changes in blood pressure is currently unclear and will require additional experimentation.

Previous work demonstrated that PVAT mediates stretch-induced relaxation of the aorta and other blood vessels (Watts et al., 2020). In light of our data indicating that PVAT-dependent relaxation in response to acetylcholine was attenuated in adult female rats following prenatal exposure to methamphetamine, we investigated the impact of prenatal methamphetamine on stretch-induced relaxation of the aorta. In contrast to acetylcholine-induced relaxation, prenatal methamphetamine had no impact on PVAT assisted relaxation in aortas from either male or female rats. Previous work has shown that NO produced by PVAT has a role in acetylcholine-induced

relaxation (Xia et al., 2016). This is consistent with our own findings, that uncoupling of cholinergic receptors from eNOS in PVAT reduces acetylcholine-induced relaxation following prenatal methamphetamine exposure **[Fig. 4.2 A] [Table 1]**. Incubation with L-NAME completely abolished acetylcholine-induced relaxation, confirming that this response was completely NO dependent **[Fig. 4.2 E] [Table 1]**. Also, we found no difference in the protein expression of eNOS in PVAT of control or treatment groups **[Fig. 4.5]**. These results suggest that prenatal exposure to methamphetamine attenuates acetylcholine induced relaxation through a mechanism that does not alter eNOS expression. A detailed study of PVAT is required to determine the underlying mechanism.

Ang-II induced contraction was significantly greater in aortas from male rats (but not female rats) that were prenatally treated with methamphetamine regardless of the presence or absence of PVAT. This effect was blocked by L-NAME, indicating that it occurred as a result of changes in NO-dependent signaling. Unlike prenatal exposure to nicotine (Xiao et al., 2008), methamphetamine exposed offspring had no effect on the mRNA transcripts encoding Ang-II receptors namely Agtr1a, Agtr1b and Agtr2 in the aortas (devoid of PVAT). Ang-II receptors are present both in endothelium and smooth muscle of the blood vessels. In the vascular smooth muscle, after binding to AT receptors, there is increase in the calcium concentration that leads to the contraction of the blood vessel. Unlike the mechanism in the vascular smooth muscle, it is thought that the binding of Ang II to AT_1 receptors decouples eNOS functioning leading to decreased NO induced relaxation in normal blood vessels (Pueyo & Michel, 1997). These findings indicate that prenatal methamphetamine induces uncoupling of eNOS through endothelial AT1 receptors and decreases nitric oxide-dependent inhibition of angiotensin-II induced contraction **[Fig. 4.9]**.

The AT receptors are coupled to eNOS through a Gi / adenylate cyclase / protein kinase A-dependent mechanism in the endothelium of thoracic aorta (Hannan et al., 2004; Yayama et al., 2006) **[Fig. 4.9]**. In contrast to this, M1 and M3 cholinergic receptors are Gq coupled and regulate eNOS through a calcium / calmodulin-dependent mechanism in the endothelium (Boulanger et al., 1994; Schneider et al., 2003) **[Fig. 4.10]**. This differential receptor coupling mechanism is consistent with our findings and may help to explain why prenatal methamphetamine decreases angiotensin-II induced eNOS activation while not affecting acetylcholine-induced eNOS activation in the endothelium.

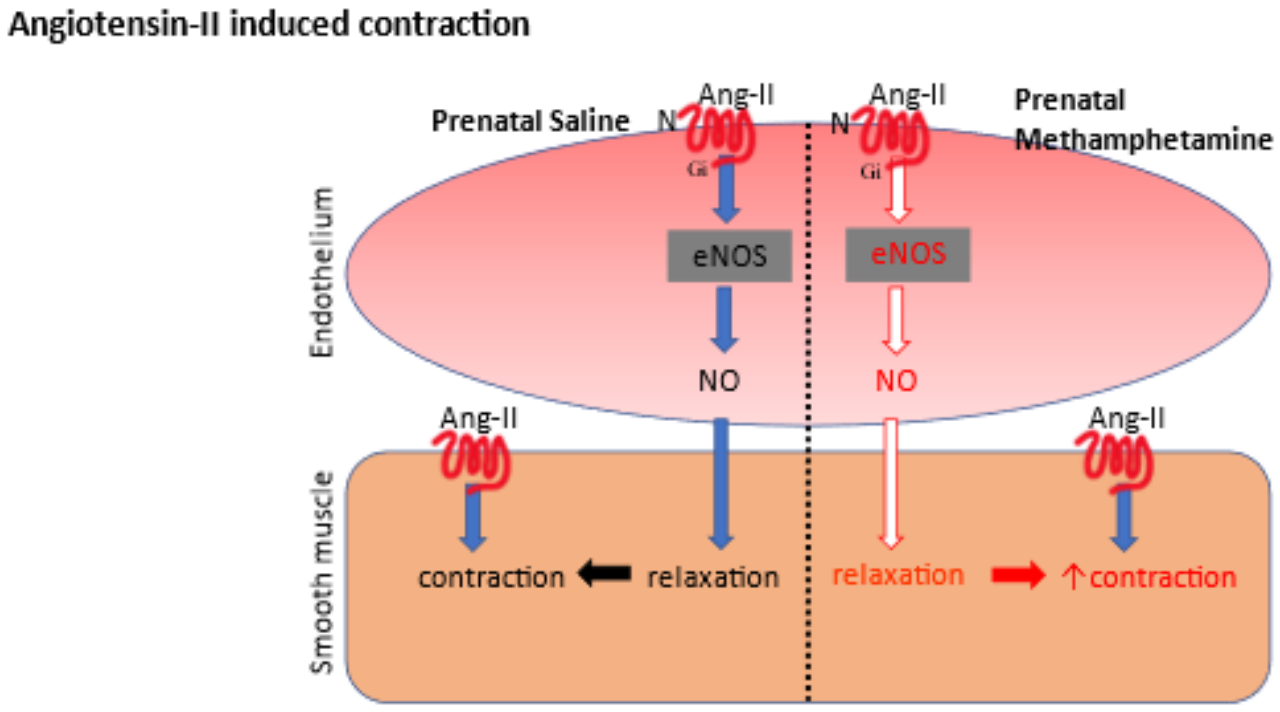

Fig. 4.9. Mechanism of action of angiotensin II on endothelium and vascular smooth muscle cell of aorta.

In vascular smooth muscle, angiotensin-II increases intracellular calcium concentration through AT receptors and leads to the contraction of aorta. But in the endothelium, AT receptors are coupled to eNOS through a Gi / adenylate cyclase / protein kinase A – dependent mechanism that leads to the release of basal amount of nitric oxide into the smooth muscle. This produces a basal level of relaxation of the aorta. However, prenatal exposure to methamphetamine leads to uncoupling of angiotensin II receptors from eNOS in the endothelium of aorta, decreasing the amount of nitric oxide produced, suppressing the relaxing effect thereby potentiating angiotensin II-induced contraction of the aorta. Ang-II: angiotensin II; eNOS: endothelial nitric oxide synthase; NO: nitric oxide. The changes indicated in red color**.**

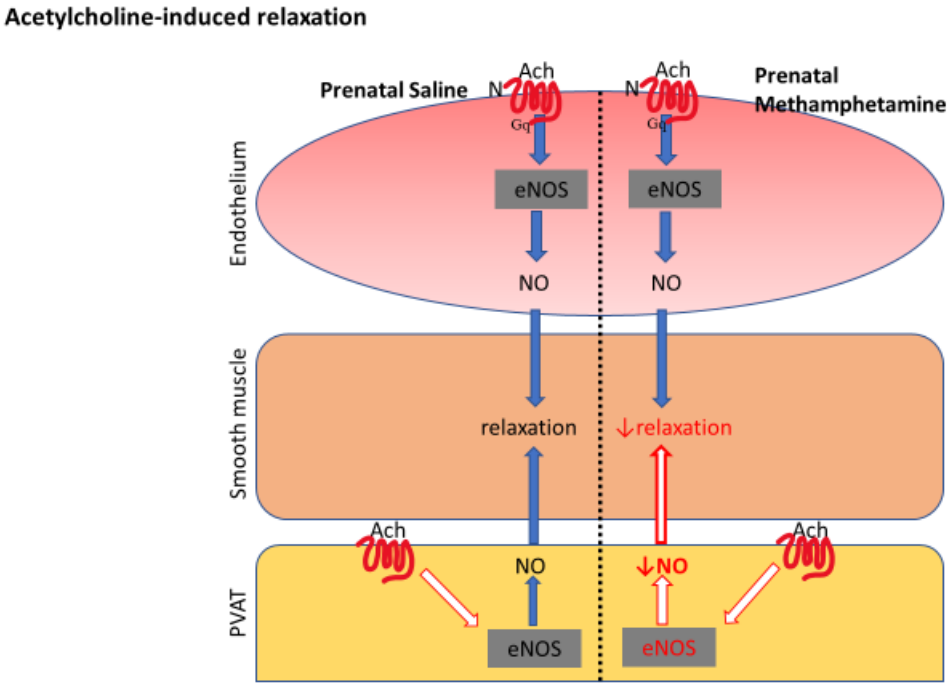

Fig. 4.10. Mechanism of action of acetylcholine on endothelium and PVAT of aorta.

Muscarinic receptors are coupled to G_q and regulate eNOS through a calcium / calmodulin-dependent mechanism in endothelium and PVAT of aorta. However, prenatal methamphetamine uncouples muscarinic acetylcholine receptor from eNOS activation in PVAT leading to decreased acetylcholine-induced relaxation of aorta.

The current study indicates that prenatal exposure to methamphetamine leads to PVAT dysfunction, disruption of NO signaling, and potentiation of angiotensin II-induced contraction of the aorta in a sex-dependent manner. Although prenatal exposure to methamphetamine affected the vascular function of aorta (large artery), there were no changes in small resistance mesenteric arteries that control blood pressure. Dysfunctional PVAT may lead to obesity, atherosclerosis, and other cardiovascular diseases (Liang et al., 2020). Among these, PVAT has a crucial role in the pathophysiological process of development of atherosclerosis in large arteries and can be a potential target for therapeutic intervention (Qi et al., 2018). However, further studies are required to determine why this vascular dysfunction occurs primarily in large arteries and only in male offspring (but not female offspring) when they are exposed to methamphetamine prenatally.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Methamphetamine is one of the most commonly abused illicit drugs. Most studies regarding the impact of prenatal methamphetamine on the offspring have focused on behavioral, neurological and locomotor changes. Earlier studies in our lab indicated that exposure to methamphetamine as an adult (Rorabaugh et al., 2017) or prenatal (Rorabaugh et al., 2016) exposure increases myocardial sensitivity to ischemic injury in adult female rats. Adult exposure to methamphetamine induces changes in myocardial gene transcription in hearts of female rats and most of these genes were related to circadian clock (Chavva et al., 2021). However, we are unaware of any studies indicating that prenatal methamphetamine alters changes in gene expression in the hearts of adult offspring. Further work is needed to determine the extent to which prenatal exposure to methamphetamine alters gene expression in the adult heart and to determine whether prenatal methamphetamine alters DNA methylation or other epigenetic mechanisms.

In continuation to the previous studies, the current study provides evidence that methamphetamine exposure during the first or second half of the gestation period worsens cardiac ischemic injury in adult female rats. Thus, if a woman decides to stop using methamphetamine around mid-term of her pregnancy, her child is still prone to cardiac ischemic injury as a young adult. The current study also proves that prenatal exposure to methamphetamine does not sensitize the heart to ischemic injury long-term if the rats were made abstinent to methamphetamine after birth. However, we do not know if it takes one-year or even less for the offspring to have normal sensitivity to I/R injury when exposed to methamphetamine prenatally.

Prenatal exposure to CNS stimulants like cocaine and nicotine disrupt the vascular function of the adult offspring in sex-dependent manner (Rorabaugh, 2021). This is consistent with our data

demonstrating that prenatal exposure to methamphetamine alters the vascular function of the aorta (but not small resistance mesenteric arteries) in a sex-dependent manner. We found that basal blood pressure was unaffected by prenatal exposure to methamphetamine. However, our data do not preclude the possibility angiotensin II -induced blood pressure (or increases in blood pressure induced by other vasoconstrictors) may be potentiated in adult animals that were prenatally exposed to methamphetamine. In fact, prior work demonstrated that adult rats that were prenatally exposed to cocaine and nicotine have normal basal blood pressure. However, the increase in blood pressure that is induced by norepinephrine is potentiated in cocaine (Xiao, Huang, et al., 2009) exposed rats and by angiotensin II in nicotine exposed rats (Xiao et al., 2008). Our data suggest that further work is needed to determine whether prenatal exposure to methamphetamine potentiates angiotensin II-induced hypertension.

Our data also indicated that prenatal exposure to methamphetamine attenuates acetylcholine-induced relaxation in aortas of male offspring only when PVAT is intact, and this effect is mediated through NO signaling pathway. Further studies are required to know if prenatal methamphetamine leads to structural and functional changes in PVAT. In addition to this, it is also required to know the difference in mechanism of acetylcholine and angiotensin II induced responses in the endothelium of aortas from prenatally methamphetamine exposed rats because dysfunction of large blood vessels can lead to atherosclerosis, arterial thrombosis and cardiovascular collapse.

Amphetamines act in different regions of the central nervous system on various neuro-anatomical regions of the brain that control different visceral functions like breathing, glycemia, sodium water balance, inflammation, and regulate blood pressure. (Ferrucci et al., 2019; Sulzer et al., 2005). Although methamphetamine exerts its effects on CNS and peripheral neurons, changes

in cardiovascular system occur through A1 / C1 neurons (located in rostral ventrolateral medullary region of the brain) which regulate various functions of cardiovascular system including the regulation of vascular tone, heart rate and blood pressure. High-dosing or repeated-dosing of amphetamines sensitizes the peripheral tissues and organs innervated by the A1 / C1 complex (Ferrucci et al., 2019). Therefore, relating our data to the general mechanism of action of amphetamines, we can summarize that methamphetamine causes CNS-mediated effects on the heart and blood vessels that is altering their function.

In conclusion, these data provide evidence that prenatal exposure to methamphetamine has a negative impact on the adult cardiovascular system and that these effects occur in a sex-dependent manner. Further studies are needed to understand the mechanism of the sex-dependent effects of prenatal methamphetamine on adult cardiovascular function.

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APPENDIX A: IRB APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

October 18, 2021

Hasitha Chavva
1675 6th Ave, Apt 2
Huntington, WV 25703

Dear Hasitha:

This letter is in response to the submitted thesis abstract entitled "*Characterization of Cardiovascular Function in Adult Offspring Following Prenatal Exposure to Methamphetamine.*" After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #719. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director