The Function of Tyramine in the Mouse Uterine Horn

by

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ABSTRACT

Pregnancy and childbirth are both natural occurring events, but still little is known about the signaling mechanisms that induce contractions. Throughout the world, premature labor occurs in 12% of all pregnancies with 36% of infant deaths resulting from preterm related causes. Even though the cause of preterm labor can vary, understanding alternative signaling pathways, which affect muscle contraction, could provide additional treatment options in stopping premature labor. The uterus is composed of smooth muscle, which is innervated, with a plexus of nerves that cover the muscle fibers. Smooth muscle can be stimulated or modulated by many sources such as neurotransmitters [i.e. dopamine], hormones [i.e. estrogen], peptides [i.e. oxytocin] and amines. This study focuses on the biogenic monoamine tyramine, which is produced in the tyrosine catecholamine biosynthesis pathway. Tyramine is known to be associated with peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose and the release of norepinephrine. This research has found tyramine, and its specific receptor TAAR1, to be localized within mouse uterus and that this monoamine can induce uterine contractions at levels similar to oxytocin.

DEDICATION

Dedicated to my father, Jacob Obayomi, my mother, Janet Obayomi, and my brother Ade Obayomi because without them supporting me one hundred percent through my graduate journey I would not be where I am today. I am extremely thankful for their constant words of wisdom, words of encouragement and for always reminding me that giving up is never an option.

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ABBREVIATIONS

DA	Dopamine
E2	Estradiol
ERα	Estrogen Receptor Alpha
OXY	Oxytocin
PBS	Phosphate Buffer Solution
TAAR1	Trace Amine Associated Receptor
TYR	Tyramine
UT	Untreated

Chapter 1. Pregnancy and the Modulators of Uterine Smooth Muscle

Introduction

Childbirth is a natural part of life and has been studied throughout time to provide adequate pre- and post-care in order to reduce injury or fatality to both moms and babies. One of the leading causes of death among newborns is due to pre-term labor. According to the World Health Organization, preterm labor is defined as babies who are born alive before thirty-seven weeks of pregnancy are completed. There are three sub-categories of preterm birth, based on gestational age. Those categories are: extremely preterm (less than twenty-eight weeks), very preterm (ranging from twenty-eight to thirty-two weeks), and moderate to later preterm (ranging from thirty-two to thirty-seven weeks). In some cases a birth must be induced early to protect the health of the mother or the fetus but more often it is the result of rupture of membranes, hemorrhage, hypertension or weakened cervix. Multiple as well as single births associated with assisted reproductive technologies are also a contributing factor to the overall increase in preterm births (Jackson et al., 2004). As technology improves, researchers continue to investigate the mechanisms, which control gestation and birth in hopes of reducing the frequency of pre-term labor.

Causes of pre-term labor

There are many risk factors that are believed to interact that cause the transition from the relaxed state of the uterus toward pre-term labor. Currently there are three accepted theories that explain the onset of labor. The first theory is progesterone withdrawal, which stems from a sheep study. According to Ligging et al., (1977), as parturition nears, the fetal-adrenal axis becomes more sensitive to adrenocorticotropic

hormone, increasing the secretion of cortisol. Fetal cortisol stimulates placental 17α-hydroxylase activity, which decreases progesterone secretion and increases estrogen production. The reversal in the estrogen/progesterone ratio results in increased prostaglandin formation, initiating a cascade of events that culminates in labor. In humans, the concentrations of serum progesterone do not decrease as labor approaches; and because progesterone antagonists such as RU486 initiates pre-term labor and progestational agents prevent pre-term labor, a decrease in the concentrations of progesterone or a decrease in the number of receptors is a reasonable mechanism for the initiation of labor. The second theory to explain the initiation of labor is oxytocin initiation. Intravenous oxytocin enhances the frequency and the intensity of uterine contractions that have already started and it was assumed that oxytocin also plays a role in initiating labor. However, the blood concentrations of oxytocin do not increase before labor, which makes it unlikely to initiate labor. The third theory is decidual activation. Although at term, decidual activation seems to be mediated at least in part by the fetal-decidual paracrine system (perhaps through localized decreases in progesterone concentration), in many cases of early preterm labor, decidual activation seems to arise in the context of intrauterine bleeding or an occult intrauterine infection (Romero et al., 2006). Pre-term labor is now believed to be a condition that is started by multiple mechanisms, including infection or inflammation, uteroplacental ischemia or hemorrhage, uterine overdistension, stress, and other immunologically mediated processes (Romero et al., 2006). However, a specific mechanism cannot be defined in most cases; therefore, causes that are linked to pre-term birth, that are not in the casual pathway, have been used to describe why pre-term labor occurs.

Mechanisms of Pregnancy and the Onset of Labor

Throughout pregnancy, the signaling mechanisms for myometrial contractility are altered first to promote relaxation and then again to promote contraction. During labor, the uterus undergoes phasic contractions, which soften the cervix, dilate the cervix and then expel the fetus. These processes are referred to as the latent, active and the second stages of labor. Each stage is correlated with increasing intrauterine pressure, however, the second stage is usually accompanied by voluntary or involuntary maternal pushing efforts. During the latent stage of labor, cervical dilation is less than four centimeters. During this stage significant and often painful contractions precede the onset of active labor. The active phase of labor is referred to as uterine contractions that are sufficient enough to dilate the cervix from four centimeters to ten centimeters. The cervix is dilated by fundal contractions that cause an outward pulling of the cervix over the intrauterine contents and the mechanical processes that are responsible for dilating the cervix are closely related to the creation of an aneurysm. In terms of creating an aneurysm, the fragile point of the sphere is the lower uterine segment, which is much thinner and generates comparatively less tension. When the intrauterine pressure increases, it results in a bulging of this area, followed by additional thinning; making this process mechanistically similar to the creation of an aneurysm. The second stage of labor involves the expulsion of the fetus and occurs while the uterus is undergoing phasic contractions. Delivery is usually assisted by maternal pushing efforts. Lastly, the uterus undergoes a tonic contraction in order to shear and expel the placenta during the third stage of labor. This tonic contraction is believed to reduce bleeding and failure of this

tonic contraction results in post-partum hemorrhage. These functional changes occur over months (increasing capacity), to days (upregulation of the contraction-associated proteins), to hours (onset of phasic contractions), to minutes (change to tonic contraction) (Young, 2007). These natural processes are controlled by physical, biochemical, and hormonal signals from not only the mother, but the fetus, membranes, and placenta as well. The end result of this rich mixture of influences leads to a robust, yet self-protective physiological system.

Key Hormones in Pregnancy

The endocrinology of human pregnancy involves endocrine and metabolic changes that result from physiological alterations at the boundary between mother and fetus (Kumar et al., 2012). Estrogen is an essential hormone during pregnancy. Pregnancy does not proceed without the guidance of estrogen. In women, estrogen is produced mainly in the ovaries; it is also produced by fat cells and the adrenal glands. At the beginning of puberty, estrogen plays a role in the development of secondary sex characteristics, such as breasts, pubic hair and armpit hair. Estrogen also helps regulate the menstrual cycle, controlling the growth of the uterine lining during the first part of the cycle. If the woman's egg is not fertilized, estrogen levels decrease sharply and menstruation begins. If the egg is fertilized, estrogen works with progesterone, another hormone, to stop ovulation during pregnancy (Bradford, 2016).

Progesterone is made first by the ovaries and then by the placenta during the second trimester. Progesterone has many roles such as making sure the placenta is functioning properly and that the uterine lining is healthy and thick. Studies have proven that progesterone stimulates the secretion of Th2 and reduces the secretion of Th1

cytokines, which is important for maintaining pregnancy (Csapo, 1973). During early pregnancy, the maternal levels of 17 α-hydroxyprogesterone increases, marking the activity of the corpus luteum. By the tenth week of gestation, 17 α-hydroxyprogesterone returns to baseline levels, indicating that the placenta has a decreased amount of 17α-hydroxylase activity. However, beginning in the thirty second week there is a second, more steady increase in this compound because the placenta utilizes fetal precursors. Progesterone also prepares and preserves the endometrium to allow implantation earlier. Studies have shown that the human corpus luteum makes a substantial amount of estradiol, however, progesterone and not estrogen is required for successful implantation (Rothchild, I. 1983).

Oxytocin is made in the hypothalamus and secreted into the bloodstream by the posterior pituitary gland. In the body, the two main functions of oxytocin are contracting the uterus during childbirth and lactation. Oxytocin stimulates uterine muscles to contract and also increases the production of prostaglandins, which increase the contractions even more. Manufactured oxytocin is sometimes given to induce labor if it has not started naturally. Manufactured oxytocin can also be used to strengthen contractions to help childbirth (Husslein, 1983).

Anatomy: The Female Reproductive System

The human female has a reproductive system located in the pelvis. The vagina is a muscular, hollow tube that extends from the vaginal opening to the uterus. Because it has muscular walls, it can expand and contract. The vagina has several functions such as being the pathway that a baby takes out of a woman's body during childbirth, and as the route for menstrual blood to leave the body from the uterus. The vagina connects with the

uterus at the cervix. The uterus contains some of the strongest muscles in the female body. These muscles are able to expand and contract to accommodate a growing fetus and then help push the baby out during labor. When a woman isn't pregnant, the uterus is only about 3 inches long and 2 inches wide. At the upper corners of the uterus, the fallopian tubes connect the uterus to the ovaries. The ovaries produce, store, and release eggs into the fallopian tubes in the process of ovulation. (Pineda et al., 2003).

The Uterus

Even though the uterus is composed of connective tissue, blood vessels and some afferent nerves, the smooth muscle cell is the dominant cell type. The majority of the uterine wall is made up of smooth muscle that is organized into bundles, which in turn are organized into fasciculata (Young, 1999). The pregnant uterus is a unique organ and is only functional for a small portion of a woman's life, if at all. It is essential that the uterus function well not only for the preservation of that organism but also for the preservation of the species. The uterus is responsive to local environment and will change function in response to different endocrine signals. The uterus is unique because it can accommodate many functional modifications needed for a normal pregnancy. The first function of the uterus occurs during the implantation stage, which is when the uterus accepts and implants the fertilized ovum. The second major function of the uterus is the capacity to become larger in order to contain the fetus and the placenta, permitting for thirty-eight weeks of growth and development. During these first two stages, there is very little hyperplasia, however, the degree of hypertrophy is significant from a seventy five gram uterus to a one thousand, three hundred gram uterus, with supplementary angiogenesis and vascular changes that are needed to supply nutrients to itself and

provide nutrients to the fetus. During fetal development it is important that the uterus not contract. The third function is that the uterus has to sense a signal from the fetus in order to prepare for labor. This stage, in general, is referred to as an up regulation of the contraction-associated proteins (Young, 2007). Recent studies from Mendelson's lab have demonstrated the signal is a surfactant protein (SP-A) and originates from the type II alveolar cells (Condon et al., 2004).

Uterine Contractions

Uterine contractions are a major part of childbirth and its function is dependent on the actions of smooth muscle. There are four main types of smooth muscle tissue which are distinguished by the type of contraction, such as whether the contraction is tonic or phasic and whether or not the muscle is excited by electrical stimulation, such as generating an action potential (Bülbring et al., 1987). The uterine muscle is very excitable and generally undergoes spontaneous rhythmic contractions that vary in frequency and amplitude. In order for a multicellular muscle to contract as an organ, the contraction of individual cells have to be coordinated by the sequential depolarization of cells. The uterus contracts as a three-dimensional system of cells and the individual responses are communicated to neighboring cells in a specific pattern. When one cell is depolarized it leads to the activation of only neighboring non-depolarized cells so that a wave of activation proceeds in a certain direction. This process is required for organized contractions and consequently the transportation of the uterine contents. However, during gestation, this coordination is much less effective than during labor, which allows gestation to be maintained (Reimer et al., 1998). Depolarization of the uterine muscle cell, when the membrane potential is made more positive relative to the resting state,

happens when an action potential is initiated through the regenerative influx of cations. In the uterine smooth muscle cells, the depolarizing action potential current is carried by calcium ions, which enter via voltage-gated calcium channels (Wray 1993). A variety of hormones, neurotransmitters, and pharmacologic agents influence the contractile state of uterine smooth muscle cells. Ultimately, the ability of any of these agents to affect response is governed by the final common pathway of calcium ion mobilization, which couples excitation to contraction. (Fuchs 1995).

Female Mouse Reproductive System: Uterine Horn

The mouse uterus has a different anatomy than that found in humans such that it is made up of two horns and a single body (corpus). The uterine horns begin at the oviducts and come together at the body of the uterus. The body of the mouse uterus is composed of a cranial portion, which contains two cavities separated by a medial septum, and a caudal portion, which leads to the cervix. The wall of the uterus consists of, from inside to outside, the endometrium, myometrium (inner circular and outer longitudinal smooth muscle layers), and adventitia (Uterus – Overview). The two uterine horns are joined caudally in a Y fashion to form the undivided uterine body and cervix. When autopsied, the length of a horn of the intact reproductive tract is approximately three times greater than the combined length of the corpus and cervix, which is about 3-5 mm. The cervix is projected about 1-2 mm into the cranial portion of the vagina. The mid-dorsal and mid-ventral outer surfaces of the cervix are fused with the adjacent inner surfaces of the vagina. Each uterine horn is attached along its length to the dorsolateral body wall by the mesometrial ligaments, which extended caudally to the dorsal wall of

the corpus (Leppi, T. J. 1964). This will serve as the birthing track for multiple mouse pups.

Reproductive Characteristics of Female Mice

Female mice reach sexual maturity at five to eight weeks and have an estrous cycle of approximately four days. The gestation period is 18.5 to 21 days, and the litter size ranges from two to more than twelve pups. Female mice have a productive breeding life of seven to eight months (Silver, 1995). These mice have reproductive states consisting of cycling, pregnant, pseudo pregnant, anestrus (seasonal non-cycling) and reproductively senescent. The first stage of the estrous cycle is called proestrus and lasts for thirteen hours, the second stage is the estrous –ovulation stage and lasts for fifteen hours. The third stage is metestrus and lasts for thirteen hours and the final stage of the estrous cycle is diestrus and lasts for fifty-six hours.

Muscle: Smooth Muscle

Smooth muscle is a major component of the uterus and is organized as an involuntary and non-striated muscle. Smooth muscle is divided into two subgroups; the single-unit and the multiunit smooth muscle. Within the unitary cells the whole sheet contracts as a syncytium (a multinucleate mass of cytoplasm that is not divided into cells). Smooth muscle can be found within the walls of blood vessels (vascular smooth muscle) such as in the aorta, small arteries, arterioles and veins. Smooth muscle is also found in the urinary bladder, uterus (uterine smooth muscle), male and female reproductive tracts and in the gastrointestinal tract. The structure and function is essentially the same in smooth muscle cells from different organs, however, the stimuli that induces the muscle differs significantly in order to perform individual effects in the

body at certain times (Mecham et al., 1995). The dense bodies and the intermediate filaments that are networked throughout the sarcoplasm, cause smooth muscle fibers to contract. A series of axon-like swellings, referred to as varicosities, from autonomic neurons form motor units through the smooth muscle (Mecham et al., 1995). The molecules myosin and actin, interact together to cause contraction, and take up a substantial portion of the volume of the cytoplasm of smooth muscle cells. Smooth muscle contains significant proteins such as: calmodulin, calponin and caldesmon. Calmodulin has a regulatory role in smooth muscle, calponin is a load bearing protein and caldesmon enhances the ability of smooth muscle to maintain tension (Aguilar et al., 2010).

Smooth Muscle Contraction

In order for uterine contraction to occur, myosin and actin filaments within the smooth muscle must slide over one another. In order for this to happen, energy is required by the hydrolysis of ATP. Myosin works as an ATPase using ATP to generate a molecular conformational change of part of the myosin and produces movement. The movement of the filaments over each other occurs when the globular heads protruding from myosin filaments attach and interact with the actin filaments to from cross-bridges. The myosin heads tilt and drag the actin filament a short distance that is about ten to twelve nanometers. The myosin heads then let go of the actin filament and then changes to move to another site on the actin filament that is a larger distance away. Unlike cardiac and skeletal muscle, smooth muscle does not contain the calcium-binding protein troponin. Contraction is started by a calcium-regulated phosphorylation of myosin, rather than a calcium-activated troponin system. Smooth muscle may either contract phasically

or tonically. Phasic contractions are defined as rapid contraction and then relaxation and tonic contractions are defined as slow and sustained contraction. Cross-bridge cycling cannot happen until the myosin heads are activated which allows for the cross-bridges to form. When the light chains are phosphorylated, they become active and contraction will occur. Myosin light-chain kinase (MLCK) phosphorylates the light chains. In order to control contraction, MLCK functions only when the muscle is stimulated to contract. Stimulation increases the intracellular concentration of calcium ions. The Calcium ions bind to calmodulin, and form a calcium-calmodulin complex. This complex binds to MLCK to activate it, allowing the chain of reactions for contraction to occur (Aguilar, 2010).

Hormones, Neurotransmitters and Molecules: Endocrinology Review

The endocrine system controls the way that the body functions. This system is a collection of glands that produces hormones that travel to all parts of the body in order to maintain the tissues and organs. Hormones go directly into the blood stream in order to control metabolism, growth and sexual development. The glands that make up the endocrine system are: the hypothalamus, pituitary gland, pineal gland, thyroid gland, parathyroid glands, adrenal glands, pancreas, thymus, testes (male) and ovaries (female) (Thyroid UK - An Overview of the Endocrine System).

HPA Axis

The HPA axis is a set of relationships and signals that occur between the hypothalamus, the pituitary gland and the adrenal glands. The 'H' in HPA stands for Hypothalamus, which is a very small part of the brain that is located centrally. The hypothalamus receives messages from all over the body and it also keeps the body

balanced by sending out messages to the nervous system via the brain. The hypothalamus sends messages from the brain to the adrenals, the pituitary and other organs, so it is generally considered to be the starting point in the HPA axis. The hypothalamus contains neurosecretory neurons, which synthesize peptides and catecholamines; these are released into the circulatory system and act as hormones. Some hypothalamic hormones are released into the systemic circulation to target distant tissues. Other hypothalamic hormones are released in the portal circulation for delivery to the anterior pituitary where they stimulate or inhibit release of the anterior pituitary hormones (HPA Axis Dysfunction). All the hypothalamic hormones are peptides except dopamine, which is a catecholamine.

The 'P' in HPA axis stands for the pituitary gland. The pituitary gland is smaller than the hypothalamus, however, it produces a large number of hormones that the body needs. The pituitary gland has two lobes, the posterior lobe and the anterior lobe. The posterior pituitary contains mostly axon terminals of the hypothalamic neurosecretory cells. These axon terminals are surrounded by the inferior hypophyseal artery capillary bed. Antidiuretic and oxytocin are the two hormones released by the posterior pituitary. The Antidiuretic hormone is responsible for osmotic homeostasis. Oxytocin mostly acts at the uterine smooth muscle and the smooth muscle in the mammary glands. There are six types of cells in the anterior pituitary, named after the primary peptide/protein hormones they produce: ACTH (adrenocorticotropin), TSH (thyroid stimulating hormone), FSH and LH (follicle stimulating and luteinizing hormone or gonadotropins), GH (growth hormone) and prolactin (lactotropin). Upon stimulation of the anterior

pituitary by hypothalamic hormones, these hormones are released and diffuse into the second portal capillary bed (Mitrovic, n.d).

The "A" in HPA axis stands for the adrenal glands. The adrenal gland consists of two endocrine glands: the adrenal medulla, which secretes catecholamines; and the adrenal cortex, which secretes steroid hormones. The adrenal medulla is in effect an enlarged and specialized sympathetic ganglion whose neuronal cell bodies do not have axons, but release catecholamines directly into the blood. Adrenal medullary secretion is under sympathetic control by way of the greater splanchnic nerve.

The HPG Axis

The female reproductive system is regulated by the HPG axis, which stands for the hypothalamic- pituitary-gonadal axis. It starts in the brain, where the hypothalamus and the pituitary are located and allows the brain to communicate with ovaries using hormones. In terms of reproduction, the two main jobs of the hypothalamus involve the gonadotropin-releasing hormone (GnRH). This hormone is responsible for initiating puberty and regulating the hormones that are involved in female reproduction. GnRH is the first hormone in the HPG pathway and once it is produced it is released into a series of capillaries in the brain, hypophyseal-portal system. These blood vessels connects the hypothalamis to the anterior pituitary making it possible for them to communicate with each other. The binding of GnRH stimulated these cells to produce luteinizing hormone (LH) and the follicle-stimulating hormone (FSH), and then these hormones are secreted into the bloodstream. Once into the bloodstream these hormones signal the ovaries to produce estrogen and inhibin both of which play essential roles in the female reproductive cycle.

In terms of the productive cycle, the preparation of the ovarian follicule is through a positive feedback loop that involves estrogen and luteinizing hormone. Once the ovarian follicule is released into the uterus, the ovary starts to produce progesterone. Progesterone has a negative effect on the positive feedback loop of estrogen and luteinizing hormones because it inhibits the hypothalamus. Progesterone is produced by the fetus if conception occurs.

Estrogen

In females, synthesis of estrogens begins in theca interna cells in the ovary, by the synthesis of androstenedione from cholesterol. Androstenedione is a substance of weak androgenic activity, which serves predominantly as a precursor for more potent androgens such as testosterone as well as estrogen. This compound crosses the basal membrane into the surrounding granulosa cells, where it is converted immediately into estrone and sub sequentially into estradiol (Yogeeswar, 2005). There are two estrogen subtypes, ER alpha and ER beta. The alpha receptor contains a region that promotes transcription activation and the beta receptor is similar to the alpha receptor however it contains a repressor domain. The three major naturally occurring estrogens in women are estrone (E1), estradiol l (E2), and estriol (E3).

Estradiol is the predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. During menopause, estrone is the predominant circulating estrogen and during pregnancy estriol is the predominant circulating estrogen in terms of serum levels (Files et al., 2011) Though estriol is the most plentiful of the three estrogens it is also the weakest, whereas estradiol is the strongest with a potency of approximately eighty times that of estriol (Files et al.,

2011). Thus, estradiol is the most important estrogen in non-pregnant females who are between the menarche and menopause stages of life. The effects that estrogen has on the uterus includes myoplasia of myometrium and cervix, increases uterine vascularity, regenerates the endometrium after menstruation and is responsible for proliferative hyperplasia of endometrium and it has a stimulant effect on the glands of endocervix and their mucus secretion.

Many of the effects of estrogens on the uterus are regulated by ER alpha, which is the main estrogen receptor in the mature organ. In the undeveloped uterus, ER alpha and ER beta are expressed at similar levels in the epithelium and stroma, and 17 beta-estradiol (E2) treatment reduces ER beta in the stroma. The transcriptional effects of E2 in the body are regulated by two different estrogen receptors, ER alpha and ER beta. A definitive role for ER alpha, stimulated by E2, was confirmed in adult female ER alpha knockout mice, where there was loss of estrogen responsiveness (Lubahn et al., 1993) as well as in mice with disruption of the estrogen-responsive ring finger protein gene (Orimo et al., 1999). Estrogen regulates target genes in a cell-specific manner which has various effects on different types of cells in the uterus. In the immature, twenty-one day-old mouse uterus, the cellular proliferation rate is very low (Li, 1994). Proliferation starts at puberty in response to cycling estrogen, although the immature uterus is fully capable of responding to E2 where estrogen can induce both epithelial and stromal cell proliferation (Martin et al., 1973). Although ER alpha is expressed in the epithelium, proliferation of epithelial cells in response to E2 is thought to be indirect through growth factors secreted by the stroma in response to estrogen. ER alpha plays an important role in differentiation by regulating target genes such as that for the progesterone receptor.

Progesterone

According to (Kin et al., 2010), progesterone is an endogenous steroid hormone involved in the menstrual cycle, pregnancy, and embryogenesis of humans. Progesterone is produced in high amounts in the ovaries, by the corpus luteum, from the beginning of puberty to menopause. Progesterone has a lot of physiological effects that are amplified when the estrogens are present (Kastner et al., 1990). Progesterone is sometimes referred to as the "hormone of pregnancy", and it has many roles that relate to the development of the fetus. Progesterone transitions the endometrium to its secretory stage to prepare the uterus for implantation (Patel et al., 2015). At the same time, progesterone has an effect on the vaginal epithelium and cervical mucus, making it thick and impenetrable to sperm (Patel et al., 2015). If pregnancy does not occur, progesterone levels will reduce, leading the female human to menstruation. If ovulation does not occur and the corpus luteum does not develop, levels of progesterone may be low, which could lead dysfunctional uterine bleeding. During implantation and gestation, progesterone appears to decrease the maternal immune response to allow for the acceptance of the pregnancy. Progesterone also reduces contractility of the uterine smooth muscle. A decrease in progesterone levels is possibly a mechanism that facilitates the onset of labor. Progesterone acts on the uterus to cause myo-hyperplasia and by increasing the strength but diminishing the frequency of uterine contraction.

Oxytocin

Oxytocin is a nine-amino acid peptide that is synthesized in hypothalamic neurons and then transported down axons of the posterior pituitary for secretion into the blood (Bannink, 2012). Oxytocin is secreted within the brain from tissues such as the ovaries

and testis and also stimulates the contraction of the uterus during labor. Studies have shown that oxytocin mediates three main effects: stimulation of milk ejection, stimulation of uterine smooth muscle contraction at birth and the establishment of maternal behavior. In terms of stimulating uterine contraction, at the end of gestation, the uterus has to contract forcefully and for a long period of time in order to deliver the fetus. During the later stages of gestation, the amount of oxytocin receptors on uterine smooth muscle cells increases. Oxytocin is released during labor when the fetus stimulates the cervix and vagina, and it enhances contraction of uterine smooth muscle to facilitate birth.

Dopamine

Dopamine is a naturally occurring catecholamine, an immediate biochemical precursor of the norepinephrine found in adrenergic neurons and it is also a neurotransmitter in the central nervous system, where it is released from dopaminergic neurons to act on specific dopamine receptors (Craig et al., 2004). The effect of dopamine on the myometrium of the uterine smooth muscle is to inhibit contractions. Research by Czerski et al, (2005), showed that the addition of dopamine to the incubation bath at a concentration of 0.26·10-4 mol/L did not cause any statistical differences in uterus motility. But a concentration of 1.3·10-4 mol/L caused a decrease in both the frequency (27%) and strength (63%), and a total lack in contractility at a concentration of 2.6·10-4 mol/L

Tyramine

Tyramine is a naturally occurring trace amine that is synthesized from the amino acid tyrosine. In invertebrates, the biogenic amine, tyramine, carries out many of the functions usually associated with noradrenaline and adrenaline in vertebrates (Evans

1980; Roeder et al. 2003; Roeder 2005). Tyramine was initially considered as only a metabolic precursor for octopamine, which cross-reacted with receptors that were specific for octopamine. However, evidence is now emerging for specific roles for tyramine in insects and nematodes, independent from those of octopamine. Thus, tyramine has been shown to cause opposite behavioral effects to octopamine in a number of insect preparations (Lange 2008). Furthermore, genetic studies have shown a role for tyramine in insect olfaction (Kutsukake et al. 2000) and on the inhibition of egg laying and the modulation of reversal behavior and the suppression of head oscillations in response to touch in *C. elegans* (Alkema et al. 2005). In humans, tyramine acts to release catecholamines made by the adrenal glands into the bloodstream. Some of the substances that can be released include dopamine, norepinephrine and epinephrine (Starke, 1974). When these hormones are in the bloodstream, systolic blood pressure and heart rate can rise. This rise in blood pressure can often be dangerous for people who take monoamine oxidase inhibitors. Since the enzyme monoamine oxidase is the mechanism the human body typically uses to rid itself of excessive amounts of tyramine, if MAOIs are taken, tyramine levels may build up, leading to increased risk of a stroke. This is why many people taking MAOIs are advised to avoid foods containing tyramine (Phillips & Arevalo, 2017).

Neurotransmitters

Each neurotransmitter is synthesized from a specific amino acid through a series of steps that require specific cofactors. Neurotransmitters are endogenous substances that act as chemical messengers by transmitting signals from a neuron to a target cell across a synapse. Neurotransmitters are generally classified into two main categories related to

their overall activity; excitatory or inhibitory. Excitatory neurotransmitters exert excitatory effects on the neuron, thereby, increasing the likelihood that the neuron will fire an action potential. Inhibitory neurotransmitters exert inhibitory effects on the neuron, thereby, decreasing the likelihood that the neuron will fire an action potential. Some neurotransmitters can exert both excitatory and inhibitory effects depending upon the type of receptors that are present. In addition to excitation or inhibition, neurotransmitters can be broadly categorized into two groups defined as small molecule neurotransmitters or peptide neurotransmitters. Many peptides that exhibit neurotransmitter activity also possess hormonal activity since some cells that produce the peptide secrete it into the blood where it then can act on distant cells. Catecholamine neurotransmitters exhibit peripheral nervous system excitatory and inhibitory effects. The excitatory effects are exerted upon smooth muscle cells of the vessels that supply blood to the skin and mucous membranes. The main effects of the catecholamines are exerted as neurotransmitters upon their stimulated release from presynaptic nerve terminals in the appropriate target organ. However, release of the catecholamines from adrenal medullary cells to the systemic circulation allows them to function as hormones as well. Regardless of their site of release, the catecholamines exert their effects by binding to receptors of the G-protein coupled receptor (GPCR) family. The catecholamines are also known as adrenergic neurotransmitters and the neurons that secrete them are referred to as adrenergic neurons. The adrenergic receptors are all members of the GPCR family. There are two distinct types of adrenergic receptor identified as the α (alpha) and β (beta) receptors. The α_1 receptors induce contraction of smooth muscle in the uterus (Brief Overview of Human Nervous System).

Blood Brain Barrier

The blood-brain barrier protects the neural tissue from changes in blood composition and toxins. Elsewhere in the body, the extracellular concentrations of hormones, amino acids and potassium influence numerous changes, especially after meals, exercise or stressful situations. Since many of these molecules control neuronal excitability, a similar change in the composition of interstitial fluid in the central nervous system can lead to uncontrolled brain activity. The endothelial cells forming the blood-brain barrier are highly specialized to allow precise control over the substances that enter or leave the brain (Blood Brain Barrier and Cerebral Metabolism). In terms of pregnancy, there is controversy regarding whether peripherally administered oxytocin crosses the blood-brain barrier. In theory, oxytocin is unable to cross the blood brain barrier because of its large size and because it is hydrophilic, however, some animal studies have reported low levels of oxytocin found in the brain following administration in the blood (Ermisch et al., 1985). Additionally, there are some electrophysiology-based animal studies that indicate that maternal oxytocin plays a neuroprotective role within the fetal brain during the birth process. This would mean that it would have to cross both the placental barrier and fetal blood-brain barrier (Khazipov, 2008). Findings from these studies suggest that maternal oxytocin inhibits certain excitatory fetal neurons from firing thereby protecting them during the birth process.

Investigation of Tyramine

Biogenic amines (BAs) are defined as low molecular weight organic bases with biological activity. They are formed and degraded as part normal metabolism within plants and animals and perform important physiological functions (Ladero, 2010, Lee et

al., 1991, Nielsen et al., 1971, Toda et al., 1978). Tyramine is a biogenic amine that is derived from phenylalanine, which occurs in the body in trace amounts. Phenylalanine is an essential amino acid that is converted to tyrosine primarily in the liver by phenylalanine hydroxylase. Blood borne tyrosine, derived from dietary proteins and from phenylalanine metabolism, enters the brain by a low affinity amino acid transport system. Tyrosine in brain extracellular fluid is taken up into catecholamine neurons by high and low affinity amino acid transporters. The relative circulating levels of tyrosine and phenylalanine can affect central catecholamine metabolism, as these amino acids compete for transport into the brain, and for transport into the neuron (Berry et al., 1996). After the conversion to tyrosine, it is then metabolized to catechol derivatives, which may play important roles as neurotransmitters. The route of formation of the catecholamines is from the decarboxylation of tyrosine into tyramine, and then conversion to dopamine (Fig. 2.).

Tyramine can participate in cardiovascular regulations through its indirect sympathomimetic effects and interactions with the metabolism of catecholamines. The intake of food rich in tyramine by patients receiving MAO inhibitors leads to hypertensive crisis (Horwitz et al., 1964). Tyramine is detected in all types of cheeses but is in highest concentration in samples of Camembert and Brie cheeses. The "cheese effect" is the hypertensive crisis induced by dietary tyramine with monoamine oxidase inhibitors (MAOIs) drugs. Tyramine is a powerful agent and has 1/20-1/50 the potency of adrenaline in the production of a temporary hypertension. Tyramine is the toxic agent in cheese for patients treated with potent monoamineoxidase inhibitors, such as tranylcypromine. Normally this amine is rapidly degraded to its non-toxic derivative, p-

hydroxyphenylacetic acid, but in the presence of the enzyme inhibitor it persists in body fluids for a consider-able period of time. In humans, intravenous or subcutaneous injection of 20-80 mg of tyramine will cause a marked rise of systolic blood-pressure, slowing of the heart, with increased cardiac output. The effects begin within 10-12 minutes after subcutaneous injection, and last 5-35 minutes. After MAO inhibition, tyramine will displace catecholamines from synaptic vesicles, increasing their release and resulting in acute hypertension (Asatoor et al., 1963).

Concluding Summary

The role of tyramine in the mammalian reproductive cycle is unknown but preliminary research conducted in this study suggests that it has an important role. The presence of tyramine in mammalian tissues has been established beyond controversy. As a phenylalanine derivative, tyramine is structurally related to catecholamines and to therapeutically active medications. Tyramine has some but not all of the properties necessary to be considered a neurotransmitter. Its mechanism of action is mainly related to its ability to release catecholamines. In this study, tyramine is being investigated for its role in causing contractions within the mouse uterus. These studies have identified that the primary tyramine receptor, TAAR1 (trace amine-associated receptor 1), is present within the mouse uterus of pseudo-pregnant mice which increases upon contraction of the uterine muscle. The data presented in this thesis illustrates the role of tyramine within the mouse uterus and by understanding its source of modulation, inhibiting the production of tyramine may be a possible mechanism to help control pre-mature labor.

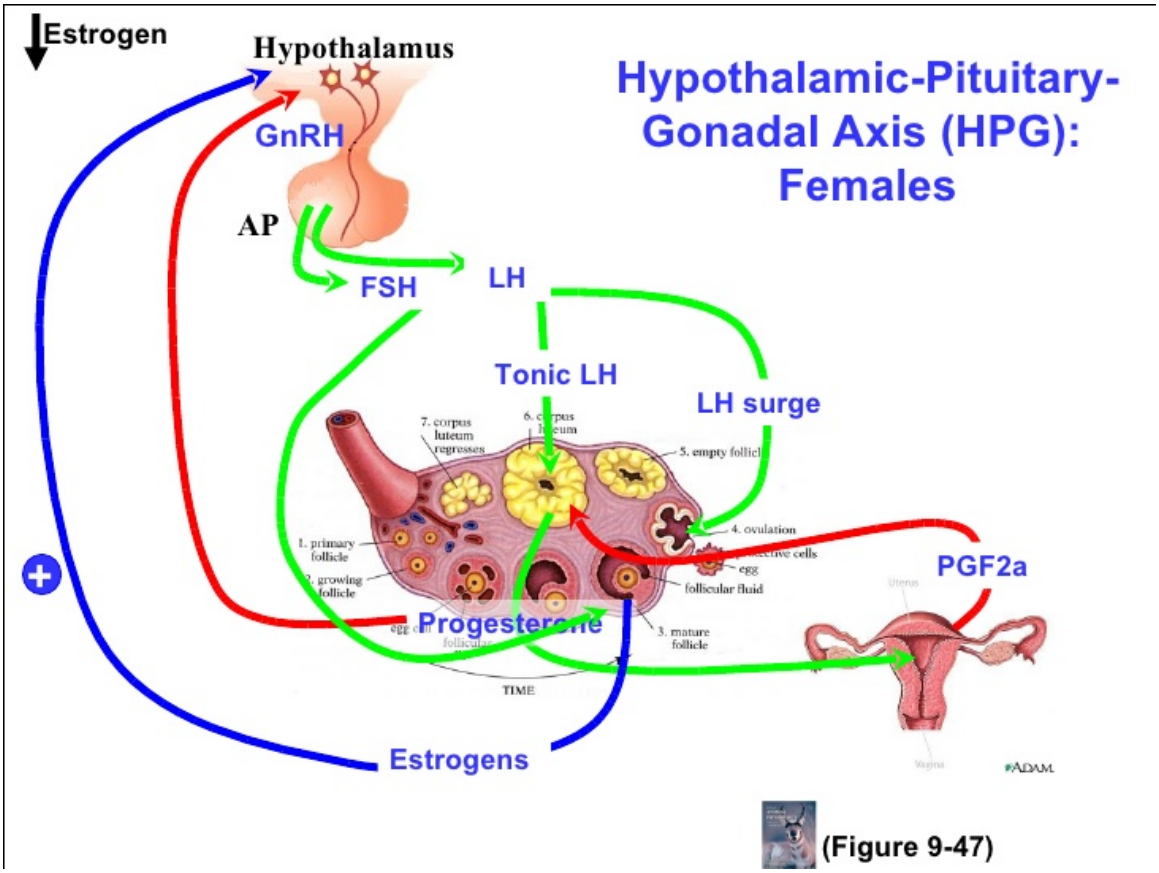

Fig. 1. Diagram of the HPG Axis.

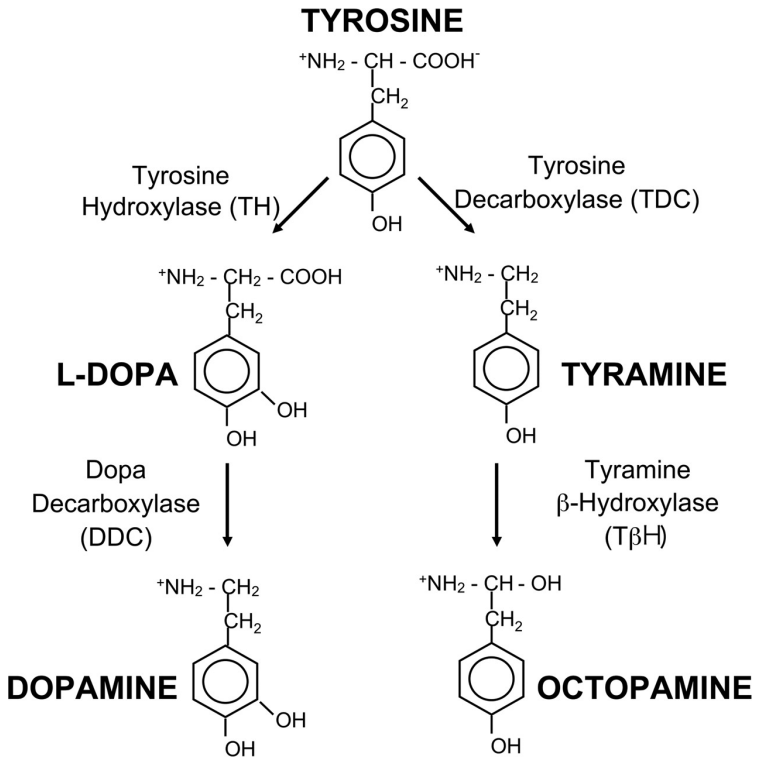

Fig. 2. Biochemical synthesis of Tyramine from Tyrosine.

Chapter 2. Localization of Tyramine in the Mouse Uterine Horn

Abstract

Pregnancy and the birthing process are natural events but still little is known about the signaling mechanism(s) that induce contractions. Globally, premature labor occurs in 12% of all pregnancies resulting in 15 million babies born preterm. Even though the cause of preterm labor can vary, understanding the signaling pathway that regulates muscle contraction could provide additional treatment options to stop premature labor. The uterus is composed of smooth muscle and in conjunction with the associated nerve fibers, forms a plexus which covers the muscle fibers. The plexus has swollen areas called varicosities that contain neurotransmitters. Within the uterine tissue, the smooth muscle receives opposing inputs from the sympathetic and parasympathetic parts of the ANS. Smooth muscle can be stimulated or modulated by many sources such as neurotransmitters (i.e. norepinephrine), hormones (i.e. epinephrine) and chemicals (i.e. nitrous oxide). This study researches an alternative modulator of smooth muscle activity, a monoamine produced in the catecholamine biosynthesis pathway called tyramine. During catecholamine biosynthesis, dopamine, tyramine, octopamine, and norepinephrine are all derived from the tyrosine precursor (Fig. 2.). Tyramine is known to be associated with peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose and release of norepinephrine. This research has found tyramine and its specific receptor TAAR1 to be localized at uterus in relation to muscular contraction and has a frequency and amplitude similar to that of oxytocin.

Introduction

The female reproductive tract is made up of a series of embryonically related yet specialized organs that coordinate sexual activity, conception, fetal growth and parturition. Many of these functions depend on the coordinated contraction of smooth muscle. Motility along the length of the reproductive tract is synchronized by a combination of phasic contractile activity and nervous control, which are heavily influenced by hormones and other modulators such as peptides and biogenic amines (Garvina et al., 2014).

The growth and development of the female reproductive tract are regulated in part by estrogen and progesterone (Pillai et al. 1999, Yan et al. 2008). The physiological effects of estrogen and progesterone are mediated by interaction of the hormone with specific intracellular estrogen receptors (ER) and progesterone (PR) receptors (Meikle et al. 2001). Throughout pregnancy, Progesterone is important because it prevents contractions by promoting smooth muscle relaxation. Estriol is a form of estrogen that is also predominant during childbirth. As estriol increases, it prevents the synthesis of progesterone and prepares the smooth muscles of the uterus for labor. Estriol and other estrogens increase the sensitivity of smooth muscles in the uterine wall to the hormones that will stimulate uterine contractions. Estrogen, or E2, increases the number of uterine oxytocin receptors in late pregnancy, preparing the uterus for contractions associated with labor. Oxytocin is the strongest stimulator of uterine contractions while dopamine will decrease uterine contractions in a dose dependent manner. These are all know modulators of uterine smooth muscle contraction but this project focused on a newly identified modulator, tyramine.

The first aim of this research project was to identify if tyramine exists in the mammalian mouse uterine horn. It was hypothesized that if tyramine was present in the uterine horn, then pseudo-pregnant female mice at reproductive age would exhibit localization of tyramine and its specific Trace amine-associated receptor 1 (TAAR1) in their uterine smooth muscle by immunohistochemistry with fluorescence and colorimetric 3,3′-Diaminobenzidine (DAB) microscopy, using fixed whole or sectioned histological sections. Results confirmed that tyramine and TAAR1 are not only present in pseudo-pregnant uterine tissue but were specifically localized in non-contracted regions preceding contraction.

Materials and Methods

Animals

Use of mice in this study was approved by Arizona State University's Institute of Animal Use and Care Committee (IACUC) under the 15-1388T protocol. Wild type (C57BL/6, Jackson Lab) female mice, between the ages of 6 to 8 weeks, were placed in cages which contained male mouse urine for 24 hours in order to stimulate pseudo-pregnancy. After 24 hours, the female mice were deeply anesthetized with chloroform, euthanized by cervical dislocation and the uterine horn was removed and placed into formaldehyde made 4% with phosphate buffered saline (PBS). Each experiment was repeated three times with three different mice.

Immunohistochemistry without Stimulants

A protocol was established to isolate the uterine horn from 6-8 week old female wild type C57/BL/6 mice that were made pseudo-pregnant by exposure to male mouse urine over a 24hr period (Fig. 4.). The tissue was collected and 0.5cm sections that had

been processed and stabilized through fixative and permeabilization buffer were immunolabeled with antibodies to p-Tyramine and TAAR1. Imaging was performed using whole tissue sections and a dipping objective lens so the periphery of the uterine horn could be observed for specific localization of signaling modulators, their receptors or the muscle confirmation.

Mouse uterine horns were collected from pseudo-pregnant and non-pseudo pregnant wild type C57BL/6 mice (aged 6-8 weeks). Prior to dissection, all mice were deeply anesthetized with chloroform and then euthanized by cervical dislocation. Each uterine horn was cut into 2 pieces and placed into formaldehyde made 2% with dilution using 1x phosphate buffered saline (PBS) overnight in the refrigerator. The following day the fixative was removed and replaced with permeabilizing solution (2% formaldehyde, 1% Tween-20 in 1x PBS) and incubated overnight in the refrigerator. Following the fixation, tissues were washed for three times for five minutes in PBS containing 1% BSA. The tissues were then incubated overnight under gentle agitation with either mouse anti-p-tyramine (1:1000; Millipore), rabbit anti-TAAR 1 (1:000; ThermoFisher Scientific), mouse anti-rabbit-ER alpha (1:000; Abcam), or anti-chicken-tyrosine hydroxylase antibody (1:500; Aves). Following incubation in primary antibody, the tissues were washed three times for five minutes each in 1x PBS containing 1% BSA. The tissues were next incubated overnight in either Alexa 488 (1:1000; ThermoFisher Scientific) anti-mouse, -rabbit or -chicken secondary antibody as well as phalloidin conjugated to Alexa 568 (1:500 ThermoFisher Scientific). Following incubation in secondary antibody, the tissues were washed three times for five minutes each in 1x PBS. Lastly, the tissues were incubated for 15 minutes under gentle agitation with DAPI DNA stain (1:1000,

ThermoFisher Scientific). The tissues were washed for five minutes in 1x PBS and then imaged with a 40x, 0.8NA dipping lens on the Leica SP5 confocal microscope housed in the WM Keck Bioimaging Laboratory at Arizona State University.

Immunohistochemistry with Stimulants

In order to evaluate the effect of various smooth muscle modulators, tissue samples were additionally collected as described above and then placed in heated and oxygenated ACSF (artificial cerebral spinal fluid). Each individual piece was incubated with either 100 nM tyramine, 10 nM dopamine, 10 nM estradiol, or 10 nM oxytocin for five minutes and then placed in 2% formaldehyde overnight at 4^0C. The formaldehyde was replaced with permeabilizing solution (2% formaldehyde, 1% Tween-20 in 1x PBS) and incubated overnight at 4^0C. The tissues were then washed for three times and followed the IHC procedure as described above.

Paraffin Embedded Tissue Histology

Mouse uterine horns were collected from pseudo pregnant and non-pseudo pregnant wild type C57BL/6 mice (aged 6-8 weeks) as described above and fixed in 2% formaldehyde overnight at 4^0C. The fixative was removed and the tissue was rinsed three times in 1x PBS followed by a standard sequential dehydration in increasing concentrations of ethanol: 50%, 70%, 80%, 90%, 95%, 100% , followed by 100% toluene, 100% toluene: paraplast (1:1 ratio), and 100% paraplast. The tissues were next placed in an embedding mold and allowed to solidify. Tissues were cut into10 micrometer transverse sections using the Leica RM1950 microtome and placed on charged slides. The slides were then cleared using xylene and then sequentially rehydrated through a series of ethanol baths as shown in Fig. 3. After step 9, the tissues

were incubated in either the rabbit-anti-TAAR 1 (1:000; ThermoFisher Scientific) or mouse-anti-ER alpha antibody (1:000; Abcam) overnight in a humidified chamber. Following incubation, the slides were washed three times for five minutes each in 1x PBS containing 1% BSA. The slides were incubated in either anti-mouse or anti-rabbit HRP (horseradish peroxidase 1:500, Pierce) overnight. Following incubation, the slides were washed three times for five minutes each in 1x PBS. The slides were next incubated with DAB (3,3′-Diaminobenzidine, VectorLabs) for ten minutes. After washing with Barnstead water, the slides were counterstained with hematoxylin, sequentially dehydrated through a series of increasing ethanol baths, followed by xylene and mounted with permount. The slides were imaged on the ThermoFisher Scientific Evos FL auto live cell imaging system in the WM Keck Bioimaging Laboratory at ASU.

Frozen Tissue Sections

Uterine horns were collected, as described above, from pseudo-pregnant and non-pseudo pregnant wild type C57BL/6 mice (aged 6-8 weeks). The tissues were fixed in 4% formaldehyde overnight at 4^0C. The next day they were incubated in 30% sucrose overnight at 4^0C. Then the tissues were placed into individual molds containing Tissue-Tek O.C.T. (VWR), quickly frozen in 2-proanol chilled in a liquid nitrogen container and then stored in a -80^0C freezer. The tissues were sectioned with a Leica CM1950 cryostat and labeled with anti-TAAR1 and anti-ER alpha followed by anti-mouse and anti-rabbit Alexa 488 antibodies as described above.

Results

Multiple experiments were conducted to confirm the presence of tyramine and its specific receptor TAAR1 in the mouse uterus. These experiments were performed using

wild type C57BL/6 mice that were induced to exhibit pseudo-pregnancy. Mice can become pseudo-pregnant after mating with a vasectomized male or being exposed to male urine (Fig. 4.). From an endocrinology point of view, pregnancy and pseudo-pregnancy are identical such that there is an increase in prolactin and the corpora lutea becomes active and begins to secrete progesterone while the uterus starts undergoing changes that would normally prepare it for implantation (Dewar 1957).

The first aim of this project was to confirm that there was localization of p-tyramine and its specific receptor TAAR1 in mouse uterine tissue. Confocal image results showed a specific localization of p-tyramine around areas of uncontracted muscle (Fig. 5a-c.) The muscle was labeled with phalloidin that was conjugated to Alexa 568 so that the muscle could not only be easily identified but would show whether the tissue was contracted. This image brought attention to whether the role of p-tyramine was either preceeding or following a contraction. Similar immunolabeling was applied using the antibody to TAAR1 which also had increased localization at the interface of contracted and non-contracted muscle (Fig5 d-f.). Colocalization was also applied to the tissue to observe the pattern of localization between p-tyramine and TAAR1. These images showed clear colocalization between tyramine and its receptor TAAR1 (Fig. 5g-i.). The figure inset at the top left of figure A is the secondary antibody control to ensure that there was not any non specifc binding of the primary antibody.

Once it was established the both tyramine and TAAR1 were localized within the mouse uterine tissue it was important to observe any changes that may occur in localization as a result of different stimulation. Using pseudo-pregnant uterine muscle, tissue samples were collected and placed in an oxygenated solution of heated artificial

cerebral spinal fluid and then treated for five minutes with either tyramine, dopamine or oxytocin. It was hypothesized that the Tissues that were not treated showed low levels of tyramine but when treated with either tyramine, dopamine or oxytocin, there was heightened localization, specically in areas that showed little contraction (Fig. 6.). Under closer examination of the oxytocin treated sample a unique pattern emerged where the muscle appeared very contracted and showing signs of muscle damage. In these regions there was a decreased localization of p-tyramine suggesting that oxytocin was sufficient to generate the contraction without the use of tyramine (Fig. 6.).

Previous studies have shown that dopamine will modulate smooth muscle and cause relaxation of the muscle fibers. The image data shown in Fig. 6. showed an increase in p-tyramine concentration when tissue was treated with dopamine which would suggest that a potential role for tyramine is to generate contraction if dopamine induces relaxation. This would explain why there was also an increase of p-tyramine in dopamine treated tissues, the tissue may have a feedback mechanism utilizing tyramine to alternates signaling between a state of contraction and relaxation. To test this idea further, tissues were again situmated with the modulators; p-tyramine, dopamine or oxytocin for five minutes and then immunolabeled with an antibody to tyrosine hydroxylase. This enzyme is a necessary step in the biochemical conversion of tyrosine to dopamine (Fig. 2.). Confocal images showed low levels of tyrosine hydroxylase in untreated tissues but in tissues stiulated with tyramine or oxytocin there was heightend localization and moderate localization in tissue treated with dopamine (Fig. 6.). In addition to labeling with tyrosine hydroxylase, the tissue was also labeled with the antibody to p-tyramine.

There was clear co-localization of p-tyramine with the tyrosine hydroxylase which supports the idea that tyramine and dopamine may act as antagonists of one another.

Imaging using whole tissue sections was very useful in observing the localization and state of muscle contraction of the uterus as an intact unit but there were still questions about where tyramine or TAAR1 were localized throughout the uterine horn. To address these questions, tissue was again collected and processed using either cryo-sectioned or paraffin embedded histological sections. Cryo-sectioned tissue was immunolabeled with antibodies to estrogen receptor alpha (ERα) or TAAR1. The tissue was labeled with ERα because many of the effects of estrogens on the uterus are mediated by ERα, the predominant ER in the mature uterus, it was important to compare the localization patterns between ERα and TAAR1. Confocal images showed general localization of both throughout the uterine tissue in both pseudo- and non-pseudo pregnant mice (Fig. 7.). Previous research showing estrogen receptor localization in reproductive tissue has been more commonly conducted using paraffin embedded tissue to obtain more precise localization and resolution that is lost due to light scatter in fluorescence images. Uterine tissue was processed initially to observe the morphology of the uterine horn cross section using hematoxylin and eosin staining (Fig. 8a-e.). This was also combined with immunostaining using anti-p-tyramine and 3,3′ -Diaminobenzidine to produce the brown colorimetric counterstain (Fig. 8.). Because tyramine is an amine, it does not produce clean localization within the cells so tissue was again processed as paraffin embedded sections and then immunolabeled with either antibody to ERα or TARR1 (Fig. 10.). Tissues labeled with ERα showed specific localization within cells along the cells of the lumen and within many cells throughout the peritoneum. In tissues that were labeled

with antibody to TAAR1, there was increased localization within cells along the lumen and within the peritoneum as compared to the tissues labeled with ERα. This data suggests that in pseudo-pregnant mouse uterine tissue that there may higher levels of tyramine earlier in pregnancy as compared to estradiol (E2) where it is known that levels of E2 will increase as pregnancy progresses**.**

Discussion and Conclusions

The first aim of this research project was to identify whether or not tyramine exists in the mammalian mouse uterine horn. It was hypothesized that, if tyramine was present in the uterine horn, then pseudo-pregnant female mice at reproductive age would exhibit localization of tyramine and its specific trace amine-associated receptor 1 (TAAR1) in their uterine smooth muscle. This was visualized by immunohistochemistry with fluorescence and colorimetric 3,3′-Diaminobenzidine (DAB) microscopy, using fixed whole or sectioned histological sections to confirm its presence within the uterine horn. The data collected from this study confirmed that tyramine and TAAR1 are not only present in pseudo-pregnant uterine tissue, but they appear to be specifically localized in non-contracted regions preceding contraction.

TAAR1 is a G-protein coupled receptor preferentially activated by the trace amine tyramine. It has shown to be distributed throughout the brain, mainly associated with areas containing monoaminergic cell bodies and projections, including the limbic areas and the prefrontal cortex. Within the brain, the signal transduction path through TAAR 1 occurs through coupling of the G protein receptor with subsequent activation of adenylate cyclase, generation of cAMP, and activation of protein kinase A and protein kinase C. These enzymes interact with transporters such as the dopamine transporter

(DAT) increasing uptake (Berry, 2016). TAAR 1 has also been found to be intracellular, possibly associated with unknown intracellular membranes. These mechanisms explain the opposing affects seen between tyramine and dopamine stimulation (Fig. 5,6.) and why TAAR1 was found localized within cells in the histological sections (Fig. 10.).

The data presented demonstrates that various modulators affect the way that tyramine is localized. In Figure 6, tyramine was differentially localized after the tissue was treated with physiological amounts of either tyramine (100nM), dopamine (10nM) and oxytocin (10nM). Previous studies have shown that tyramine has physiological effects on peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose, and the release of norepinephrine (Ladero, 2010). Dopamine has a relaxant effect on tissue and oxytocin promotes contractions that are strong enough to expel the fetus during birth. When the uterine horn was treated with tyramine, the tissue became more tight and organized; indicating that contractions were occurring and labeling the tissue with the p-tyramine antibody showed that the intensity of the tyramine signal increases significantly, especially in areas that directly precede contraction. The actin filaments within the muscle fibers were visualized using the toxin phalloidin, which was conjugated to Alexa 568. Using phalloidin it was possible to observe the difference in muscle fiber structure such that if they were more separated and are not as tightly organized, the tissue was in a more relaxed state or non-contracted. After the dopamine treatment, the muscle appears more relaxed and the amount of p-tyramine localization increased suggesting that its increase was to offset the muscle relaxation generated by the dopamine. When labeled with the p-tyramine antibody, the tyramine signal is almost non-existent in the overly contracted areas and the signal starts to become stronger in the area

that is not as contracted (Fig. 6.) which further suggests it may have a role in muscle contraction. This is further supported when observing the co-localization of p-tyramine and tyrosine hydroxylase (TH). Tyrosine hydroxylase is the enzyme that converts tyrosine to L-DOPA (the precursor for dopamine). It was observed that tyrosine hydroxylase would increase in concentration in response to increased tyramine localization (Fig. 7.) seemingly to counter its effect. Another key observation is that in dopamine treated tissue, the p-tyramine and the tyrosine hydroxylase are localized to the same area. Alternatively, in tissue that was treated with oxytocin, the localization of the p-tyramine decreases yet the localization of tyrosine hydroxylase significantly increases trying to counteract the extreme contractile effect that the oxytocin had on the tissue. During normal pregnancy, the levels of oxytocin typically stay low and do not dramatically increase until the time of delivery when the cervix has reached 8-10 cm dilation in humans (Fig. 11.).

The data from this research indicates that tyramine and its receptor TAAR1 are localized within the mouse uterine tissue and suggests that it may have a role in muscle contraction. The next aim of this project is to identify the function tyramine has in the mouse uterus, which will require a physiological approach. Chapter 3 discusses the methods developed to study mouse uterine tissue, the observations made from these experiments and the future directions of this study.

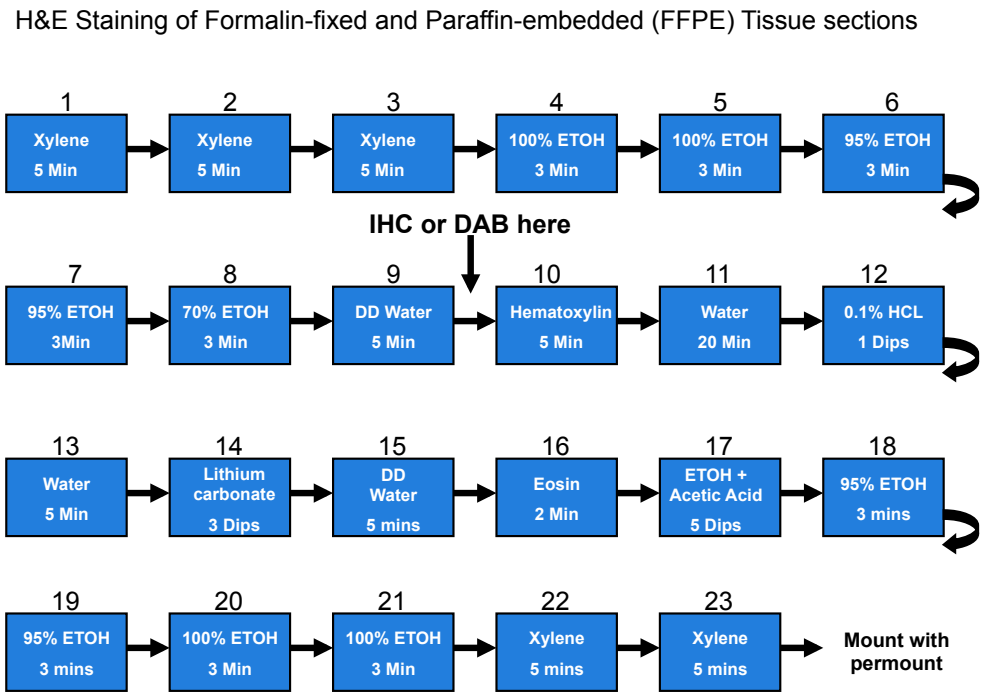

Fig. 3. Hematoxylin and Eosin staining protocol. The steps used to clear and rehydrate paraffin embedded tissue sections. Also included are the steps to add immunohistochemistry and to dehydrate and mount the slides in permount resin.

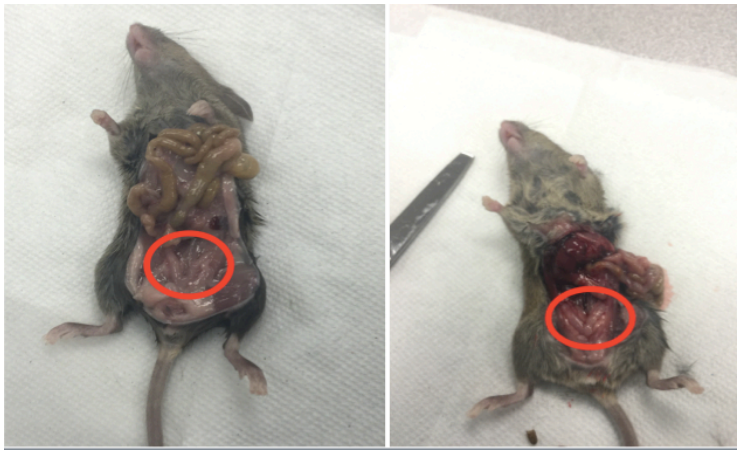

Fig. 4. Non-pseudo pregnant vs. pseudo pregnant uterine mouse horn. The mouse on the left is not pseudo-pregnant and the uterine horn shows no swelling or enriched blood flow unlike the mouse on the right.

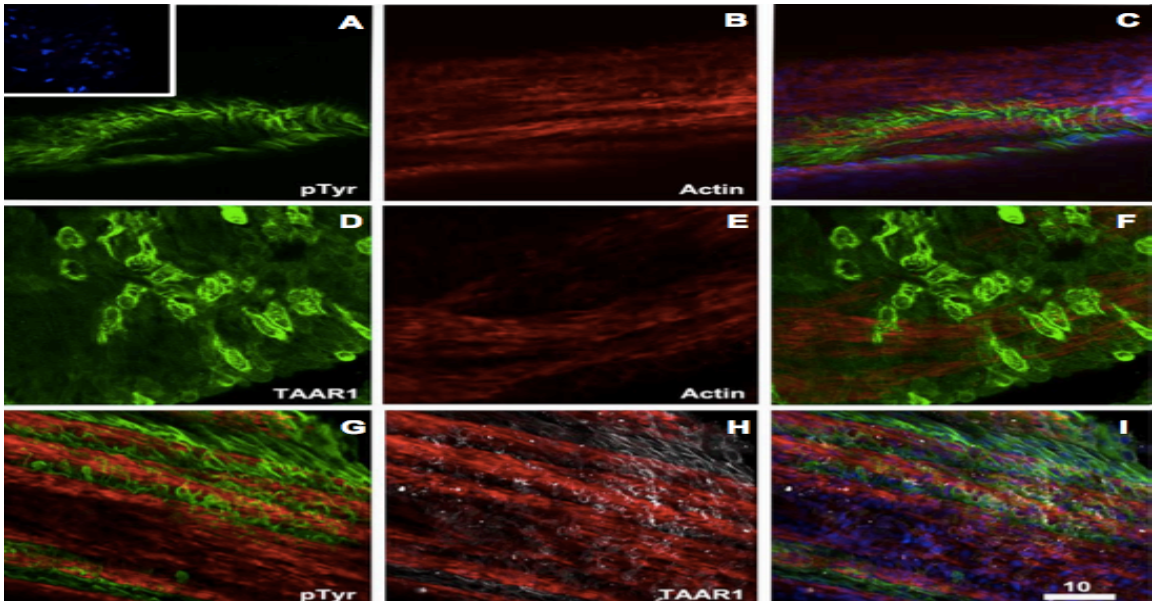

Fig. 5. Localization of p-Tyramine and TAAR1 in intact mouse uterine horn. Confocal Images of Mouse Uterus Images show the localization of anti-pTyramine [A], phalloidin [B], and both p-Tyr and phalloidin [C] in untreated mouse uterine tissue. Figures D – F show the localization of anti-TAAR1 [D], phalloidin [E] and both TAAR1 and phalloidin [F]. In figures G-I actin is labeled with phalloidin [red] and co-localized with either p-Tyr [G] or TAAR1 [H]. Figure I shows localization of p-Tyr, TAAR1 and phalloidin. Blue is DAPI staining of DNA. Inset on fig. A. is a second antibody control.

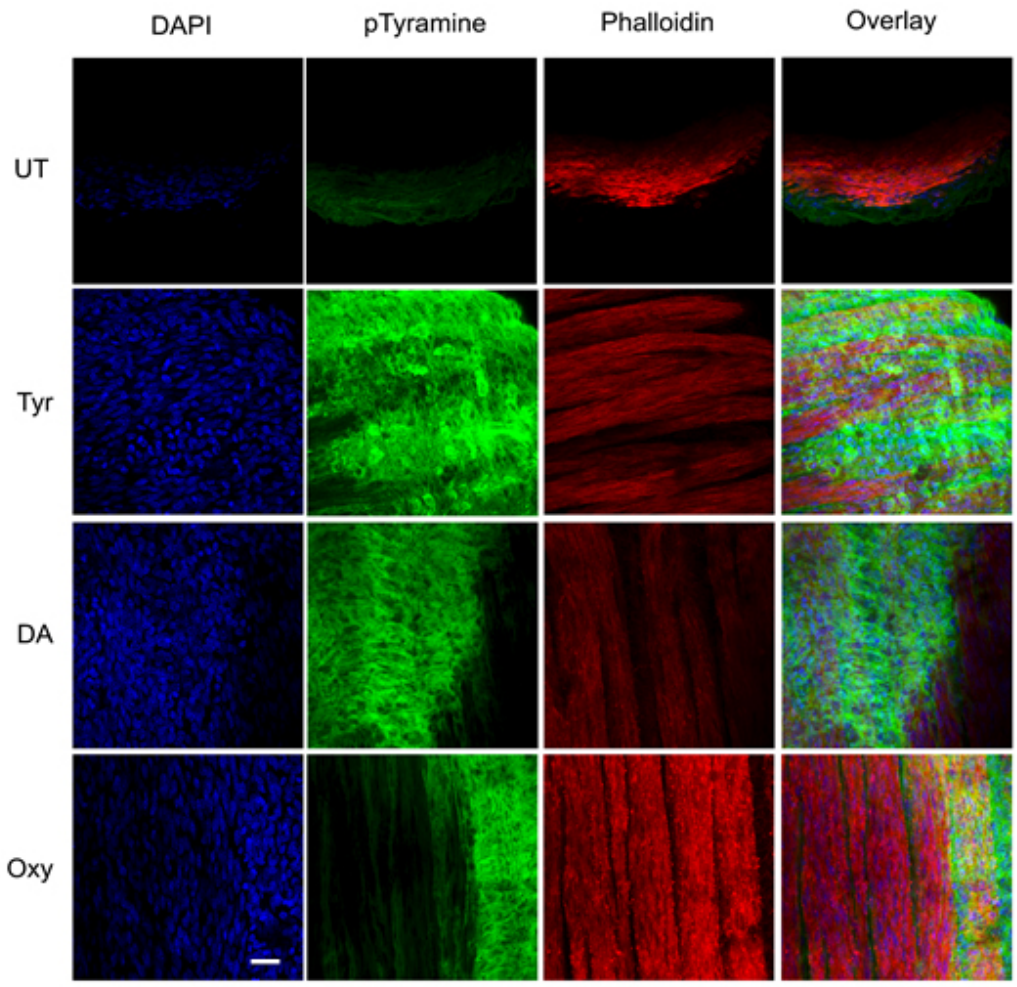

Fig. 6. Tyramine localization on un-stimulated and stimulated mouse uterine tissue. Confocal images depict the localization of DNA [blue], p-Tyramine [green], actin [red] and combined overlay of either untreated or uterine tissue stimulated with tyramine, dopamine or oxytocin. Scale bar is 10μm.

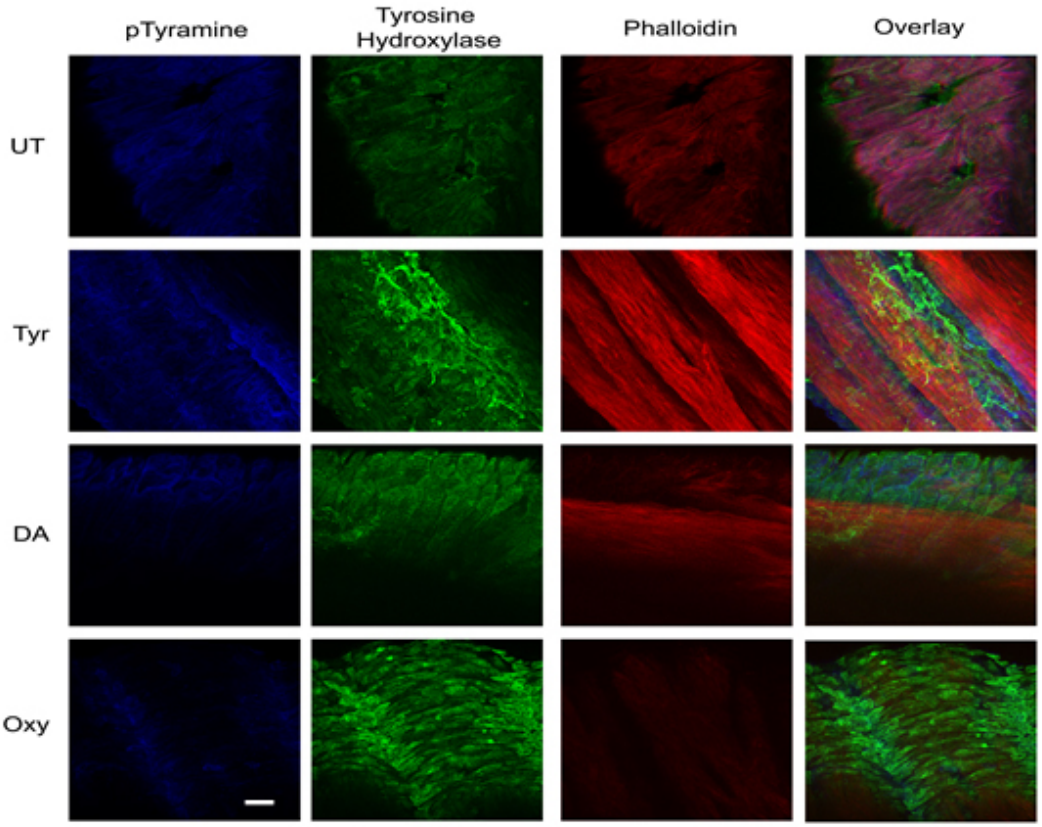

Fig. 7. Co-localization of Tyrosine Hydroxylase and p-Tyramine in mouse uterine tissue. Confocal images depict the localization of p-Tyramine [blue], Tyrosine Hydroxylase [green], actin [red] and combined overlay of either untreated or uterine tissue stimulated with tyramine, dopamine or oxytocin. Image bar is 10 μm.

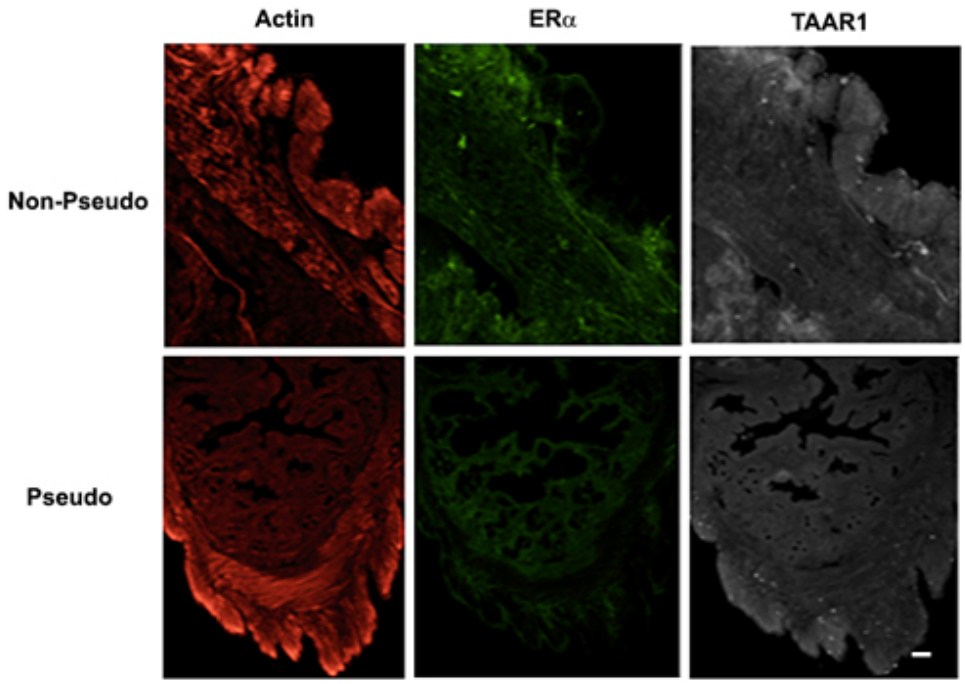

Fig. 8. Frozen sections and the co-localization of TAAR1 and ERα on pseudo and non-pseudo pregnant mouse uterine muscle. Confocal images depict the localization of Actin [red], ERα [green], and TAAR 1 [grey]. Scale bar is 20 μm.

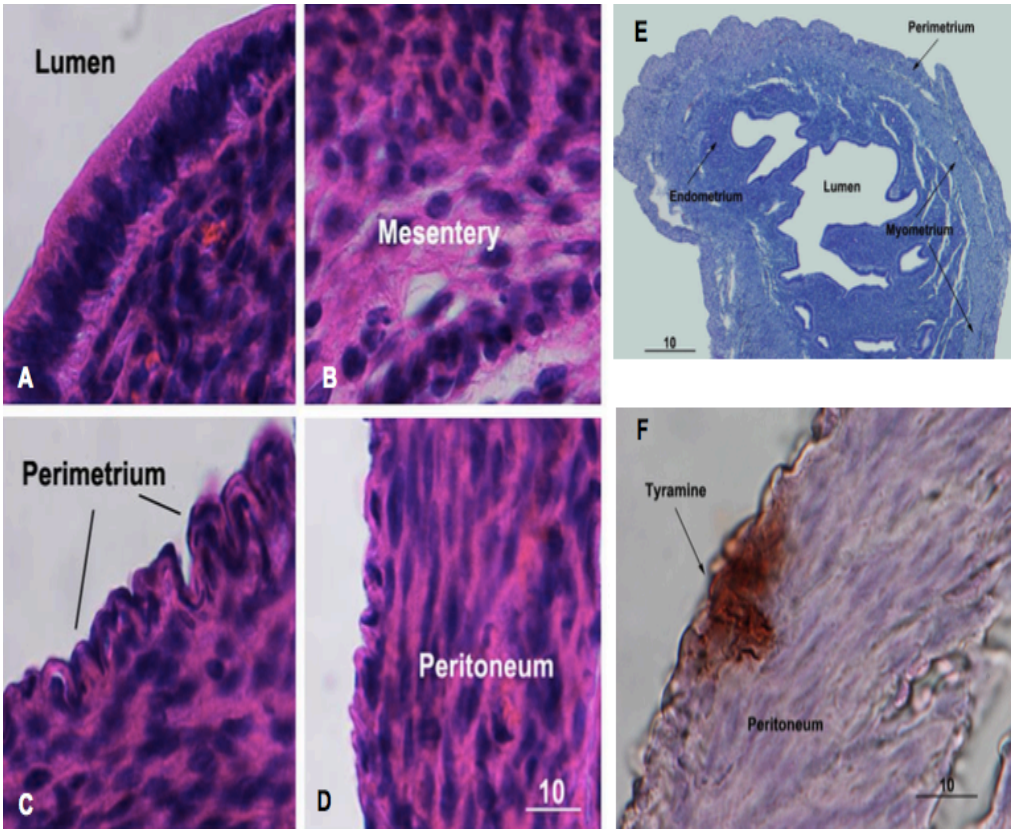

Fig. 9. Stained histological sections of mouse uttering horn. Figures A – D are 10μm transverse sections through a 6-week-old female mouse uterine horn that was stained for hematoxylin and eosin. The hematoxylin stains the nuclei [blue] while the eosin stains cytoplasmic regions, which include the muscle fibers [pink]. Fig. E. shows a complete cross section. Fig. F. was stained for H&E and immune-labeled with antibody against p-tyramine and treated with 3,3′-Diaminobenzidine to produce the brown pigment.

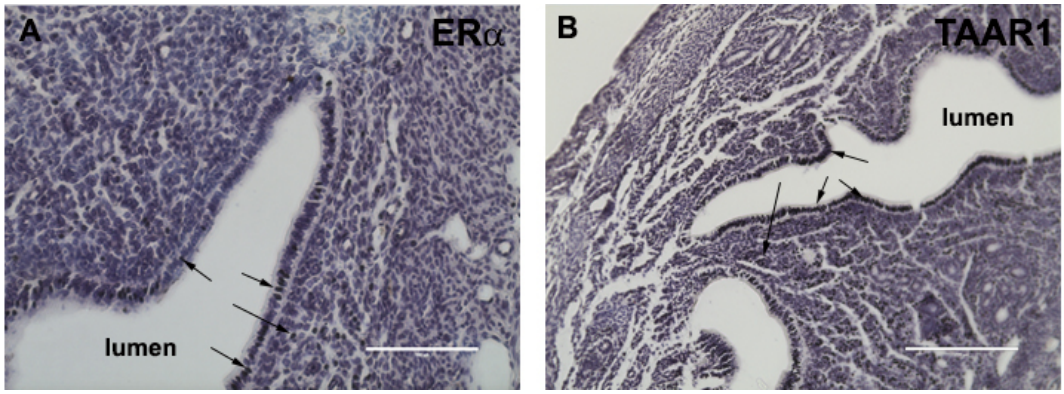

Fig. 10. ERα and TAAR1 localization in the mouse uterus. Cross section of mouse smooth muscle uterine tissue counterstained with hematoxylin and dark grey regions (arrows) in Fig. A are ERα and in Fig. B is TAAR1 localization stained with 3,3′-Diaminobenzidine. Scale bar is 100μm.

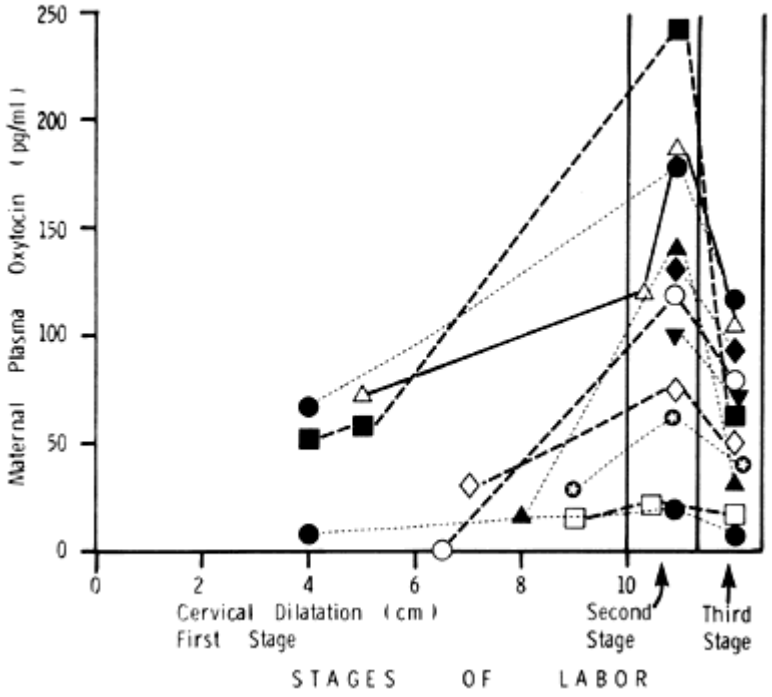

Fig. 11. Oxytocin levels during delivery. (Figure source Cassels, 2012)

Chapter 3. The Role of Tyramine in the Mouse Uterine Horn

Abstract

Smooth muscle tissue makes up the walls of the reproductive tract in the uterus. Usually, smooth muscle surrounds an organ with a hollow space or lumen, so that the muscles can exert pressure on the lumen. One of the hallmarks of smooth muscle tissue is the fact that its activity is not regulated by somatic (voluntary) motor neurons. Instead, smooth muscle tissue has an endogenous rhythm, and the force of contractions can be increased or decreased by hormones and other factors like neurotransmitters, peptides and amines. This portion of the study focuses on the physiological effects induced by tyramine in mouse uterine muscle.

Introduction

The second aim of this project was to find the function of tyramine within mouse uterine muscle. Previous studies have shown that tyramine is associated with peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose and the release of norepinephrine. Because tyramine has been shown to be a modulator of smooth muscle, it was probable that tyramine could also function as an alternative modulator of smooth muscle within the uterus. It was hypothesized that if tyramine was a modulator of smooth muscle activity in the uterine horn, then uterine muscle form pseudo-pregnant female mice at reproductive age would exhibit contraction as measured by force transduction upon stimulation using tyramine.

Materials and methods

Animals

The use of mice in this study was approved by Arizona State University's Institute of Animal Use and Care Committee (IACUC) under the 15-1388T protocol. Wild type (C57BL/6, Jackson Lab) female mice, between the ages of 6 to 8 weeks, were placed in cages which contained male mouse urine for 24 hours in order to stimulate pseudo-pregnancy. After 24 hours, the female mice were deeply anesthetized with chloroform, euthanized by cervical dislocation and the uterine horn was removed and placed heated, oxygenated artificial cerebral spinal fluid (ACSF). Each experiment was repeated three times using three different mice

Force Transduction

The BIOPAC Smooth Muscle Contraction apparatus (BIOPAC, Systems Inc.) was used to measure the contractile force of the mouse uterine smooth muscle (Fig. 12.). After the force transducer was calibrated, the uterine horns were collected from pseudo pregnant and non-pseudo pregnant wild type C57BL/6 mice (aged 6-8 weeks). After dissection, the uterine horns were cut into 0.5cm length sections and placed in an oxygenated buffer reservoir containing heated ACSF to prevent the tissue from becoming hypoxic. The tissue was transferred from the reservoir to the test chamber, where it was attached to two heart clamps connecting one end to a holder within the chamber and the other end to the transducer. The test chamber also contained ACSF buffer that was heated and oxygenated. The tissue remained untreated for 15 minutes so the value for the baseline contractions could be recorded. The tissue was then treated so the buffer within the test chamber had a 100 nM concentration of tyramine. After 15 minutes, the tissue

and saline that was in the test chamber was discarded, and a new piece of tissue and new saline solution was added to a clean test chamber. This procedure was repeated with treatments of oxytocin (10 nM), estrogen (10 nM), octopamine (10 nM) and dopamine (10 nM). Force transduction experiments were repeated at least three times individually as well as sequential treatments such as tyramine followed by dopamine.

Results

The uterine muscle exhibits peristaltic contractions that have a defined duration, amplitude and frequency. In the mouse, untreated uterine muscle from pseudo pregnant mice had a contraction duration averaging 36 seconds, a frequency of 60 seconds and intensity at 0.1 grams. The concentration of tyramine used to stimulate the uterine muscle was 100 nM, which is the physiological concentration known to stimulate smooth muscle (Fig. 13.). Upon stimulation, the average duration was reduced to 15 seconds, with a frequency of 60 seconds and intensity at 0.51 grams. Because octopamine is within the biochemical conversion pathway following tyramine, it was also tested. Smooth muscle that was stimulated by 10 nM of octopamine did not induce any contractile activity. As a positive control, oxytocin was used to induce contractions within the tissue at a 10 nM physiological concentration. This resulted in an average duration of 15 seconds, a frequency of 60 seconds and intensity at 1.9 grams. As a negative control, dopamine was used to inhibit contractions using a 10 nM physiological concentration. As expected, dopamine did not induce any contractile activity. In addition to the physiological stimulation of the uterine tissue using a physiological concentration of tyramine, tissue was also tested to observe the effect that increasing concentrations of tyramine would have on muscle contraction. It was found that contractile strength would

increase as the concentration of tyramine increased (data not shown). The results of these measurements demonstrated that tyramine can induce contractions at a level that is comparable to oxytocin.

These experiments were repeated using non-pseudo pregnant mouse uterus. For untreated uterine smooth muscle, there was an average duration of 22 seconds, a frequency of 60 seconds and intensity at 0.19 grams. Again, a concentration of 100 mM tyramine was used to treat the uterine tissue that resulted in an average duration of 32 seconds, a frequency of 60 seconds and an intensity of 0.56 grams. The concentration of E2 used to stimulate the uterine muscle was 10 nM, resulting in an average duration of 38 seconds, a frequency of 60 seconds and an average intensity of 0.60 grams. The positive control stimulus of oxytocin was conducted at a 10nM physiological concentration, resulting in an average duration of 36 seconds, a frequency of 60 seconds and an intensity of 2.1 grams (Fig. 16.). These values were graphed and compared (Fig. 16.). From the graph it is evident that tyramine affects the uterine horn at a similar intensity as that induced by E2 in the pseudo pregnant uterine muscle and that there is an approximate two fold increase in the effect of oxytocin as compared to stimulus in non-pseudo pregnant uterine muscle.

Discussion

The second aim of this project was to find out what tyramine's function is within the mouse uterine horn. Results from this study did support the hypothesis that tyramine has the ability to modulate smooth muscle function and can induce contractions. These results also confirmed the data from Chapter 2 that showed p-tyramine and TAAR1 localization precede contraction. The force transduction data for the non-pseudo and the

pseudo pregnant uterine muscle differed in the intensity and the duration of the contractions. Tissues that were not treated with any modulator had low intensity contractions, which were expected. Overall, the non-pseudo pregnant tissues that were treated with modulators had intensities that were less than the pseudo pregnant tissues that were not treated. The oxytocin and the dopamine treatments were used as the positive and negative control since both of those hormones are widely known to either contract or relax uterine tissue, which was reinforced in these results. The epinephrine treatments did not have any significant contractile activity (data not shown). According to (Segal et al., 1998), physiological concentrations of epinephrine decrease uterine activity by means of $ß_2$-receptor activation. The octopamine also did not induce any contractile activity, which was also expected since tyramine functions independently of octopamine. Estradiol treatments did increase the intensity of the contractions in the mouse smooth muscle tissue. The contractile strength generated by tyramine was found to be similar to the contractile strength induced by estrogen, which suggests a possible role of tyramine to also prepare the smooth muscle tone in preparation for delivery. This correlates with previous reports that estradiol increases uterine contractions and increases uterine blood flow to sustain contractions in the human uterus (Sastry et al., 1997). Tissues that were treated with 100nM physiological concentration of tyramine (Sotnikova et al., 2010) induced contractile responses that were more intense than the baseline response but not as intense as that induced by oxytocin. An interesting observation is that the frequency of contraction remained constant between untreated and treated in both pseudo and non-pseudo pregnant mice. Besides the direct effect of modulation on contractile intensity it was found that smooth muscle from pseudo pregnant mice have

half the duration of contraction as compared to the non-pseudo pregnant mouse tissue. Additionally, smooth muscle contractile duration in non-treated (no modulator added) mouse tissue had an opposite effect where the duration in non-pseudo pregnant mice is half of the value found for pseudo pregnant mice. This suggests that another potential modulator may be involved through the process of generating pseudo pregnancy yet it does not appear to change stimulant effect of tyramine but it does affect the intensity of oxytocin.

Future Directions

The results obtained in this study formed only the beginning of understanding the role of tyramine in the mouse uterus. They have also opened the way for many more questions that can be investigated such as identifying other elements and mechanisms that may be involved in tyramine signaling. Identifying these proteins or molecules will require the use of immuno-purification and mass spectrometry to identify upstream/downstream targets. This technique would also help validate the antibodies, p-tyramine and TAAR1 that are used in the study. It would also be necessary to also quantify the levels of tyramine in the uterine smooth muscle tissue at each stage of the mouse estrous cycle and at various time points through the lifespan of the mouse. Future projects would also include measuring tyramine levels throughout pregnancy and specifically while the mouse is giving birth. Recently the Baluch lab acquired LifeAct transgenic mice. These mice contain a transgene encoding a 17-amino acid peptide called LifeAct, which binds F-actin. This peptide is co-expressed with GFP which enables the actin cytoskeleton to be imaged both *in vivo* and *in vitro*. Actin is a key component of muscle tissue and this transgenic model allows researchers the ability to visualize muscle

contractions. Various receptors or cell surface protein antibodies can be directly conjugated to fluorophores and imaged live to observe recruitment during contractile activity.

These studies have identified that tyramine is a modulator of smooth muscle uterine activity and the primary tyramine receptor, TAAR1 (trace amine-associated receptor 1), is present within the mouse uterus and increases in localization preceding contraction of the uterine muscle. As experiments continue in the study of tyramine modulation, researchers will have a better understanding of pregnancy and how it is regulated. Through this understanding, it may be possible to create future treatments such as the inhibition of tyramine production, to help control pre-mature labor.

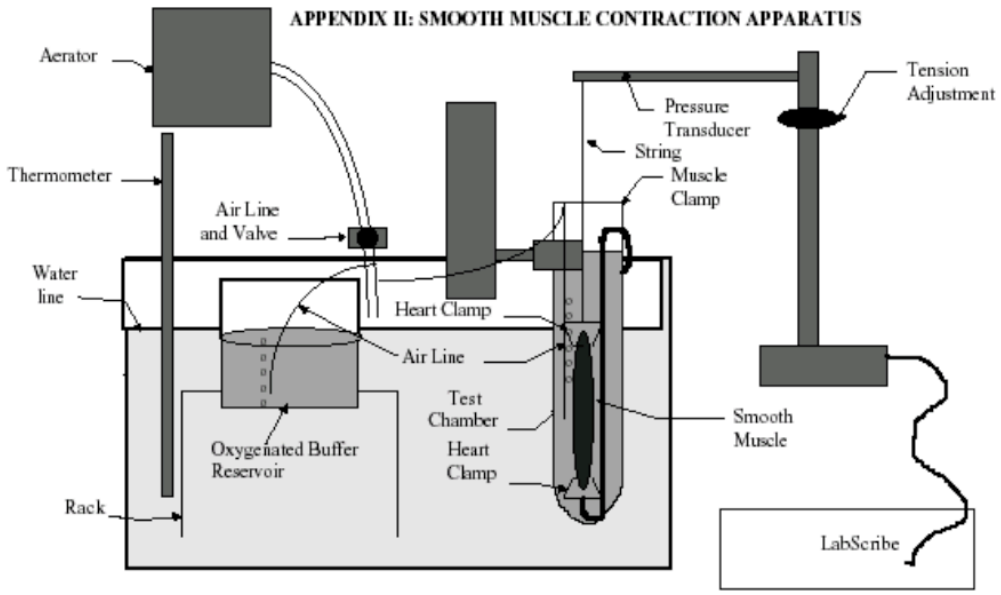

Fig. 12. BIOPAC system to measure contractile force.

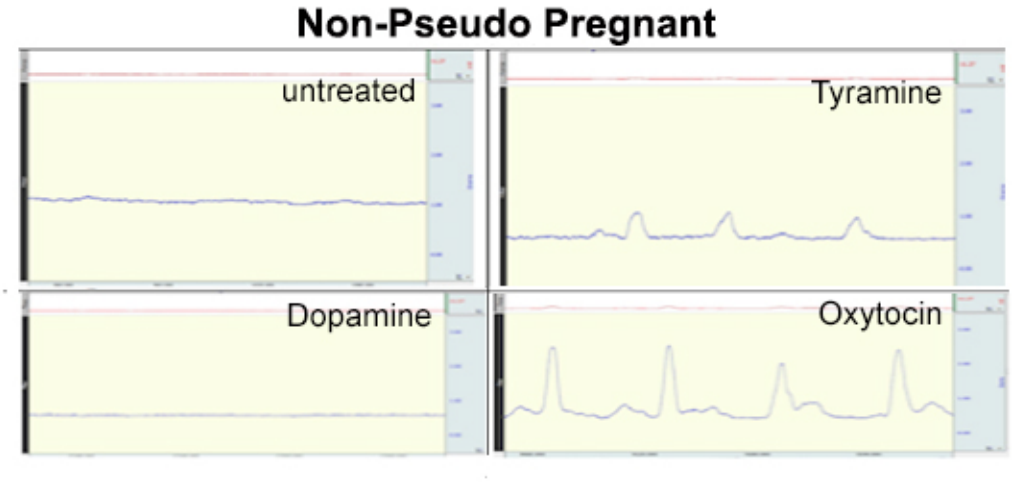

Fig. 13. Force transduction measurement graphs from stimulated non-pseudo pregnant mouse uterine muscle. The X-axis is Time (s) and the Y-axis is the intensity (g)

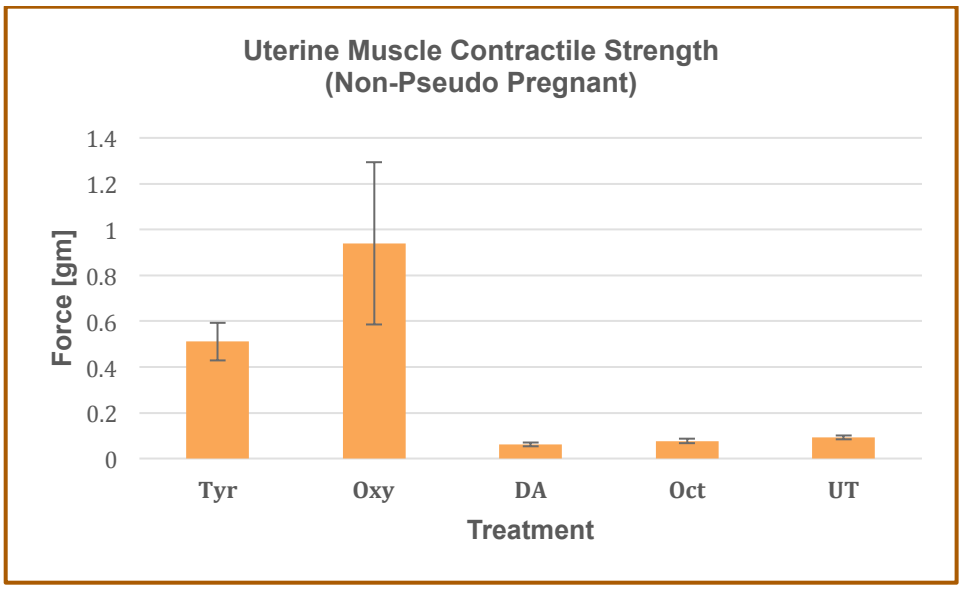

Fig. 14. Force measurements compared between stimulant types from non-pseudo pregnant uterine muscle. [Significance measured using a one way ANOVA, $p<0.05$]

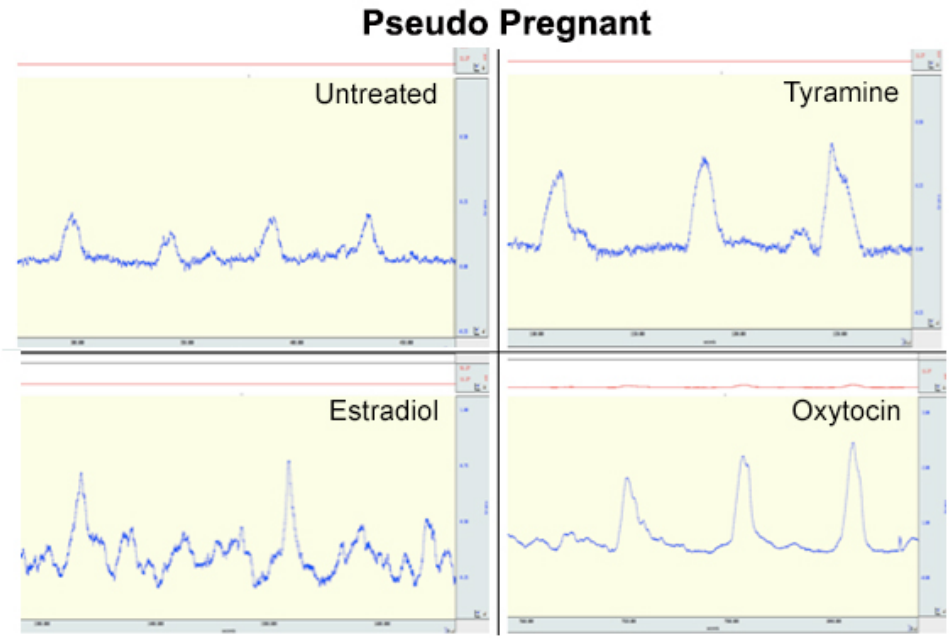

Fig. 15. Force transduction measurement graphs from stimulated pseudo pregnant mouse uterine muscle. The X-axis is Time (s) and the Y-axis is the intensity (g)

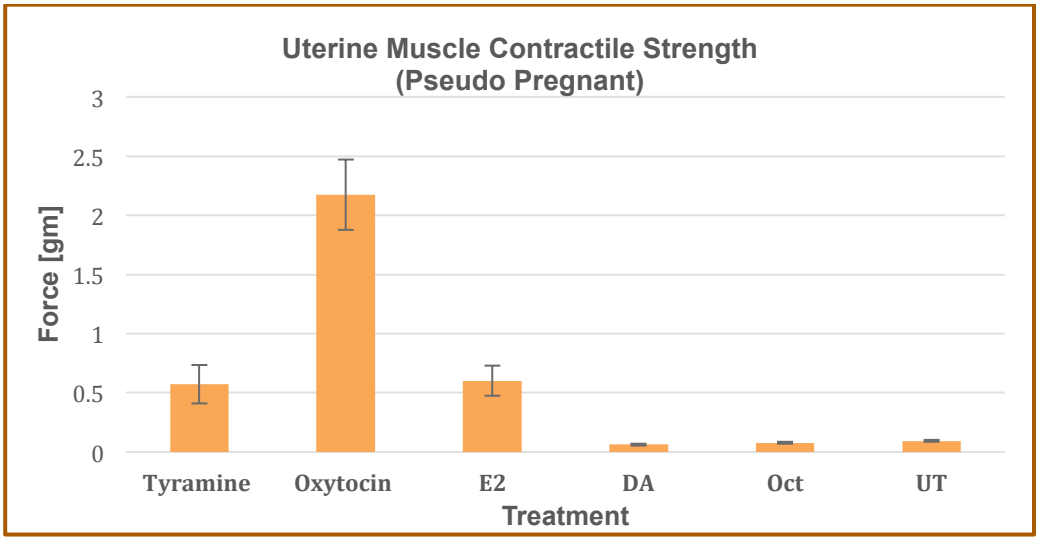

Fig. 16. Force measurements compared between stimulant types from pseudo pregnant uterine muscle. [Significance measured using a one way ANOVA, $p<0.05$]

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APPENDIX A

ANIMAL SUBJECTS

Use of mice in this study was approved by Arizona State University's Institute of Animal Use and Care Committee (IACUC) under the 15-1388T protocol. Wild type C57BL/6 mice were obtained by the Jackson Laboratory in Sacramento, California. Mice were housed and bred in the ASU Department of Animal Care and Technologies (DACT) vivarium in Life Sciences B wing. Female mice, between the ages of 6 to 8 weeks, were used for this study and were placed in cages that contained male mouse urine for 24 hours in order to stimulate pseudo-pregnancy.