EFFECT OF TRANSLOCATOR PROTEIN (TSPO) STIMULATION OF STEROIDOGENESIS BY TSPO-SPECIFIC DRUG LIGAND: IMPLICATIONS FOR HYPOGONADISM AND SPERMATOGENESIS

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ABSTRACT

The Leydig cells, localized in the interstitial compartment of the mammalian testis, produce testosterone (T) in response to luteinizing hormone (LH) released from the anterior pituitary. As most men age, serum T levels decrease, which is clinically referred to as hypogonadism. Fatigue, erectile dysfunction, and reduced bone density and muscle mass are some of the symptoms linked to these declining T levels. Age-related decline in T also occurs in aging Brown Norway rats. Previous studies reported that with aging, LH levels do not change, but Leydig cells become less responsive to LH, resulting in reduced T production. Currently, the only readily available treatment for hypogonadism is T replacement therapy (TRT). However, exogenous T administration has been shown to suppress LH release and thus Leydig cell T production. This results in reduced intratesticular T levels and therefore in suppressive effects on spermatogenesis to the point of azoospermia. Thus, TRT is an inadequate therapy for men who wish to father children.

We wished to develop a method by which to elevate serum T levels without affecting intratesticular T levels. To accomplish this, we used the current understanding of the T biosynthetic pathway to pharmacologically stimulate a key protein in this pathway called Translocator Protein (18-kDa TSPO) using a TSPO-specific drug ligand. We compared the effects on serum and intratesticular T levels of administering the TSPO-specific ligand with administering exogenous T. Old (18-24 mo) rats have significantly reduced serum T levels compared to young (3-6 mo) rats. We administered the TSPO drug ligand, N,N-dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27), via daily ip injection to aged rats at a concentration of 1 mg/kg body weight over the course of 10 days. Control rats received vehicle for the same time period. Another group of aged rats were given exogenous T via T-containing

Silastic implants. Serum T levels were significantly lower in old control rats than in young control rats. When administering FGIN-1-27, serum T levels in old rats rose significantly, and administering exogenous T rose to the level of young rats. Administering exogenous T to old rats reduced intratesticular T levels significantly from control levels. In striking contrast, administering FGIN-1-27 to old rats resulted in a significant increase in intratesticular T levels. Taken together, these results show that administering FGIN-1-27 can increase serum T in hypogonadal old rats, as can administering exogenous T. However, in contrast to exogenous T, administering FGIN-1-27 can do so without reducing intratesticular T levels, suggesting that this approach would not suppress spermatogenesis and might even enhance this process.

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LITERATURE REVIEW

Background

Whether or not to classify aging as a disease has been controversial. Nevertheless, it is a naturally occurring phenomenon, which is evident from the whole organism down to the cellular level. There are a number of diseases and conditions that are characterized by senescence such as heart disease, high blood pressure, cancer, arthritis, dementia, osteoporosis, diabetes, and Alzheimer's disease among others ("Aging: Associated Diseases & Information", 2016). It is clear just from this list that aging affects all organ systems whether symptoms are immediately present or not.

The male reproductive system is a prime example of a bodily system that progressively shows signs of aging. The Leydig cells, which reside in the interstitial compartment of the testis, produce testosterone (T) in response to luteinizing hormone (LH) released from the anterior pituitary. With aging, these cells produce less T resulting in a condition that is clinically referred to as hypogonadism. It is a syndrome characterized by a significantly reduced serum T levels (Basaria, 2014). Low sperm production isn't usually thought of as indicative of hypogonadism, though there are a lot of infertile males who have low T. Insufficient production of androgen is accompanied by many uncomfortable symptoms that include but are not limited to: decreased muscle mass, increased weight gain, and low bone density, cognitive changes, erectile dysfunction, increased fatigue, and low libido (Kumar et al., 2010; Ullah et al., 2014)

T is a cholesterol-derived steroid hormone produced by Leydig cells and has an effect on a number of cell types throughout the body. Being the principal sex hormone produced by males, it has significant masculinizing capabilities. This is supported by research that has been done on transgender patients. Cross sex hormone therapy is often employed by transgender men (who are

biologically female) to induce virilization in effort to promote development of masculinizing characteristics while suppressing feminizing ones (Unger, 2016). Studying normal male development in utero through puberty and into adulthood further supports T's role as the primary male sex hormone. As in the male rat, humans have two populations of Leydig cells that develop. In utero, there is a population of "fetal" Leydig cells that produce T independent of LH stimulation to elicit the development of the male reproductive tract (Chen et al., 2009). After birth, a population of adult Leydig cells, which requires LH in order to produce T, eventually replaces the population of fetal Leydig cells. T and its derivatives are responsible for the secondary sex characteristics (deepening of the voice, facial and pubic hair, increased muscle mass/strength etc.) and external genitalia development, which notably occurs during pubescent years (Chen et al., 2009). T is produced indefinitely throughout a man's life after puberty. Despite this, many men experience a decline in T production (which is shown by the serum and intratesticular concentrations) noticeably starting in the fourth or fifth decade of life. Although many of these low T men are older (40-50+), there are low T men that are young heightening the clinical relevance of this condition (Yin & Swerdloff, 2010).

Despite the declining serum T levels that accompany aging, LH levels either rise slightly or remain unchanged (Surampudi et al., 2012). These age-related changes occur due to problems with LH signaling. With reductions in LH signaling and inadequate levels of enzymatic and non-enzymatic antioxidants to combat reactive oxygen species (ROS), oxidative stress is thought to be at least partially responsible for the decline of T levels. Steroidogenesis is known to be highly reliant on ATP and its production is accompanied by production of ROS (Midzak, Chen et al., 2011). Such oxidative stress is presumably exacerbated by ROS that is given off by cytochrome P450 enzymes that catalyze steroid hydroxylations (Hanukoglu, 2006; Midzak, Chen et al.,

2011). Research has also shown that exposure to pollutants can result in intracellular deficits ultimately leading to hypofunctional Leydig cells. Thus, external exposures combined with ROS accumulation over time can lead to defects in Leydig cells' ability to produce T.

<u>Hypothalamus-Pituitary Gonadal Axis</u>

T production occurs throughout the life of a typical male, but there are two notable events that occur with its synthesis. It is first synthesized in utero to stimulate development of the male reproductive system (Chen et al., 2009). Fetal Leydig cells produce T independent of LH stimulation and are exclusively found in the male fetus. These cells decline after birth and are eventually replaced by adult Leydig cells at puberty (Griswold & Behringer, 2009; Hardy et al., 2009). Genetic and environmental factors contribute to the onset of puberty, but it is at this time that T is synthesized in very large quantities. The onset of puberty is marked by an influx of neuroendocrine hormones, which acts on the hypothalamus to initiate signaling by the hypothalamic-pituitary-gonadal (HPG) axis. Kisspeptin has been identified as a key regulator for the onset of puberty and necessary for normal sex hormone production in both males and females (Irwig et al., 2004; Novaira et al., 2014; Rhie, 2013; Skorupskaite et al, 2014). Kisspeptin receptor knockout studies in mice have confirmed this. Both male and female kisspeptin knockout mice had low gonadotropin levels and were infertile (Novaira et al., 2014).

During puberty, kisspeptin binds to its receptor on target cells located in the arcuate/infundibular nucleus and preoptic area of the hypothalamus, which then stimulates the release of gonadotropin-releasing hormone, or GnRH. It is still unclear what causes the heighted kisspeptin production observed at the onset of puberty. Nevertheless, its action is necessary to stimulate the hypothalamus to secrete GnRH (Novaira et al., 2014). The hypothalamic neurons are associated with the anterior pituitary via the hypophyseal portal system, which are a

collection of blood vessels that allow rapid communication between the glands. Upon secretion by the hypothalamus, GnRH travels through this collection of vessels to the anterior pituitary and elicits the production and secretion of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones are then released into systemic circulation where they travel throughout the body. GnRH release is pulsatile, which leads to the pulsatility of gonadotropin secretion (Irwig et al., 2004; Novaira et al., 2014; Rhie, 2013; Skorupskaite et al, 2014). FSH acts on the Sertoli cells found in the seminiferous tubules of the testis and facilitates the production of sperm. LH induces the Leydig cells found in the interstitial compartment of the testis to produce T. When T is produced, it travels through the serum to negatively feedback on its own production by suppressing GnRH, LH, and FSH release from the hypothalamus and anterior pituitary. This action by T also contributes to the pulsatility of the hormones and thus its own production. A summary of the HPG axis is shown in Figure 1.

<u>Steroidogenesis – Cellular Mechanism for Testosterone Biosynthesis</u>

There has yet to be a consensus on the full T biosynthetic pathway, but it is thought that many of the key players have been identified. The proposed pathway (shown in Figure 2) begins as LH from the anterior pituitary arrives at the interstitial compartment of the testis via the bloodstream. The LH receptor, a member of the seven-transmembrane G protein-coupled receptor (GPCR) superfamily, resides on the plasma membrane of Leydig cells. Each receptor is composed of a G protein and three subunits (α, β, and γ), which reside on cytoplasmic face of the plasma membrane. The Gα subunit interacts with both the G protein and the Gβ and Gγ subunits, which are typically bound together. In the inactive state, GDP is bound to the Gα subunit. LH binding induces to a conformational change in the receptor that leads to the dissociation of GDP and successive binding of GTP to the Gα subunit (Midzak et al., 2009). This subunit, now active,

interacts with an effector called adenylyl cyclase (AC). This enzyme uses local ATP supplies to generate cyclic adenosine monophosphate (cAMP), a key second messenger in the pathway. At some point, the Gα subunit uses its ATPase activity to hydrolyze the bound GTP breaking the stimulatory interaction between it and AC. The GPCR subsequently returns to its resting state. (Midzak et al., 2009). But as long as GTP is bound to the Gα subunit (assuming the rest of the cascade is unaffected), the pathway remains active.

Once produced, cAMP leads to an amplification of the signal throughout the cell. Four molecules of cAMP activate protein kinase A (PKA) by binding to PKA's regulatory subunits releasing the catalytic subunits that can go on to phosphorylate downstream substrates (Liu et al., 2006). The remaining steps are heavily under investigation, and therefore the exact mechanism remains unclear.

Arguably the most important finding associated with LH-stimulated cAMP signaling is that it has been linked to the formation of a large protein complex, commonly referred to as the transduceosome. This complex facilitates the transfer of cholesterol from the cytoplasm to the mitochondrion, the rate-determining step of steroid synthesis (Privalle et al., 1987; Simpson et al., 1978). Thus, the more efficiently this step occurs the more efficient the overall process becomes. Cholesterol is recruited from one of three sources. It can be generated de novo in the smooth endoplasmic reticulum (ER), the mobilized from lipid droplets, or utilized from the plasma membrane (Rone et al., 2009). The majority of cholesterol in a cell is embedded in the plasma membrane and recycles in and out of the cell, but the cholesterol that is mobilized to the mitochondria is thought to originate from cytosolic storages of lipid droplets (Rone et al., 2009). The mobilization of cholesterol can be modeled in two phases (Midzak et al., 2009). The first involves cholesterol esterase which becomes active upon phosphorylation by cAMP-activated

PKA (Beckett & Boyd, 1977; Trzeciak & Boyd, 1973). This enzyme then catalyzes the hydrolysis of lipid droplet cholesteryl esters creating a pool of cytosolic unesterfied cholesterol that can be transported to the mitochondria and utilized for steroidogenesis (Shen et al., 2003). The second phase consists of transduceosome-mediated movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) and the continued movement via the metabolon, another important complex associated with the mitochondrion, to a key metabolic enzyme found mitochondrial matrix.

The transduceosome and metabolon that drive the second phase have been of high research interest, but the role of each player within these complexes remains highly controversial. Researchers believe the transduceosome is composed of many proteins localized to the OMM including OMM proteins, translocator protein (TSPO), previously named peripheral benzodiazepine receptor, and voltage-dependent anion channel (VDAC). Studies have shown that there are cytosolic proteins that also assemble with TSPO and VDAC to form the transduceosome. These include steroid acute regulatory (StAR) protein, acyl-CoA binding domain-containing protein 3 (ABCD3), protein kinase A regulatory subunit I alpha (PKA-RIα), and 14-3-3 adaptor proteins (Issop et al., 2013; Rone et al., 2012; Zirkin et al., 2018). There has been considerable progress in establishing functions to each of these proteins, but the exact interactions that facilitate cholesterol's movement is still under investigation.

The proposed mechanism begins with Golgi-associated protein, ABCD3 (previously PAP7). Its ability to bind both TSPO and PKA-RIα allows it to detach from the Golgi and bring cAMP-dependent PKA-RIα found in the cytosol to the mitochondria via interaction with OMM protein TSPO or VDAC1 or both (Aghazadeh et al., 2012; Li et al., 2001, Liu et al., 2006; Issop et al., 2013). This moves PKA-RIα into the proximity of the StAR protein allowing for efficient

phosphorylation of StAR's Ser-194 residue resulting in its maximum activity. StAR mRNA is pre-made and is translated upon cAMP signal transduction (Aghazadeh et al., 2012; Reinhart et al., 1999). This 37-kDa protein has two distinct regions, hydrophobic STAR-related lipid transfer (START) domain that binds cholesterol and an N-terminal mitochondrial signal sequence that is removed after reaching the OMM. Together, these regions of the protein facilitate the movement of cholesterol from the cytosolic environment to the OMM (Aghazadeh et al., 2012; Issop et al., 2013; Liu et al., 2006; Rone et al., 2009). TSPO is an 18-kDa OMM protein that also contains two distinct domains as well that are important for steroidogenesis, a benzodiazepine-binding (drug) site and a cholesterol recognition amino acid consensus (CRAC) site (Midzak, Akula, et al., 2015). The benzodiazepine-binding site has been found to bind a many compounds, which appear to stimulate the translocation activity of TSPO making this protein a promising pharmacological target (Midzak, Akula, et al., 2015; Midzak, Zirkin et al., 2015). TSPO's high affinity CRAC motif promotes the capture of cholesterol from StAR. Fluorescence resonance energy transfer (FRET) analysis has shown that these proteins indeed interact (West et al., 2001). Hormonal stimulation leads to TSPO aggregation/polymerization, which has been correlated with higher cholesterol binding affinity and movement into the mitochondria (Bogan et al., 2007; Issop et al., 2013). VDAC's beta-barrel secondary structure is thought to form a pore with TSPO facilitating the movement of cholesterol across the OMM.

The 14-3-3 proteins are small acidic cytosolic proteins that regulate various cellular functions including DNA replication, transcription, mitosis, apoptosis, cellular signaling, and cytoskeletal structure and function. These proteins are believed to be integrally involved in the T biosynthetic pathway. Researchers believe they interact with the transduceosome to negatively regulate the translocation of cholesterol and thus steroid production (Aghazadeh et al., 2012; Aghazadeh et

al., 2014). The γ isoform is found in both homodimers and heterodimers with other 14-3-3 isoforms under basal conditions. But upon hormonal stimulation, concentrations of this protein increase and the dimers dissociate allowing the monomers to bind other substrates (Aghazadeh et al., 2014). These monomers bind the START domain of StAR in steroidogenic cells keeping it in its unphosphorylated and only partially active form (Aghazadeh et al., 2014). Continued stimulation leads to a more substantial increase in 14-3-3γ levels, which through a dominant negative mechanism dissociates from StAR and re-dimerizes (Aghazadeh et al., 2014). This allows StAR to become fully active by phosphorylation of its Ser-194. The ε isoform is another 14-3-3 protein but works through a different mechanism. VDAC1 and StAR are thought to compete for 14-3-3ε binding. VDAC1 binding and association with TSPO is believed to promote the intercalation of 14-3-3ε between the two proteins physically blocking the movement of cholesterol into the mitochondria (Aghazadeh et al., 2012). Somehow these interactions are balanced in normal cells so that this protein's inhibitory function is prevented.

Once cholesterol bypasses the negative effects of these 14-3-3 proteins and has entered the OMM pore of the transducesome, it moves on the metabolon. There is considerable overlap between these two protein complexes. The metabolon is an 800-kDa protein complex composed of transducesome proteins, TSPO and VDAC1, IMM protein ATPase family AAA Domain-containing protein 3 (ATAD3), and matrix protein Cytochrome P450 11A1 (CYP11A1). ATAD3 is an IMM protein that interacts with both the TSPO/VDAC1 pore and CYP11A1. It establishes a route for cholesterol to move through the IMM to CYP11A1 which is associated with the matrix face of the IMM (Issop et al., 2013; Rone et al., 2012). CYP11A1 is an enzyme that catalyzes the conversion of cholesterol into pregnenolone (Issop et al., 2013; Rone et al., 2012). Figure 3 is a schematic that shows the proteins of the transduceosome and the metbolon.

Pregnenolone is shuttled to the smooth endoplasmic reticulum (ER) where the remaining enzymatic events occur. It is quickly converted to progesterone by 3β-Hydroxysteroid dehydrogenase (3β-HSD). Progesterone and its successor are acted on by cytochrome p450 17 (CYP17) forming 17α-hydroxprogesterone and androstenedione respectively. Finally, 17β-Hydroxy steroid dehydrogenase (17β-HSD) catalyzes the last step converting androstenedione to T (Midzak et al., 2009).

Leydig cell development

There are two distinct populations of Leydig cells found in mammals: fetal Leydig cells (FLCs) that develop in utero and adult Leydig cells (ALCs) that develop after birth (Chen et al., 2009; Chen, Stanley et al., 2010). The FLCs produce T and its metabolites during gestation at as early as day 15.5 to promote the formation of the male external genitalia and urogenital system (Warren, 1989). FLCs are able to synthesize T without LH stimulation. This has been confirmed by studies that investigated LH receptor null mice in utero, which produce T at similar levels as wildtype mice (Zhang et al., 2001). FLCs are eventually lost after birth and replaced by ALCs. Despite this, rat studies have demonstrated that FLCs can persist for a significant amount of time after birth (Ariyaratne & Mendis-Handagama, 2000; Kerr & Knell, 1988; Zirkin & Ewing, 1987).

A number of experiments have been conducted to provide support for the presence of Stem Leydig Cells (SLCs). The morphology of these cells has been described as spindle-shaped and these cells are observable around day 7 post-birth. When isolated and cultured with growth factors, these mesenchymal-like cells can be maintained for an extended period of time supporting self-renewal characteristic of stem cells (Davidoff et al., 2004; Ge et al. 2006). Changing the medium to one that contained LH and a number of other factors caused the SLCs

to become 3βHSD, one of the smooth ER enzymes, and produce T suggesting that they had subsequently differentiated (Ge et al. 2006). A lineage tracing experiment was performed to see if this differentiation could occur in an adult rat in vivo. In this experiment, an alkylating agent called ethane dimethane sulphonate (EDS) was used to selectively kill all the ALCs. Researchers labeled isolated putative SLCs, labeled them with a fluorescent dye (carboxyfluorescein diacetate succinimidyl ester) and injected them into parenchyma at the cranial pole of the testis. Ten days post-treatment they extracted the rats' testes. There was an emergence of large population of fluorescently labeled cells that were 3βHSD positive compared to rats injected with saline, which suggesting that the SLCs were indeed stem cells (Ge et al. 2006).

There has been a four-stage model established using rats. The model begins with the "mensenchymal like" SLCs, which do not express key Leydig cell markers (i.e.3β-HSD and the LH receptor) denoting their "stemness" and providing evidence that they are in an undifferentiated state. The next stage, progenitor Leydig cells (PLCs), is defined by commitment to the Leydig cell lineage because they begin to express Leydig cell specific markers and produce T (Shan et al., 1993). These cells are small and spindle-shaped making them very similar morphologically to the SLCs. They are highly proliferative, but their numbers concurrently decline (Hardy et al., 2009). While PLCs decline, morphologically distinct cells denoted as immature Leydig cells (ILCs) emerge. The transition to this stage is evident as the cells are round shaped and have increased levels of steroidogenic enzymes (Shan et al., 1993; Zirkin & Ewing, 1987). T is not the primary steroid produced as T metabolic enzymes, 3α-hydroxysteroid dehydrogenase and 5α-reductase, are highly expressed (Murono, 1989; Shan et al., 1993). But cells eventually reach the ALC stage. Their turnover rate is very low and T

biosynthesis is several-fold higher than both PLCs and ILCs due to higher production of androgen and lower T metabolism (Chen et al., 2009; Ge et al. 2006).

Because ALCs do not proliferate often, it is clear that there are age-related changes that occur within these cells that lead to reductions in T production, as previously mentioned. With that in mind, there has been interest in determining if SLCs also age. One study treated young and old Brown Norway rats using EDS to eliminate the ALCs and measure T production when the ALCs repopulated the testis. Surprisingly, the restored ALCs from aged animals was comparable to that of young rats even at 10 weeks post-EDS treatment, and this heightened T production was not the result of higher LH exposure (Chen et al., 1996). Even more interesting is that at 30 weeks post-EDS treatment, the ALCs that repopulated the testes of the old rats produced T that was significantly lower than the level observed at 10 weeks (Chen, Guo et al., 2015). This observation has two implications. (1) The SLCs from which these new ALCs arise could be aged and/or (2) there is a difference between the young and old animal environments in which the new ALCs develop (Chen, Guo et al., 2015). Additionally, another study treated neonatal Sprague-Dawley rats with EDS which eliminated the existing FLCs (Su et al., 2018). The ALCs that repopulated the testes 21 days later first showed an increase in T production but after 56 days showed a substantial decline in T synthesis. These ALCs were somehow defective suggesting that development of ALCs is a highly sensitive process. A broader understanding on the intrinsic and extrinsic factors that regulate the differentiation of SLCs and development of ALCs could provide further implications on how aging occurs in hypogonadal men.

<u>Testosterone Signaling and its Role in Spermatogenesis</u>

Once produced by the Leydig cells and secreted by the testes, T circulates in the blood in one of three forms. The majority of it, estimated to be 97%, is either bound weakly to the blood

plasma protein, albumin, or tightly to sex hormone binding globulin (Ullah et al., 2014). The other 3% or so travels through the blood freely (Ullah et al., 2014). T levels vary widely from person to person and with age. Despite this, investigation of serum levels versus levels in the testes has revealed that intratesticular T (ITT) levels are much higher. In fact, studies have found that the ITT levels are at least 30 fold higher in rats and 100 fold higher in humans (Coviello et al., 2004; Hill et al., 2004). This makes sense, of course, because T is produced by the Leydig cells, which reside in the testes, so local concentration would be expected to be higher than that of systemic circulation. Research has shown that when ITT falls below a critical value spermatogenesis is dramatically affected (Hill et al., 2004). In one important experiment, Sprague-Dawley rats were administered T-containing silastic implants of varying sizes (Zirkin et al, 1989). The size of the implant correlated with increases in measured serum T and ITT. After dropping below certain concentration of T (in this case ~20ng/ml), there was a significant reduction in sperm count indicating that a certain amount of T is necessary in order to maintain spermatogenesis (Zirkin et al, 1989). This has also become clear through experiments in which T was administered to healthy men. The administration of exogenous T inhibits the production of endogenous T due to negative feedback on the hypothalamus and pituitary thereby substantially reducing ITT levels (Coviello et al., 2004). These low ITT levels lead to significant reductions in sperm count and germ cell loss (Coviello et al., 2004; Hill et al., 2004).

Taken together, the above observations denote that T, particularly ITT, is integrally involved in the maintenance of spermatogenesis. Androgen receptors have been found in peritubular cells, Sertoli cells, and Leydig cells (Coviello et al., 2004). Sertoli cells have been determined to be the main cells in which ITT carries out its effects. T diffuses through the plasma membrane due to its hydrophobicity and can subsequently bind to its androgen receptor (AR) displacing heat shock

proteins that had previously been sequestering it to the cytosol. The T-AR complex then translocates to the nucleus where it can bind to DNA and induce expression of T-dependent genes (Hill et al., 2004; Walker, 2011). The exact biological mechanism by which T regulates spermatogenesis remains unclear. Interestingly though, T deprivation or AR knockout specifically in Sertoli cells can result in infertility in three different ways. One of these impairments results from a failure of round spermatids to transform into elongated spermatids as this process is dependent on T (Holdcraft & Braun, 2004; O'Donnell et al., 1994). Another is that fully mature spermatozoa are cannot detach from the Sertoli cells and the germ cells are endocytosed by the Sertoli cells (O'Donnell et al., 1994). And finally, the blood testis barrier is disrupted making the developing sperm susceptible to attack by the immune system (Meng et al, 2011; Willems et al., 2010). Further, ITT has been shown to modulate the expression and localization of AR (Hill et al., 2004). In one experiment, T/estradiol implants were administered to male rats, which resulted in reduced ITT levels via negative feedback on the HPG axis. Immunostaining illustrated a dramatic loss of AR protein localization to the nucleus despite unchanged AR mRNA levels (Hill et al., 2004). Local administration of T rescued this mutate phenotype suggesting the T somehow controls AR localization in Sertoli cells. These data support the assertion that T is essential to the maintenance of homeostatic level of spermatogenesis.

Hypogonadism

Hypogonadism, or androgen deficiency syndrome, can manifest from alterations upstream or within the T biosynthesis pathway. Its classification as primary or secondary hypogonadism is dependent on where in the pathway the problem originates. Primary hypogonadism is when the Leydig cells have reduced responsiveness to normal LH levels. Thus, the problem arises at the

level of the testes or more specifically the Leydig cells. Secondary hypogonadism is characterized by dysfunction within the hypothalamic-pituitary gonadal axis. This is marked by reduced levels of T in the bloodstream, which is brought about by inadequate levels of gonadotropins, LH and FSH. Due to low T production, men with secondary hypogonadism often have reduced spermatogenesis as well (Basaria, 2014; Kumar et al., 2010).

An important longitudinal study published in 2001 analyzed both total and free T in a homogenous population of healthy men. This study revealed that aging was likely the primary contributor to declines of in T observed over time (Harman et al., 2001). Although most commonly attributed to changes that arise over time, hypogonadism can stem from other deficits brought about by chronic disease, serious injury, obesity, medications, phthalate exposure, cancer treatment (chemotherapy and radiation), pituitary disorders, or Klinefelter's syndrome (Basaria, 2014; Gao et al., 2017; Ha et al., 2016; Kumar et al., 2010; Motohashi et al., 2016). Because of the symptoms that present with androgen deficiency syndrome, research largely seeks to understand the intrinsic and extrinsic contributions to Leydig cell aging in order to develop therapies for reversal and even potential prevention methods.

Intrinsic and Extrinsic Contributions to Leydig cell Aging

Model Organisms for Leydig cell Aging

It is essential to the validity of disease modeling studies to conduct experiments using animals that manifest the disease or condition of interest in a similar manner to that of humans. Thus, obtaining a model organism that reflects the age-related changes that are characteristic of men with reduced T is the goal for studying hypogonadism. There are a number of rat strains that show declines in serum T over time, these being Fisher 344, Wistar, Sprague-Dawley, and Brown Norway. Despite this, most of these strains have characteristics that do not represent that

pattern of aging seen in men. The most important of these is the paired decline in gonadotropin levels suggesting that the decreased T phenotype stems from defects within the HPG axis, or secondary hypogonadism. As such, these rat strains can be treated via the administration of human chorionic gonadotropin (HcG), which functions like LH, to rescue lowered serum T levels (Zirkin et al., 2018). This contrasts, of course, with humans. As mentioned earlier, the levels of LH and FSH are unchanged in men with primary hypogonadism (the largest and thus most relevant population) denoting that the deficit comes from Leydig cell dysfunction. Further, some strains have physiological manifestations, such as weight gain and tumors of the HPG axis, that occur with aging making it difficult to distinguish hypogonadism from other diseases and conditions. The Brown Norway is a rat strain that shows the same age-associated changes in cellular environment and decline in serum T without low gonadotropin levels and minimal ambiguous physiological presentations making it a good model organism (Zirkin et al., 2018).

Intrinsic: Shifts in Redox state

The free radical theory of aging states that over time cells experience a shift in the ratio of pro-oxidants and antioxidants in such a way that oxidative damage to intracellular macromolecules, such as DNA, proteins, and/or lipids, can occur (Cui et al, 2012; Luo et al., 2006; Rebrin & Sohal, 2008). Such damage can have functional consequences within cells. Bearing this in mind, it is possible that decreases in protective antioxidants and increases in pro-oxidant molecules could lead to reductions in important upstream signaling molecules and eventually T production (Diemer et al., 2003; Quinn & Payne, 1984; Quinn & Payne, 1985). In fact, in aged Leydig cells, levels of non-enzymatic antioxidants, such as glutathione (GSH), and enzymatic antioxidants, namely GSH peroxidase and superoxide dismutase 1 and 2 (SOD-1, 2), are substantially reduced (Cao et al., 2004; Luo et al., 2005).

The role of antioxidants in Leydig cells aging has been studied by analyzing the transcription factor known as nuclear factor erythroid 2-related factor (Nrf2). This protein regulates the expression of many phase II enzymatic antioxidant genes (Yang et al., 2016). Nrf2 displays cellular latency as, under basal conditions, it is sequestered to the cytoplasm by another protein called Keap1. This protein acts to inhibit Nrf2's translocation into the nucleus to active transcription via its U3 ubiquitin ligase activity that targets Nrf2 to the proteasome for degradation. Only upon activation by some stimulation, such as oxidative stress, does the dissociation of Nrf2 occur which allows it to enter the nucleus and bind the antioxidant response element (ARE) promoting the expression of these antioxidant genes (Yang et al., 2016).One study created Nrf2 null mice and observed T production of their Leydig cells over time. It was found that initial loss of Nrf2 didn't affect serum levels, as young (3 months) Nrf 2-/- mice had serum T levels that were comparable to control mice (Nrf2+/+) (Chen, Jin et al., 2015). But by middle age (8 months), these mice displayed reductions in steroidogenic function with gradually declining T levels through old age (21-24 months). Moreover, knockout of Nrf2 (and its downstream antioxidants) was paired with increased oxidative stress through measurement of protein nitrotyrosines providing more evidence for intrinsic Leydig cell aging theory that oxidative stress leads to reduced steroidogenesis.

Further, cells metabolize carbohydrates via the electron transport chain to generate large amount of energy in the form of ATP. Free radicals or ROS are produced as by-products of normal metabolism (Cui et al, 2012). Leydig cells undergo the same metabolism to produce energy and residual ROS, but they are also steroidogenic, which makes them even more susceptible to oxidative stress. The phase I cytochrome P450 enzymes also can generate ROS contributing to the imbalance in the redox state of the cell (Hanukoglu, 2006). Research supports

these assertions as studies show levels of superoxide content, a form of ROS, and lipid peroxidation, an example of oxidative damage, are elevated in aged Leydig cells (Chen et al., 2001; Peltola et al., 1996).

Moreover, the mitochondrial theory of aging stems from the free radical theory citing that aging results from oxidative damage to macromolecules but specifically in the mitochondria (Cui et al, 2012). This theory is somewhat supported by the observed decline in functional cytochrome P450 11A1 (CYP11A1) levels. This enzyme resides in the mitochondria and catalyzes the first step in T biosynthesis. If CYP11A1 levels drop, T production could also drop. The positive correlation between age and oxidative stress proposes a mechanism for the decline of T observed over time. One notable study looked at long-term suppression of endogenous T production via the administration of T implants (Chen & Zirkin, 1999). The implants were given to young (3 months old) and middle aged (13 month old) rats. After 8 months, the implants were removed. After 2 months, it was observed that both groups (now 13 and 23 months old) produced T at levels comparable to young rats which was substantially higher than 23 month controls (Chen & Zirkin, 1999). One could conclude that because these cells were not producing T the Leydig cells were less susceptible to oxidative stress and therefore aging.

As previously mentioned, aging men experience a substantial decline in measurable serum T levels. These declining levels are accompanied by age-related changes in the T biosynthesis pathway, namely LH receptor-stimulated cAMP production and levels of key proteins including StAR, TSPO, and some of the steroidogenic enzymes (Chen et al. 2004; Diemer et al., 2003; Luo et al., 2005). Reduced levels in StAR and TSPO have been of great research interest because of their function in the mobilization from cholesterol from cytosolic stores to the mitochondria. A shift in the redox environment of steroidogenic cells that results increased in oxidative stress is

thought to be the culprit of reduced levels of these key proteins and steroid formation (Diemer et al., 2003; Quinn & Payne, 1984; Quinn & Payne, 1985). Oxidative stress is brought about by reductions in enzymatic (GSH peroxidase, and Cu, Zn-superoxide dismutase, Mn-dismutase) and non-enzymatic antioxidants (GSH, ascorbic acid, α-tocopherol) and increased susceptibility to free radical production by both the electron transport chain and mitochondrial cytochrome P450 enzymes (Cao et al., 2004; Hanukoglu, 2006; Cui et al, 2012). Therefore, without the protective action of antioxidants, the electrophilic properties of free radicals, such as hydrogen peroxide, superoxide, and hydroxyl radicals, can lead to severe macromolecular damage, including lipid peroxidation and formation of protein and DNA adducts (Cao et al., 2004).

Among the cellular changes observed as Leydig cells begin producing less T over time, the non-enzymatic antioxidant, GSH, has been of great interest. It is the most abundant antioxidant in cells with the primary function of ridding the body of ROS and harmful xenobiotic electrophiles to prevent oxidative damage and reduce adduct formation (Forman et al., 2009; Yu, 1994). As such, a number of studies used a compound called buthionine sulfoximine (BSO) to diminish GSH levels in order to observe the effects on steroid synthesis (Chen et al., 2008; Chen, Zhou, et al, 2010). GSH is a tri-peptide thiol that requires two ATP dependent enzymes, γ-glutamylcysteine ligase and GSH synthetase, for its production (Griffith & Meister, 1979). BSO is a potent inhibitor of γ-glutamylcysteine ligase, which is important for these studies because this enzyme catalyzes the rate-determining step in GSH biosynthesis (Griffith & Meister, 1979). In one study, researchers extracted and cultured in vitro Leydig cells from Brown Norway rats; they found that by diminishing available GSH using BSO these cells produced significantly less T (Chen et al., 2008). The same study found that administering BSO directly to Brown Norway rats in vivo via injection resulted in the same decline in T synthesis (Chen et al., 2008). Another

study used MA-10 tumor Leydig cells, which are cells that produce progesterone (P) as an end product instead of T through a very similar pathway. In this study, scientists depleted GSH using two different compounds, BSO and dimethyl maleate (DEM), and observed the effects on P production. Additionally, a subset of cells was exclusively exposed to the pro-oxidant, tert-butyl hydroperoxide (t-BuOOH). They found that using these compounds to diminish GSH levels as well as exclusively subjecting cells to t-BuOOH did not result in a drop in steroid synthesis (Chen, Zhou, et al., 2010). Interestingly though, incubating the MA-10 cells with BSO or DEM while also subjecting these cells to t-BuOOH researchers observed a significant decline in P production (Chen, Zhou, et al., 2010). This could be a result of differences between the internal environments of the MA-10 Leydig cells and primary Leydig cells of Brown Norway rats. The MA-10 cells are also cells derived from Leydig cell tumors that are alternatively grown in culture, which could potentially account for the differences observed. Nonetheless, it is clear from these studies that even if oxidative stress is not exclusively responsible, it certainly plays an important role in the decline in T biosynthesis observed over time.

Intrinsic: Increase in cyclooxygenase-2

Cyclooxygenases are important enzymes involved in the production of prostacyclins, thromboxanes, and prostaglandins (PGs) (Huang et al., 2003). PGs are involved in the regulation of several biological processes such as osmotic balance, blood pressure, and immune responses among others (Huang et al., 2003). Cyclooxygenase is expressed in two isoforms. The first, COX-1, has been found to be expressed constitutively in cells whereas the second, COX-2, is expressed in response to a variety of cellular changes one of which is hormone stimulation (Chen et al, 2007). When LH binds to its receptor, arachidonic acid is released from Leydig cell membranes and can be used as a substrate for COX-2 catalyzed production of PG. Research has

shown that suppression of steroidogenesis is concomitant with PG production (Chen et al, 2007). Additionally, COX-2 levels are considerably higher in the Leydig cells of aged Brown Norway rats suggesting that COX-2 expression negatively regulates steroid production (Frungieri et al., 2006). This has been supported by a study that used COX-2 inhibitors. This study showed that, in fact, these inhibitors led to increased T production in aged Brown Norway rats providing evidence for the theory that COX-2 has conflicting effects on steroid synthesis (Frungieri et al., 2006). COX-2 expression levels have been documented in men with infertility issues that are presumably is driven by low T levels (Frungieri et al., 2006). Consequently, the age-related increases in COX-2 expression observed in Brown Norway rats could also be reflected in primary hypogonadal men and thus provide an additional intrinsic mechanism by which aging leads to declines in serum T.

Extrinsic: Phthalate Exposure

Much of the research into low serum T levels of hypogonadal men has been focused on investigating the intrinsic causes of aging. Accordingly, there has been a great deal of evidence to support the free radical theory of aging on which a lot of Leydig cell aging hypotheses are based. But, it goes without saying that there are extrinsic factors that could be at play here as humans are exposed to countless xenobiotics. The investigation of exposures to environmental toxicants provides an alternate approach to understanding how Leydig cell aging occurs. Phthalate exposure has been linked to major deficits in the structure and functioning of the male reproductive tract including morphological changes, reproductive toxicity, and low T.

Phthalate esters are potent endocrine disruptor molecules; these derivatives of phthalic acid are chemicals that are manufactured to create and promote the malleability and longevity of a broad range of consumer products that include but are not limited to: paints, personal care

products, toys, food packing containers, and pharmaceuticals etc. (Gao et al., 2017; Ha et al., 2016; Motohashi et al., 2016). Therefore, it is no question that humans are exposed to phthalates daily. Because of this, the United States Environmental Protection Agency (US EPA) has classified many phthalates as priority pollutants (US EPA, 2007). This organization expressed concern and strongly advocated for methods to prevent or reduce exposure to phthalates. Relevant exposures, such as microwaving food in plastic containers and infants' mouthing of toys, have been difficult to quantify, though, due to problems with obtaining reliable data (US EPA, 2007). Nonetheless, human phthalate exposure is ubiquitous and affects all humans throughout their lifespan.

Because of the hydrophobic nature of some phthalate esters, exposure to these chemicals begins even before birth. The placenta allows the slow transfer of short-chain esters from the maternal blood supply to the fetus (Mose et al., 2007). One study looked at how orally administering in four different doses (0, 10, 50, and 100 mg/kg) a commonly found phthalate, di(n-butyl) phthalate, to the pregnant female Sprague-Dawley rats would affect male reproductive function (Motohashi et al., 2016). This lab found time and dose dependent changes in Leydig cell mitochondrion morphology, and reductions in steroidogenic enzymes and T levels of male pups. Phthalate metabolites have been found in breast milk of pregnant women citing one important source by which infants are exposed indicating that exposure after birth can occur through the mother (Motohashi et al., 2016). Another study explored, in vivo and in vitro, the effects of a different phthalate ester, di(2-ethylhexyl) phthalate (DHEP), on testicular structures and T production (Ha et al., 2016). The in vivo approach involved the administration of DHEP to Sprague-Dawley rats by gavage for 30 days. In the in vitro approach, a mouse Leydig cell line (TM3 cells) was treated with DHEP for 24 hours. The in vivo study revealed that exposure to

DHEP could lead to morphological malfunctions of seminiferous tubules, mitochondria, chromatin, as well as germ and Sertoli cell death. Results of both studies showed that DHEP exposure was correlated reductions T production. Concomitant measurements of two groups of molecules indicated that two distinct pathways were contributing to the reductions in T levels. It was observed lipid peroxidation was increased as well as ERK pathway induced 5α-reductase 2 (an enzyme that converts T to dihydrotesterone; DHT) suggesting oxidative damage and irreversible conversion of T to DHT provided one mechanism for reduced T (Ha et al., 2016). Additionally, serum LH levels (but no changes in GnRH) were reduced in the Sprague-Dawley rats indicating that there were abnormalities with the HPG axis (Ha et al., 2016). Although these mechanisms might be different from men with low T, these results strongly support the fact that phthalate exposure affects testicular morphology and function.

The two studies cited above looked at phthalate exposures that were acute in comparison to that what would be expected over the course of a typical human lifetime. Further, these studies exposed rats to high levels of one type of phthalate, but in reality humans are exposed to many different types of phthalates (among other things). Thus, a more relevant study would be one that analyzes the effects of human applicable exposures over a long duration. One study did this by examining the effects of long-term low-dose exposure to a mixture of six phthalates: di-n-octyl phthalate (DNOP), dimethyl phthalate (DMP), di(n-butyl) phthalate (DBP), di(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), and butyl benzyl phthalate (BBP) (Gao et al., 2017). This phthalate mixture was orally administered at three low doses by gavage to male Sprague-Dawley rats daily for 15 weeks. Vital proteins in the T biosynthesis pathway, like StAR, Cytochrome 450 enzymes, and 17β-HSD, were reduced in exposed mice (Gao et al., 2017). These lowered protein levels were accompanied by significant declines in serum T and ITT

without decreasing serum LH levels (Gao et al., 2017). Hence, this study provides more pertinent evidence that low dose exposure to phthalates over an extended period of time could, in fact, pose a threat to the reproductive homeostasis and adequate T levels.

Therapy for Hypogonadism

Testosterone Replacement Therapy

Various studies have attempted to determine the prevalence of male hypogonadism, but there doesn't seem to be a clear consensus on the number of affected individuals (Araujo et al., 2004; Araujo et al., 2007; Harman et al., 2001; Morley et al., 1997; Pastuszak et al., 2016; Wu et al., 2010). This is because successful diagnosis is heavily dependent on early morning serum T measurement, as that is the time when the T levels are the highest (McGill et al., 2012). Because this isn’t a test run during routine checkups, diagnosis is reliant on men reporting symptoms to their doctors, not to mention many facilities don't have the resources necessary to perform T measurements (McGill et al., 2012). Possibly the most unfortunate characteristic of this syndrome is that many of its symptoms overlap with those of other diseases and conditions. Consequently, the diagnosis of this condition is extremely difficult. Even worse, there aren’t many therapies for androgen deficiency. The main treatment prescribed by physicians, though, is T replacement therapy (TRT).

TRT is primarily used to treat the burdensome symptoms of androgen deficiency, such as lower urinary tract symptoms, erectile dysfunction and lowered energy, sex drive, muscle mass, bone loss etc. (McGill et al., 2012). Advances in TRT have led to the development different ways in which T can be administered. Injections, transdermal patches and gels are the main modes of delivery, but these methods aren’t without issues. The injections usually elicit substantial fluctuations in serum T necessitating adjustment to find the appropriate dosage (Beattie et al., 2015; Surampudi et al, 2012). The transdermal approaches circumvent this

problem by releasing constant T, but they have drawbacks as well. The gel can rub off via skin contact with a partner for example (Beattie et al., 2015; Surampudi et al, 2012) and the patch can be irritating or detach from the skin due to sweating/contact with water (Ullah et al., 2014). Nonetheless, studies have shown that TRT has been effective in improving hypogonadal symptoms, particularly impotence, lower urinary tract symptoms, and erectile dysfunction (Yassin et al, 2014 ; Yucel et al., 2017).

Despite being the most commonly used therapy and its validity in treating the symptoms of male hypogonadism, TRT use has been called into question because of its proposed links to major diseases affecting men, namely prostate cancer and cardiovascular diseases/events. One early study found that male castration resulted in a regression of metastatic prostate cancer and prostate cancer was activated (measured by serum acid phosphatase levels) after androgen injections suggesting that T influenced prostate cancer progression (Huggins & Hodges, 2002). There have been studies done that have implicated TRT as a contributor to recurrence in previous prostate cancer patients and development of cancer in high-risk men (Fowler & Whitmore, 1981; Prout & Brewer, 1967). There is not enough evidence to definitively link TRT to progression or recurrence in men with a history of prostate cancer or rule out such a link (Pastuszak et al., 2016). Further, examining PSA levels is used as an initial screening procedure during the work up of males over the age of 40. A meta-analysis of randomly controlled trials found no significant association between TRT and prostate-specific antigen (PSA) levels (Kang & Li, 2015). There are other studies that support that TRT is not associated with an increased incidence of prostate cancer (Coward & Carson, 2009; Haider et al, 2009;,39; Jin et al., 2001). In fact, it appears that age is the most important and reliable characteristic associated with progression of prostate cancer (Jin et al., 2001). However, physicians as well as well-informed

cancer patients can be reluctant to use TRT to treat hypogonadism if it presents as a comorbidity necessitating more concrete data.

Like studies into prostate cancer, there has been controversy over the proposed link between TRT and cardiovascular disorders. An epidemiological study assessed the risk of cardiovascular dysfunction in hypogonadal men using TRT (Maggi et al., 2016). This study looked at a large cohort of European men and found no TRT related ties to adverse cardiovascular events in these men. However, a recent controlled clinical trial analyzed association between TRT and non-calcified coronary plaque volume, as a measure cardiovascular risk (Budoff et al., 2017). It was a double blind experiment consisting of mainly older white men conducted over the course of 12 months. After a year, there was significant correlation between TRT and non-calcified coronary plaque volume determined by computed tomography angiography (Budoff et al., 2017). There are other studies that confirm this denoting that exogenous T administration is linked to other cardiovascular events/conditions, such as myocardial infarction (heart attack), coronary artery disease, and erythrocytosis leading other adverse cardiovascular events (Bachman et al., 2010; Basaria et al., 2010; Calof et al., 2005; Coviello et al, 2004; Finkle et al., 2014). This evidently conflicts with the conclusions of the epidemiological study mentioned previously as well as many other emerging studies. It goes without saying that much of the research done to look at the correlation between TRT and these diseases is riddled with bias, particularly selection bias. For example, many of these studies looked at primarily older white men, yet this is not the only population that is affected by hypogonadism. Therefore, more research into the possible associations between TRT and other health complications are necessary.

Although not observed in all men, TRT has been shown to reduce endogenous T production ultimately leading to azoospermia (Brumner et al., 1991; Murdoch & Goldberg, 2014; Tom et

al., 1992). This makes sense as the exogenous T administration leads to a suppression of gonadotropins from the pituitary resulting in lower ITT and thus low sperm count. Adequate ITT is necessary in order to maintain spermatogenesis in men and other male mammals (McLachlan et al., 2002; Weinbauer & Nieschlag, 1993; Zirkin et al., 1989). As previously stated, hypogonadism primarily affects men beginning in their 40s or 50s. However, hypogonadism does affect some younger men. These men, unlike many of the men who are associated with this condition, may wish to father children. Because of the contraceptive properties of exogenous T, TRT is not an adequate treatment for these men. Thus, there has been increasing interest in establishing validity for other therapies that can raise serum T without suppression the endogenous T production and spermatogenesis. As such, TSPO drug ligands have become area of interest for treatment of these young hypogonadal men who wish to have children.

TSPO drug ligands

TSPO is arguably the most important protein in the T biosynthetic pathway, as it is a protein that is a member of both the transduceosome and the metabolon. Its role is to facilitate the movement of cholesterol through the mitochondrial membrane and ultimately its delivery to CYP11A1, the rate-determining step in T synthesis. However, one study has refuted the TSPO's assumed vitality. In this study, the research team was able to successfully create TSPO null mice. Examination of spermatogenesis, reproductive capacity, and T production showed that all were unaffected (Motohashi et al., 2016). Closer analysis revealed that levels of other key molecules of T pathway, namely 3β-HSD, StAR, and CYP11A1, were also unchanged (Motohashi et al., 2016). The conclusion drawn by this research team was that TSPO is not essential for T production. On the contrary, other TSPO null research has challenged the validity of this study, as these knockout experiments showed significant reductions in steroid synthesis (Fan et al.,

2015; Fan et al, 2017; Owen et al., 2017). The former group didn't provide an alternative mechanism to explain normal steroidogenesis in these TSPO knockout mice. It is possible that in TSPO deficient cells other proteins are compensating for the loss of this protein. Further, MA-10 cell research has shown that TSPO knockout increases ROS production (Tu et al., 2016). Because elevated ROS is thought to be linked to cell aging and lower steroid production (due to TSPO's function in the transduceosome and metabolon), it seems unlikely that knocking down TSPO would leave steroid production unaffected. It appears that this protein is indeed very important to adequate steroid production whether it is present or not.

It appears that, when TSPO is present, it can be utilized in order to facilitate steroid production. TSPO consists two domains, the CRAC motif that binds cholesterol and the benzodiazepine site that binds a number of compounds. In the presence of a TSPO agonist, these two regions seem to work in concert to speed the movement of bound cholesterol into the mitochondrion thereby improving the efficiency of the rate limiting step in steroid synthesis (Midzak, Akula, et al., 2015).

Low steroid concentration in the brain has been linked to neurological issues, such as depression and anxiety (Rasmusson et al., 2006; Uzunova et al, 1998). Production of steroids within the brain has been found to modulate gamma-aminobutyric acid type A ($GABA_A$) receptor activity. Studies have shown that these neurosteroids bind to $GABA_A$ receptors enhancing their activity which results in reductions in stress responses as well as analgesic, anticonvulsant, and anxiolytic effects (Barbaccia et al., 1996; Brunton et al, 2009; Patchev et al., 1994; Patchev et al., 1996). With this in mind, new research has been geared towards TSPO as a target to treat neurological disorders, especially anxiety disorders. Currently, the main treatments for anxiety disorders are benzodiazepines and selective serotonin reuptake inhibitors (SSRIs).

Many patients taking benzodiazepines report negative side effects, tolerance, and withdrawal issues (Bandelow et al., 2008). Although SSRIs are often reliable, it takes a while for patients to experience the positive effects and sometimes during this time their initial symptoms worsen (Bandelow et al., 2008). Researchers have been interested in using TSPO drug ligands to test it as an alternative treatment for these disorders. One drug ligand, XBD-173, has been shown to stimulate GABAenergic neurotransmission and liberate experimentally-induced panic attacks in rodent models without the sedation or withdrawal symptoms (Rupprecht et al., 2009). It also showed fast acting anxiolytic effects in human volunteers (Rupprecht et al., 2009). There have been clinical trials conducted to investigate its safety and efficacy with results that seem promising (Rupprecht et al., 2010).

Although there are limited published studies looking at how TSPO agonists affect steroid production in the male, TSPO could be a promising pharmacological target particularly for reversal of declining serum T levels that are observed with age. Some studies have induced hypogonadism in young rats using a GnRH antagonist (cetrorelix). Administration of TSPO ligand to these animals resulted in significantly higher ITT, illustrating that these ligands could reverse induced hypogonadism (Aghazadeh et al., 2014; Papadopoulos et al., 2015). Perhaps, the most exciting and relevant research with respect to the treatment of hypogonadal men was a reversal study that looked at how TSPO ligands affected ITT and serum T levels in primary hypogonadal rats. This study utilized an in vivo and an in vitro approach to examine the effects of two distinct TSPO drug ligands, N,N-dihexyl-2-(4-fluorophenyl)indole-3-acet-amide (FGIN-127) and benzodiazepine 4’-chlorodiazepam (Ro5-4864) (Chung et al., 2013). In the in vitro experiment, Leydig cells from both young and old Brown Norway rats were cultured with each of the TSPO drug ligands. Similarly, the in vivo study involved the injection of these drug

ligands. The approaches showed that FGIN-127 substantially increased T production by old cells and in old rats to that of their younger counterparts. FGIN-127 is currently being evaluated in labs to determine the extent of its stimulatory effects with respect to concentration and time. Further, research groups are studying how these drug ligands stimulate steroidogenesis because the precise mechanisms by which this occurs is still unclear (Papadopoulos et al., 2015). In spite of its unknown mechanism, these compounds are prime drug candidates that can be utilized for treating male hypogonadism.

INTRODUCTION

Leydig cells are testosterone (T)-producing cells localized in the interstitial compartment of the mammalian testis. These cells are stimulated by luteinizing hormone (LH) released from the anterior pituitary, which elicits cAMP-mediated transport of cholesterol through the outer mitochondrial membrane (OMM) to inner mitochondrial membrane (IMM) protein cytochrome P450 side-chain cleavage enzyme (CYP11A1). This enzyme carries out a side cleavage reaction converting cholesterol to pregnenolone. Then, pregnenolone is shuttled to the smooth endoplasmic reticulum (ER). It is quickly converted to progesterone by 3β-Hydroxysteroid dehydrogenase (3β-HSD). Progesterone and its successor are acted on by cytochrome p450 17 (CYP17) forming 17α-hydroxprogesterone and androstenedione respectively. Finally, 17β-Hydroxy steroid dehydrogenase (17β-HSD) catalyzes the last step converting androstenedione to T (Midzak et al., 2009; Miller & Bose, 2011; Rone et al., 2009).

Translocator protein (18-kD TSPO), an OMM protein, appears to be crucial for normal steroidogenesis, as it moves cholesterol from an intracellular source through the OMM to CYP11A1, the rate-limiting step in steroid synthesis (Midzak, Rone et al., 2011; Papadopoulos et al., 2015). TSPO contains two distinct domains that are important for cholesterol transport, a benzodiazepine-binding site and a cholesterol recognition amino acid consensus (CRAC) site (Midzak, Akula, et al., 2015). Structural characterization studies that have used recombinant TSPO support its proposed structural and functional significance (Murail et al., 2008). Site-specific mutagenesis has also been employed to disrupt the CRAC domain, which resulted in an inability of TSPO to bind cholesterol (Jamin et al., 2005). The benzodiazepine-binding site has been found to bind a number of compounds. In fact, one study showed that TSPO's binding of ligands stabilizes its secondary and tertiary structures (Lacapere et al., 2001; Murail et al., 2008).

Ligand binding also seems to stimulate the cholesterol translocation activity of TSPO, making this protein a promising pharmacological target (Midzak, Akula, et al., 2015; Midzak, Zirkin et al., 2015).

However, one research group has provided results refuting TSPO's assumed vitality to steroidogenesis. In this study, TSPO null mice were created. Examination of spermatogenesis, reproductive capacity, and T production revealed that all were unaffected (Motohashi et al., 2016). Closer analysis showed that levels of other key molecules of T pathway, namely 3β-HSD, steroidogenic acute regulatory protein (StAR) protein, and CYP11A1, were also unchanged (Motohashi et al., 2016). The conclusion drawn by this research team was that TSPO is not essential for T production. On the contrary, one prior study showed that selective TSPO knock down using a Cre-recombinase system resulted in significant reductions hormone-stimulated steroidogenesis (Fan et al., 2015). Additionally, two subsequently published studies showed that TSPO disruption resulted in reduction in steroid production (Fan et al, 2017; Owen et al., 2017). Specifically, Fan et al generated two TSPO mutant MA-10 cell lines using the CRISPR-Cas9 system. Both of these lines showed significant reductions in steroid formation even upon the administration of a cAMP analog, dibutyryl cAMP (Fan et al, 2017). Further the Owen et al. study showed that a human polymorphism in TSPO's CRAC domain resulted in an amino acid substitution, which presumably reduces its ability to bind cholesterol. This polymorphism is observed in humans that have anxiety disorders and often these disorders are caused by low steroid concentrations in the brain. Thus, it has been proposed that this polymorphism impairs TSPO's function leading to reduced neurosteorid production and psychological disorders. Owen et al. found that by recapitulating this in a rodent model there was indeed a reduction in steroid

production. Also, in vitro studies also showed that the corresponding TSPO mutation resulted in lowered cholesterol affinity (Owen et al, 2017).

The group that reported TSPO deletion had no effect on steroidogenesis provided no alternative hypothesis for this observation. It is possible that in TSPO deficient cells other proteins are compensating for the loss of this protein. MA-10 cell research has shown that TSPO knockout increases ROS production (Tu et al., 2016). Because there is a proposed link between elevated ROS and both cell aging and lower steroid production (due to TSPO's function in the transduceosome and metabolon), it seems unlikely that knocking down TSPO would leave steroid production unaffected. It appears that this protein is indeed very important to adequate steroid production whether it is present or not. In accordance with studies supporting TSPO's role in steroidogenesis, introduction of TSPO cDNA into TSPO deficient cells is able to rescue their steroidogenic function (Papadopoulos et al., 1997).

With aging, Leydig cells of both men and rodents produce less T. Analysis of aged Leydig cells has revealed that there are concomitant reductions in TSPO, LH receptor-stimulated cAMP synthesis, StAR, and enzymes in the mitochondria and smooth ER (Chen et al. 2004; Diemer et al., 2003; Luo et al., 2005). Low serum T results in a condition that is clinically referred to as hypogonadism and is thought to affect 4 to 5 million men in the United States, with the majority being 60 years or older (Zirkin et al., 2018). Insufficient production of androgen is accompanied by many symptoms that include but are not limited to: decreased muscle mass, increased weight gain, and low bone density, cognitive changes, erectile dysfunction, increased fatigue, and low libido (Kumar et al., 2010, Ullah et al., 2014). Primary hypogonadism describes men who show significant reductions in serum T over their lifespan despite having normal levels of LH. Therefore, the observed decline in T levels cannot be attributed to inadequate LH production and

release by the anterior pituitary but rather to the Leydig cells' insensitivity to LH (Chen et al., 2002). It has been difficult to pinpoint exactly how these defects come about, but generally aging is coupled with both intrinsic (ie. oxidative stress) and extrinsic (ie. environmental exposures) changes that could result in reduced steroidogenic function (Cui et al, 2012; Rebrin & Sohal, 2008).

This has brought about considerable interest in therapies to combat both the decline in T production and the side effects of hypogonadism. T replacement therapy (TRT) has been used largely to treat the symptoms (Ullah et al., 2014). This mode of action is met with some benefits but also has a number proposed of risks, such as increase susceptibility to prostate cancer and cardiovascular events (Bachman et al., 2010; Basaria et al., 2010; Budoff et al., 2017; Calof et al., 2005; Coviello et al, 2004; Finkle et al., 2014; Fowler & Whitmore, 1981; Maggi et al., 2016; Prout & Brewer, 1967). The correlation between TRT and these diseases is controversial, though, and its research ongoing.

Although exogenous T administration leads to an increase in serum T, it also results in suppression via negative feedback of the release of gonadotropin-releasing hormone (GnRH) and gonadotropins (LH and follicle-stimulating hormone) from the hypothalamus and pituitary, respectively. Reduced LH leads to lower ITT and eventually sperm count. In one important experiment, Sprague-Dawley rats were administered T-containing silastic implants of increasing sizes (Zirkin et al, 1989). The size of the implant correlated with increases in measured serum T. After dropping below certain concentration of T (~20ng/ml) within the testis, there was a significant reduction in sperm count indicating that a certain amount of T is necessary in order to maintain spermatogenesis (Zirkin et al, 1989). This has also become clear through experiments in which T was administered to healthy men. The TRT administration inhibits the production of

endogenous T due to negative feedback thereby substantially reducing ITT levels (Coviello et al., 2004). These low ITT levels lead to significant reductions in sperm count and germ cell loss (Coviello et al., 2004; Hill et al., 2004). Thus, adequate ITT levels are necessary in humans in order to maintain sperm numbers (McLachlan et al., 2002; Weinbauer & Nieschlag, 1993; Zirkin et al., 1989).

Hypogonadism primarily affects men beginning in their 40s or 50s. However, hypogonadism also affects some younger men (Yin & Swerdloff, 2010). These men, unlike many of the men who are associated with this condition, may wish to father children. Because of the suppressive effects of exogenous T on both the hypothalamus and the pituitary, TRT is not an adequate treatment for these men (Brumner et al., 1991; McLachlan et al., 2002; Murdoch & Goldberg, 2014; Tom et al., 1992; Weinbauer & Nieschlag, 1993; Zirkin et al., 1989). Thus, there has been increasing interest in establishing validity for other therapies that can raise serum T without suppression the endogenous T production and spermatogenesis. Stimulation of aged rats in vivo and aged Leydig cells in vitro with TSPO drug ligands have been shown to increase steroid production in some laboratory studies (Aghazadeh et al., 2014; Chen et al. 2004; Chung et al., 2013; Papadopoulos et al., 2015). As such, TSPO drug ligand administration could become a treatment method of these young hypogonadal men who wish to have children. We hypothesized herein that administering TSPO drug ligands would increase serum T without reducing ITT levels, and therefore maintaining spermatogenesis. This is in contrast to TRT, which would increase serum T levels but would reduce in ITT levels due to negative feedback on the hypothalamus and pituitary and eventually reduce spermatogenesis.

To test this hypothesis, we determined the in vivo effects of administering high affinity TSPO drug ligands FGIN-1-27, XBD-173, and Ro5-4864 to aged (21 months) Brown Norway

rats on serum T and ITT and compared these results to aged controls. We also administered exogenous T via T-containing Silastic implants to aged rats and observed how the effects on serum T and ITT compared to aged controls. We furthered these efforts by determining how T implants affected seminiferous tubule fluid (STF) T and sperm production in young Brown Norway rats. We show that administering T implants significantly increased serum T in aged rats, but decreased intratesticular T (ITT) levels significantly when compared to aged controls. Conversely, FGIN-1-27 (but not XBD-173 at 1 mg/kg body weight and Ro5 at 3 mg/kg body weight) administration to aged rats at 1 mg/kg body weight significantly increased serum T and maintained ITT levels compared to aged controls. We also show that T implants significantly reduced STF T and sperm count in young rats. The 2 cm capsule given to young rats resulted in the most significant reduction in STF T levels and sperm count when compared to controls. Because the STF T levels of young rats that received 2 cm implants were strikingly similar to ITT levels of old rats that received 2 cm implants, it can be inferred that the aged rats that received 2 cm capsules also would show a significant reduction in sperm count. On the contrary, ITT levels of the FGIN-1-27 treated old rats were significantly higher than ITT levels of aged controls. This suggests that spermatogenesis would not be suppressed in these FGIN-1-27 treated hypogonadal rats and FGIN-1-27 administration might even enhance this process. These results provide early evidence that pharmacological stimulation of TSPO could serve as a novel therapy for young hypogonadal men.

MATERIALS AND METHODS

Reagents

N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN-1-27), benzodiazepine 4’-chlordiazepam 4′-chlorodiazepam (Ro5-4864), N-benzyl-N-ethyl-2-(7-methyl-8-oxo-2-

phenylpurin-9-yl)acetamide (XBD-173) were obtained from Sigma-Aldrich (St Louis, Missouri). [1,2,6,7,16,17-3H(N)]-Testosterone was from PerkinElmer Life Sciences, Inc (Boston, Massachusetts). T antibody was from MP Biomedical (Solon, Ohio).

Effect of T-containing Silastic implants and TSPO drug ligands on T production in vivo

Young (3 months old) and aged (21 months old) Brown Norway rats were obtained through the National Institute on Aging (Bethesda, Maryland). They were housed in the animal facilities of Johns Hopkins Bloomberg School of Public Health (Baltimore, Maryland) in controlled light (14-hour light, 10-hour dark) at a temperature of 22°C with free access to food and water. All animal handling and care were in line with the protocols approved by the institutional Animal Care and Use Committee of Johns Hopkins University.

To determine the in vivo effect of TSPO ligand on T production, aged rats received FGIN-1-27 or XBD-1-27 dissolved in 10% DMSO (1 mg/kg body weight) via daily ip injection. After 10 days, serum and intratesticular fluid were collected for T measurement by radioimmunoassay (RIA). A preliminary experiment was conducted in which aged rats received Ro5-4864 dissolved in 10% DMSO (3 mg/kg body weight) via daily ip injection. After a 5-day period, serum and intratesticular fluid were collected for T measurement by RIA. To compare TSPO drug ligands to exogenous T administration, aged rats were given subdermal 2 cm T-containing Silastic implants. After 10 days serum and intratesticular fluid were collected for T measurement by RIA. Each experiment included control groups, both young and old, that were injected with vehicle (10% DMSO) with serum and intratesticular fluid collected for T measured via RIA to compare to the experimental groups. Finally, experimental rats received subdermal T implants totaling 1, 2, 6, 6, 12, or 24 cm in length. After 8 weeks, rats were euthanized by decapitation, trunk blood was collected, and serum was prepared and stored frozen at -20 C for subsequent

determination of T by RIA. Both testes were removed and weighed. Briefly, IF and STF were collected as follows. The tunica albuginea was incised at one pole, and testes were centrifuged at low speed (54 x g; 10 min; 0 C) to drain IF. Subsequently, testes were decapsulated and rinsed thoroughly to remove residual IF. The seminiferous tubules were then extruded through the hub of a syringe, and the preparation was centrifuged (6,000 X g; 15 min; 0 C) to collect STF as a supernatant above the collapsed seminiferous tubules. Immediately after collection, IF and STF were snap-frozen in liquid nitrogen and subsequently stored frozen at -80 C before assay for T. The contralateral testis from each rat was used for determining the numbers of advanced spermatids per testis by the hemacytometric counting of testicular homogenates under phase contrast microscopy (Zirkin et al, 1989).

Statistical analysis

Data are expressed as means ± SEM of at least 3 independent experiments (except for experiments with Ro5-4864, XBD-173, and spermatogenesis). For two group comparisons, a student t-test was performed. Values were considered significant at $P<0.05$.

RESULTS

Hypogonadism in Brown Norway rats

Figure 4A shows that serum T is significantly lower (~74%) in aged Brown Norway rats when compared to young rats. This is important as it displays the manifestation of hypogonadism in these rats. As shown in Figure 4B, aged rats also display a significantly lower concentration of T (~37%) within the testis compared to young rats.

In vivo effects on aged rats with T-containing Silastic implants

Two-centimeter T-containing Silastic implants were administered to aged Brown Norway rats. Control rats received vehicle. After a 10-day period, serum and intratesticular fluid were

collected for T measurement by RIA. Figure 5A illustrates that the implanted animals have significantly higher serum T levels compared the old controls, an increase of almost 5-fold. In contrast, Figure 5B displays a significant decrease in intratesticular testosterone (ITT) in rats administered implants compared to control rats (a decline of about 57%).

In vivo effects of a TSPO drug ligand (FGIN-1-27) in aged rats

To determine the effect of TSPO stimulation of T production in vivo, FGIN-1-27 was administered to aged rats via daily ip injection at a concentration 1 mg/kg body weight over the course of 10 days. Control rats received vehicle. Perhaps, the most exciting results are displayed in Figure 6A showing a 2-fold increase in serum T in FGIN-1-27 treated aged rats that was statistically significant. This was matched with a significant 1.5-fold increase in ITT in these rats compared to controls. There were no major changes in body weights of these animals denoting that there were not cytotoxic effects of any of the TSPO drug ligands.

Relationship between intratesticular T concentration and spermatogenesis in young rats

T-containing Silastic implants of increasing sizes were administered to young Brown Norway rats. After 8 weeks, STF was collected to measure T via RIA and testes were used to determine the numbers of advanced spermatids per testis by the hemacytometric counting of these cells in testicular homogenates. The effect of T-containing Silastic implants on STF T concentration and sperm counts is shown in Figure 7. The STF T concentration (7A) and sperm count (7B) in control rats were 31.2±2.5 ng/ml and 214.9±4.5 x 10^6 sperm/testis, respectively. In rats that received 2 cm implants, the STF T concentration (7.5±0.6 ng/ml) and sperm count (34.1±16.0 x 10^6 sperm/testis) were reduced to their lowest values which were both significantly less than those of control rats. There were step increases in the STF T concentration and sperm count of rats receiving implants greater than 2 cm. Comparison of Fig. 7A and 7B show that

sperm numbers could be maintained quantitatively at about 15-20 ng/ml T in the STF. As shown in Figure 4B, intratesticular T levels in old controls, though reduced from that of young controls, is about 20 ng/ml, sufficiently high to maintain spermatogenesis quantitatively. Whereas T implants reduced ITT levels to below 10 ng/ml (Fig. 5B), administering FGIN to the old rats resulted in ITT levels of about 30 ng/ml, well above the level required for spermatogenesis maintenance.

In vivo effects of other TSPO drug ligands (XBD-173 and Ro5-4864) in aged rats – preliminary studies

After observing the effects of FGIN-1-27 on serum and ITT, preliminary studies were conducted to determine if other TSPO drug ligands could elicit similar responses in hypogonadal rats. Therefore, preliminary studies were done to determine whether stimulation of TSPO using drug ligands XBD-173 and Ro5-4864 would increase serum T levels without reductions in ITT levels in old rats. XBD-173 was administered to aged rats via daily ip injection at a concentration 1 mg/kg body weight over the course of 10 days. Control rats received vehicle. Ro5-4864 was administered to aged rats via daily ip injection at a concentration of 3 mg/kg body weight over a 5 day period. Control rats received vehicle. Aged rats administered XBD-173 or Ro5-4864 did not show changes in serum T (Figures 8A and 9A) or ITT (Figures 8B and 9B) when compared to control rats.

DISCUSSION

Aging is accompanied by reduced serum T concentrations in both men and Brown Norway rats (Chen et al., 2002; Liu et al., 2005; Veldhuis et al., 2012; Zirkin & Chen, 2000). This condition, clinically termed hypogonadism, is thought to affect 4 to 5 million US men with reports stating that 20% to 50% of men over 60 are affected (Araujo et al., 2004; Araujo et al.,

2007; Bhasin, & Basaria, 2011; Harman et al., 2001; Morley et al., 1997; Surampudi et al, 2012 Zirkin et al., 2018, 72,). These men experience a number of uncomfortable symptoms including fatigue, decreased muscle mass, weight gain, osteoporosis, depression, erectile dysfunction, and low libido (Kumar et al., 2010, Ullah et al., 2014) Hypogonadal men don't necessarily have fertility issues, but there are a lot of infertile males who have low T (Kim & Schlegel, 2008; Schlegel, 2009). This is a significant issue for infertile couples that are seeking medical advisement, as 40% to 50% of these cases can be at least partially attributed to the male (Hwang et al., 2011).

It has become clear that normal steroidogenesis is initiated by the binding of LH, which promotes cAMP-mediated cholesterol transport from the cytosol to the mitochondria, the rate-limiting step, where it begins its enzymatic conversion to T. StAR and TSPO are two key proteins that drive this process. StAR is a 37-kDa protein that has two distinct regions, hydrophobic STAR-related lipid transfer (START) domain that binds cholesterol and an N-terminal mitochondrial signal sequence that is removed after reaching the OMM (Aghazadeh et al., 2012; Issop et al., 2013; Liu et al., 2006; Rone et al., 2009). TSPO is an 18-kDa OMM protein that also contains two distinct domains, a benzodiazepine-binding (drug) site and a cholesterol recognition amino acid consensus (CRAC) site (Midzak, Akula, et al., 2015). It is thought that hormone stimulation results in StAR's mobilization of cholesterol. TSPO's high affinity CRAC motif subsequently promotes the capture of cholesterol from StAR. TSPO then aggregates and interacts with another OMM protein called VDAC forming a pore in the OMM for cholesterol delivery to CYP11A1. TSPO null research has highlighted its importance to steroidogenesis, as these knockout experiments showed significant reductions in steroid synthesis (Fan et al., 2015; Fan et al, 2017; Owen et al., 2017). Interestingly, in aged Leydig

cells, there are significant reductions in TSPO, LH receptor-stimulated cAMP production, and some of the steroidogenic enzymes (Chen et al. 2004; Diemer et al., 2003; Luo et al., 2005).

The main therapy for hypogonadal men, TRT, doesn't address the source of the intracellular dysfunctions, but rather attempts to alleviate the uncomfortable symptoms. Additionally, TRT has been controversial, as it seems to be tied to other health complications, such as cardiovascular problems and prostate cancer (Bachman et al., 2010; Basaria et al., 2010; Budoff et al., 2017; Calof et al., 2005; Coviello et al, 2004; Finkle et al., 2014; Fowler & Whitmore, 1981; Maggi et al., 2016; Prout & Brewer, 1967). Advances in TRT have led to the development different ways in which T can be administered. Injections, transdermal patches and gels are the main modes of delivery, but these methods aren't without issues. The injections usually elicit substantial fluctuations in serum T necessitating adjustment to find the appropriate dosage (Beattie et al., 2015; Surampudi et al, 2012). The transdermal approaches circumvent this problem by releasing constant T, but they have drawbacks as well. The gel can rub off via skin contact with a partner for example (Beattie et al., 2015; Surampudi et al, 2012) and the patch can be irritating or detach from the skin due to sweating/contact with water (Ullah et al., 2014). Nonetheless, studies have shown that TRT has been effective in improving hypogonadal symptoms, particularly impotence, lower urinary tract symptoms, and erectile dysfunction (Yassin et al, 2014; Yucel et al., 2017).

Although not observed in all men, TRT has been shown to reduce endogenous T production ultimately leading to azoospermia (Bremner et al., 1991; Murdoch & Goldberg, 2014; Tom et al., 1992). This makes sense as the exogenous T administration leads to the suppression of GnRH release from the hypothalamus and LH from the pituitary resulting in lower ITT and thus low sperm count. Appropriate ITT levels are necessary in order to maintain spermatogenesis in men

and other male mammals (McLachlan et al., 2002; Weinbauer & Nieschlag, 1993; Zirkin et al., 1989). As previously stated, hypogonadism primarily affects men beginning in their 40s or 50s, but a subset of these hypogonadal men are younger (Yin & Swerdloff, 2010). These men, unlike many of the men who are associated with this condition, may wish to father children. Because of the contraceptive properties of exogenous T, TRT is not an appropriate treatment for these men or infertile men who are hypogonadal.

Thus, research into other possible therapies has been of great interest. The most promising of these seems to be TSPO drug ligands for its ability to raise endogenous T production by aged Leydig cells. Despite age-related reductions in TSPO, we hypothesized that pharmacological stimulation of TSPO via drug ligands would increase serum T without reducing ITT levels and therefore maintain spermatogenesis. This is in contrast to exogenous T administration, which would increase serum T levels but would reduce in ITT levels due to negative feedback on LH and eventually reduce spermatogenesis. To test this, we compared serum T and ITT levels of aged rats receiving T implants to control rats. The same was done with FGIN-1-27 administration. We also gave T implants of increasing sizes to young rats. We show that the 2 cm capsule given to aged rats significantly increased serum T but significantly reduced ITT when compared to old controls. In striking contrast, FGIN-1-27 administration to old rats not only significantly increased serum T levels but also significantly increased ITT levels.

Out of all capsule sizes, the 2 cm capsule given to young rats resulted in the most significant reduction in STF T levels and sperm count when compared to controls. We observed that STF T levels of young rats that received 2 cm implants were comparable to ITT levels of old rats that received 2 cm implants. Because these concentrations were so similar, it can be inferred that, like the young rats that received 2 cm capsules, the aged rats that received 2 cm capsules also show a

significant reduction in sperm count. In striking contrast, ITT levels of FGIN-1-27 treated old rats have ITT levels that were much higher than both of these T implanted groups and were comparable to young implanted controls indicating that sperm count was unaffected. Even further, the ITT levels of these FGIN-1-27 treated old rats were significantly higher than ITT levels of aged controls suggesting that FGIN-1-27 administration might lead to an enhancement of spermatogenesis.

Taken together, this illustrated that exogenous T administration raised serum T levels but reduced ITT levels, resulting in reduced ITT and reduced sperm production. In striking contrast, the TSPO-specific drug ligand, FGIN-1-27, administration to aged rats not only raised serum T levels but also elevated ITT concentrations compared to old controls. From our results with the 2 cm implants, this implies that maintaining or increasing ITT levels, as we show with FGIN-1-27, could maintain or even elevate sperm numbers. Because FGIN-1-27 is able to raise serum T levels without reducing ITT levels or sperm numbers, it might prove to be a useful therapy for hypogonadal men who wish to father children.

Due to exciting results using FGIN-1-27, we conducted preliminary studies in aged rats using two structurally distinct drug ligands, XBD-173 and Ro5-4864. Unfortunately, we did not observe the same effects on serum and ITT with these two ligands.

Despite the promising results described, there are a few limitations. (1) We did not compare the in vivo effects of TSPO drug ligands to both old and young rats. The significant increase we see in serum T and ITT for FGIN-1-27 compared to old rats might not have been substantial enough to be deemed statistically significant when juxtaposed with both young and old rats. (2) The lack of increase in serum T and ITT for the experiments using XBD-173 and Ro5-4864 could have been due to how the ligand was dissolved. We used 10% DMSO as the solvent which

made the ligands' dissolution more difficult. We did this to circumvent potential sensitivities the rats might have to high DMSO concentrations but could have reduced the potency of the drugs unintentionally undermining our efforts. (3) We only did short-term experiments with the TSPO drug ligands whereas the T implant experiment in young rats was done over an 8-week period. If FGIN-1-27 was administered for a longer period (or a period that matched that of the T implant experiment in young rats), its efficacy might have diminished or had cytotoxic effects in these animals. Further research on these molecules and others could provide mechanisms to stimulate endogenous T production and the restoration of male reproductive homeostasis.

FIGURES

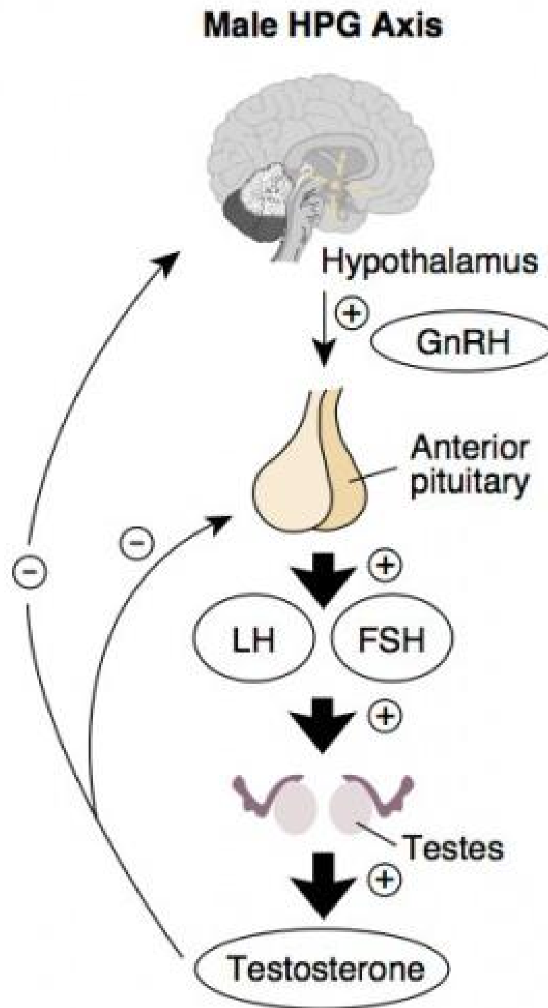

Figure 1. – Hypothalamic-pituitary-gonadal (HPG) axis Beginning at puberty, kisspeptin binds to GnRH neurons eliciting the release of gonadotropin releasing hormone (GnRH) from the hypothalamus (not shown). GnRH acts on the anterior pituitary stimulating the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), which travel via the bloodstream to the gonads. In the testis, LH binds to its receptor on the Leydig cells inducing the production of testosterone, which negatively feeds back on the release of GnRH and secretion of LH and FSH. (Hill, 2012)

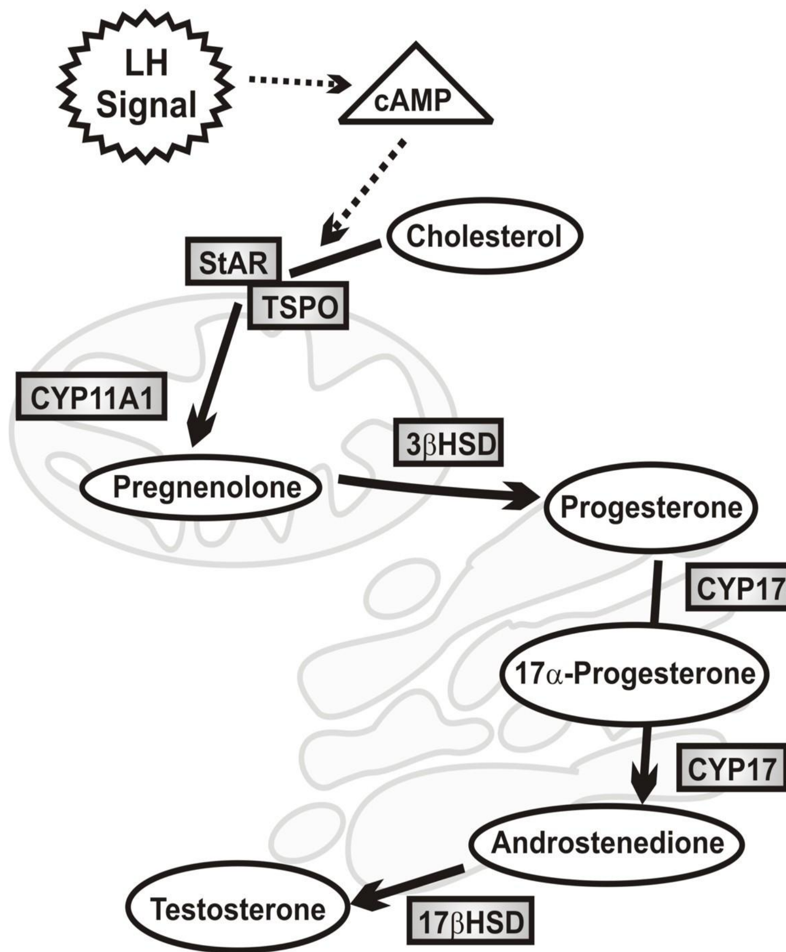

Figure 2 – Leydig Cell Testosterone Biosynthesis Pathway Luteinizing hormone (LH) binds to its receptor localized on the plasma membrane of Leydig cells, which subsequently leads to the cAMP production. Increases in cAMP levels allow StAR and TSPO to facilitate the transport of cholesterol to CYP11A1. Cholesterol is then converted to pregnenolone, which moves to the smooth endoplasmic reticulum where it undergoes a series of enzymatic reactions leading to testosterone as the final product. (Midzak et al., 2009)

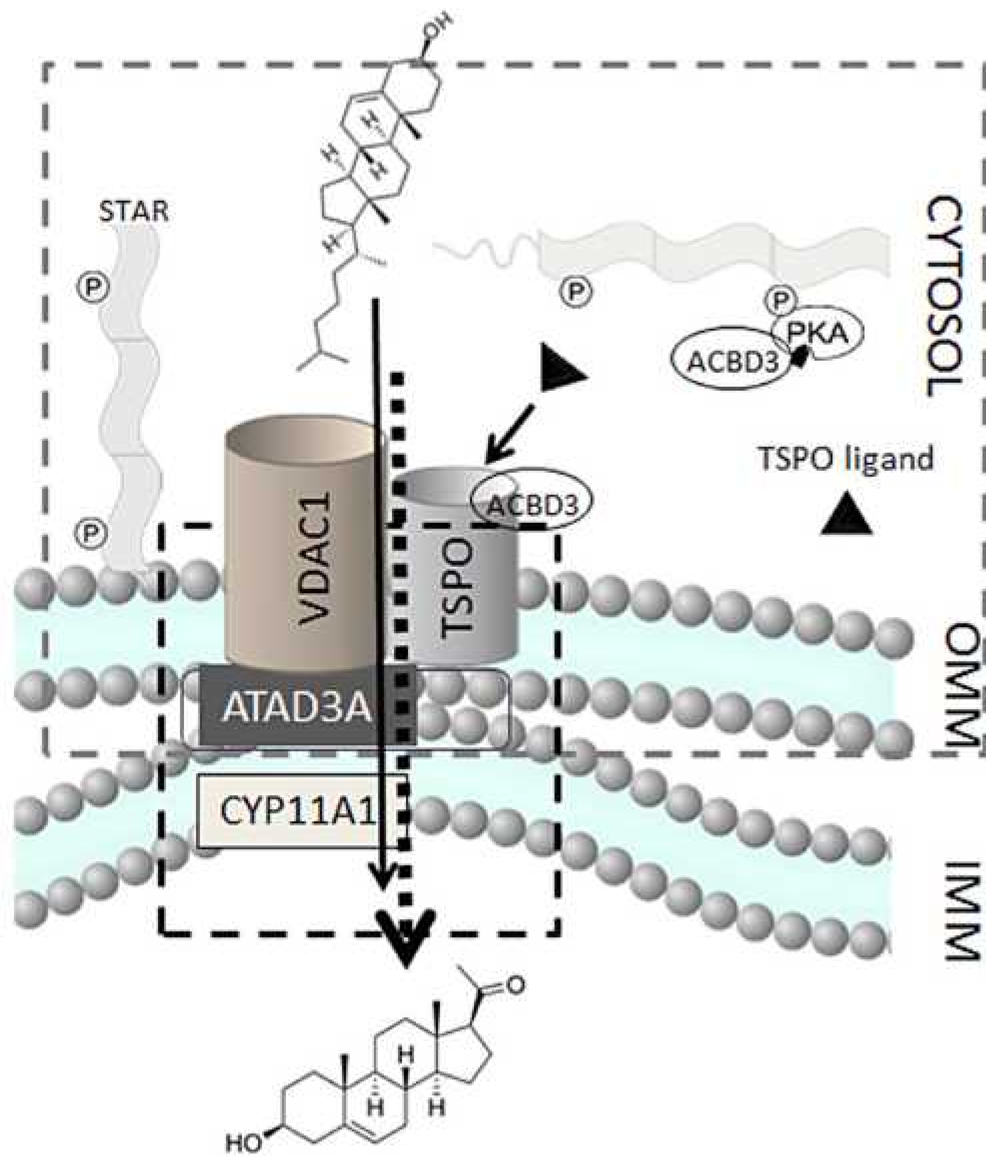

Figure 3 – Cholesterol movement into the mitochondria via two complexes, transduceosome and metabolon. In response to hormone stimulation, Golgi associated protein, ABCD3, is recruited to the mitochondria by OMM proteins TSPO and VDAC1. When ABCD3 is recruited, it brings PKA-RIα. Simultaneously, activated StAR mobilizes cholesterol and interacts with VDAC1 and TSPO completing the transduceosome complex. VDAC1 and TSPO form the metabolon with IMM protein, ATAD3, and matrix protein, CYP11A1. Cholesterol is thus mobilized from the transduceosome through both the OMM and IMM to be metabolized to pregnenolone by CYP11A1. (Papadopoulos et al, 2015)

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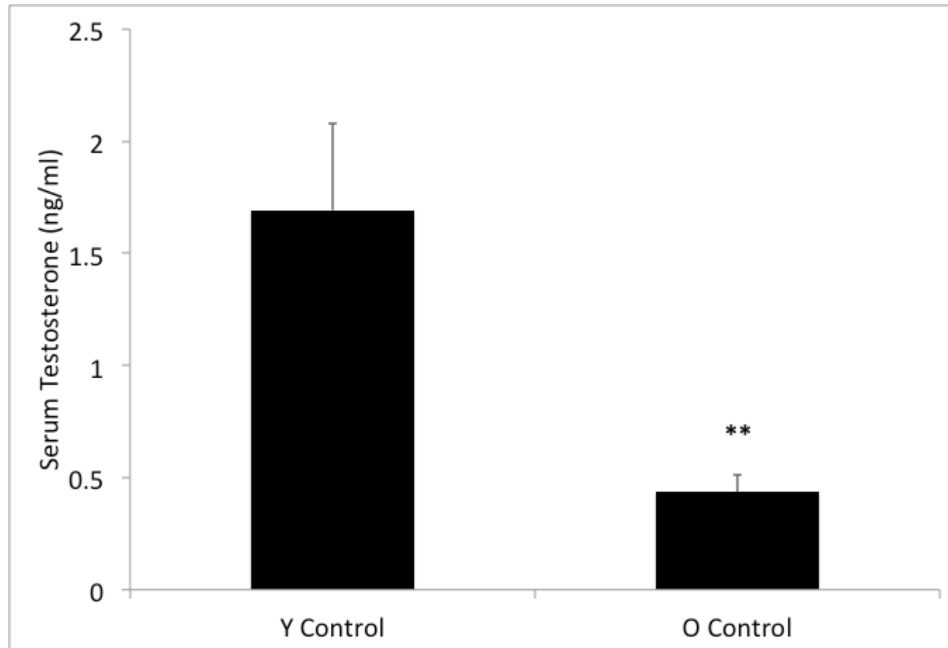

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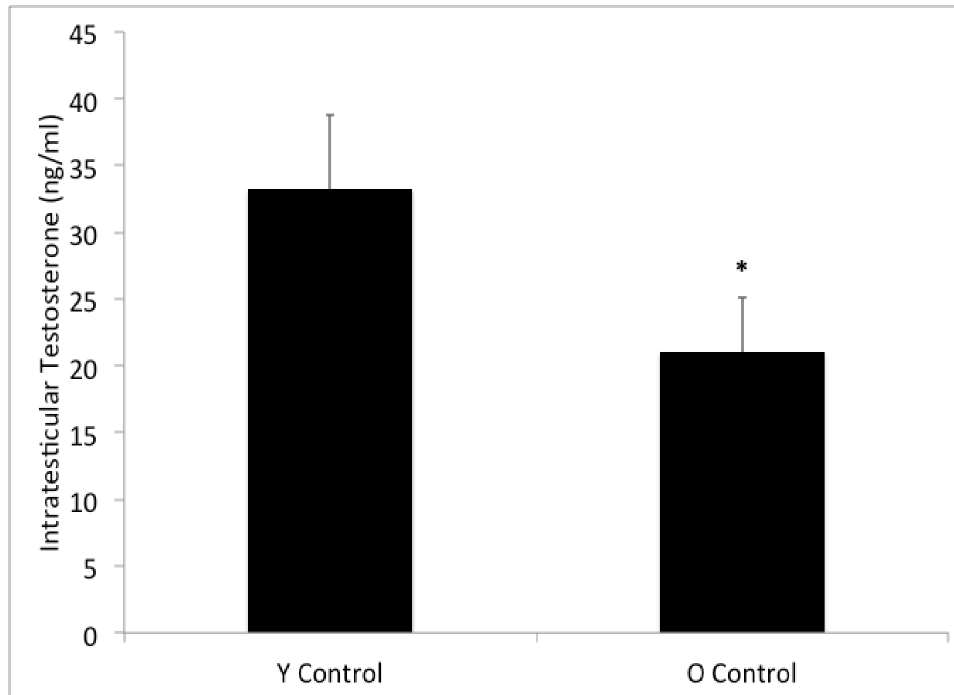

Figure 4 – Figure 4 – Serum (A) and intratesticular (B) T in young and old control rats. (A) Serum T is significantly higher in young compared to old control rats. (B) Intratesticular T is significantly higher in young control than old control rats. In each case, at least 3 independent studies were performed (mean±SEM). * $P<0.05$, ** $P<0.01$

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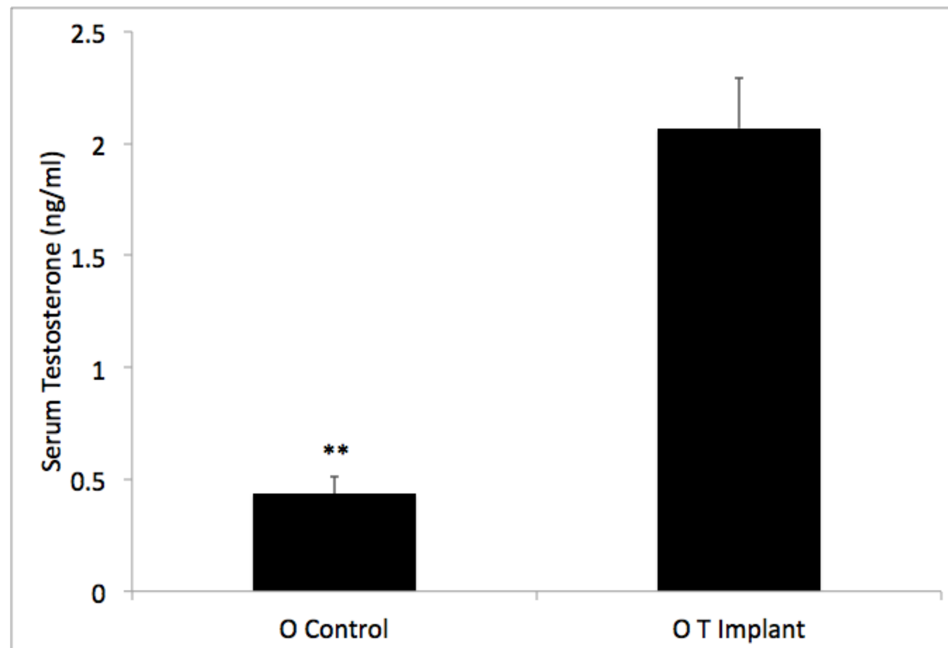

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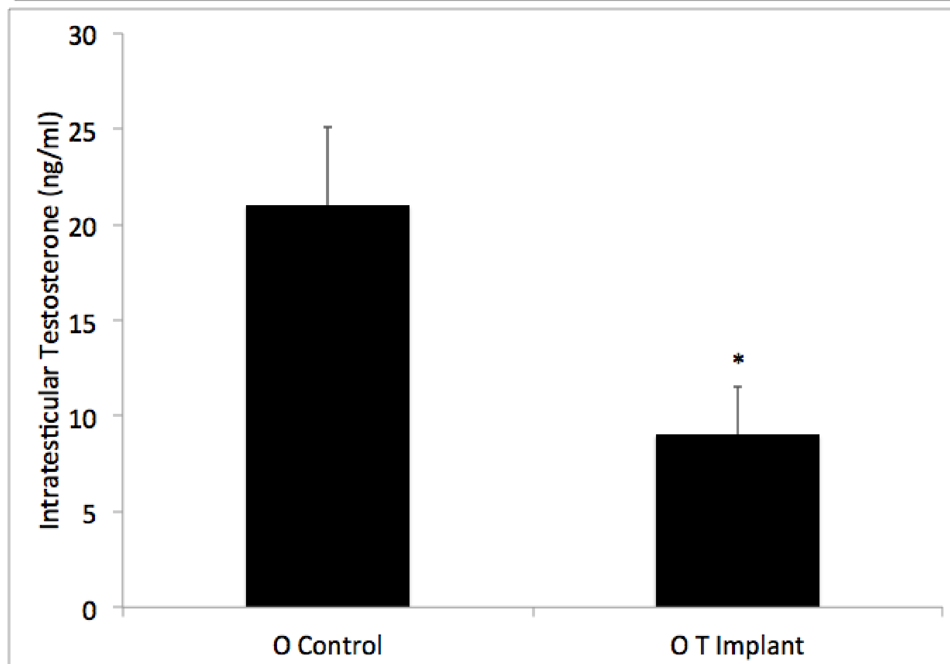
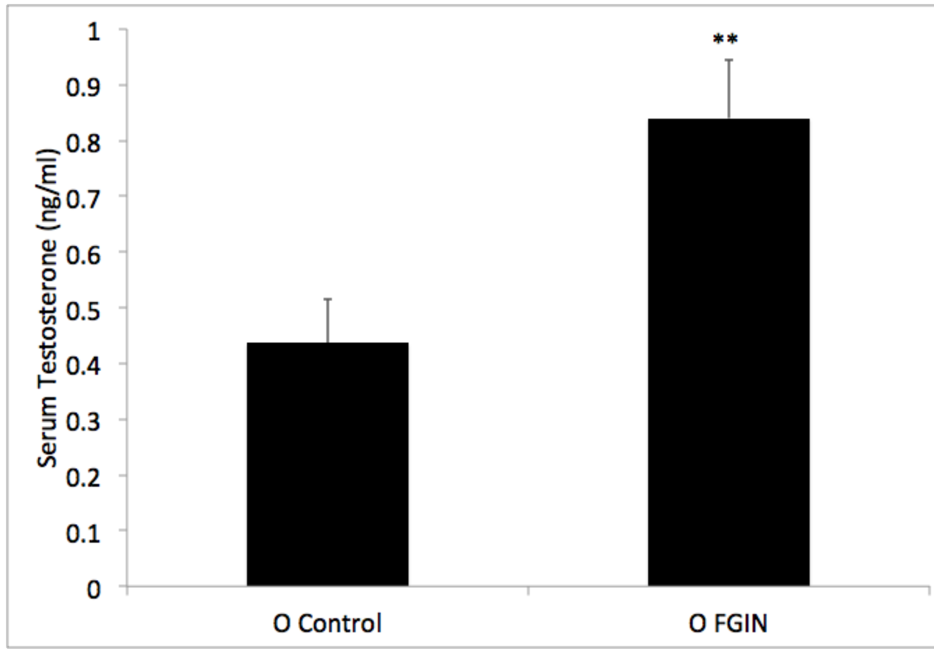


Figure 5 – Serum (A) and intratesticular (B) T in old rats administered 2 cm T-containing silastic implants or administered vehicle (control). (A) Serum T significantly increased in old rats given 2 cm T implants compared to old control rats. (B) Old rats administered 2 cm T implants show a significant decrease in intratesticular T levels compared to old rats administered vehicle. In each case, at least 3 independent experiments were performed (mean±SEM). *P<0.05, **P<0.01 compared with controls.



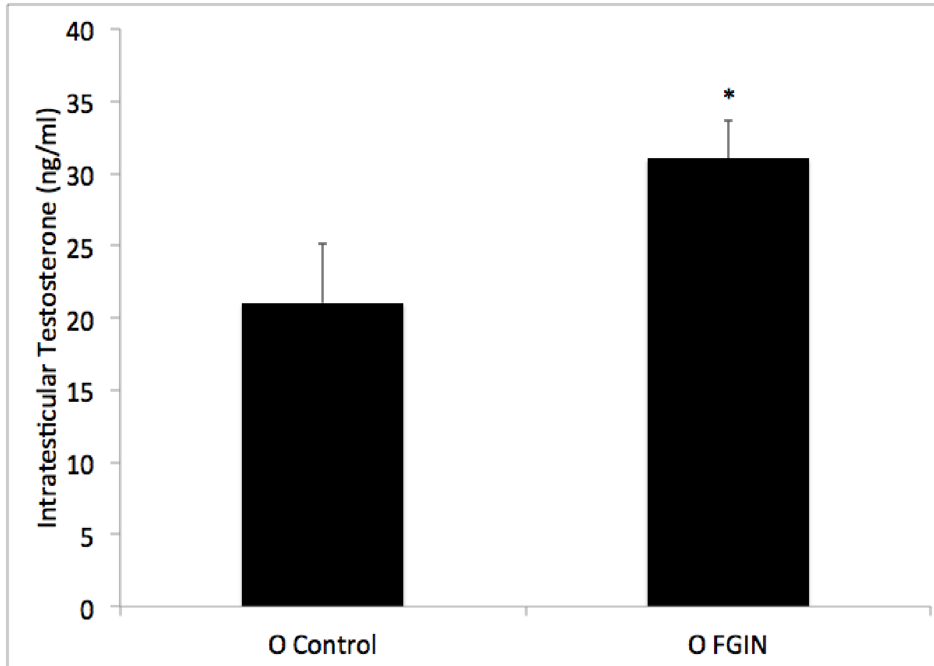


Figure 6 – Serum (A) and intratesticular (B) T in old rats administered FGIN-1-27 via daily ip injection at a concentration of 1 mg/kg body weight for 10 days. Control rats were administered vehicle for the same time period. (A) Serum T increased significantly in old rats administered FGIN-1-27 compared to old control rats. (B) In contrast to the effects of exogenously administered T (Fig. 3), intratesticular T levels increased significantly in response to FGIN-1-27 administration compared to vehicle-treated old rats. In each case, at least 3 independent experiments were performed (mean±SEM). $^*P<0.05$, $^{**}P<0.01$ compared with controls

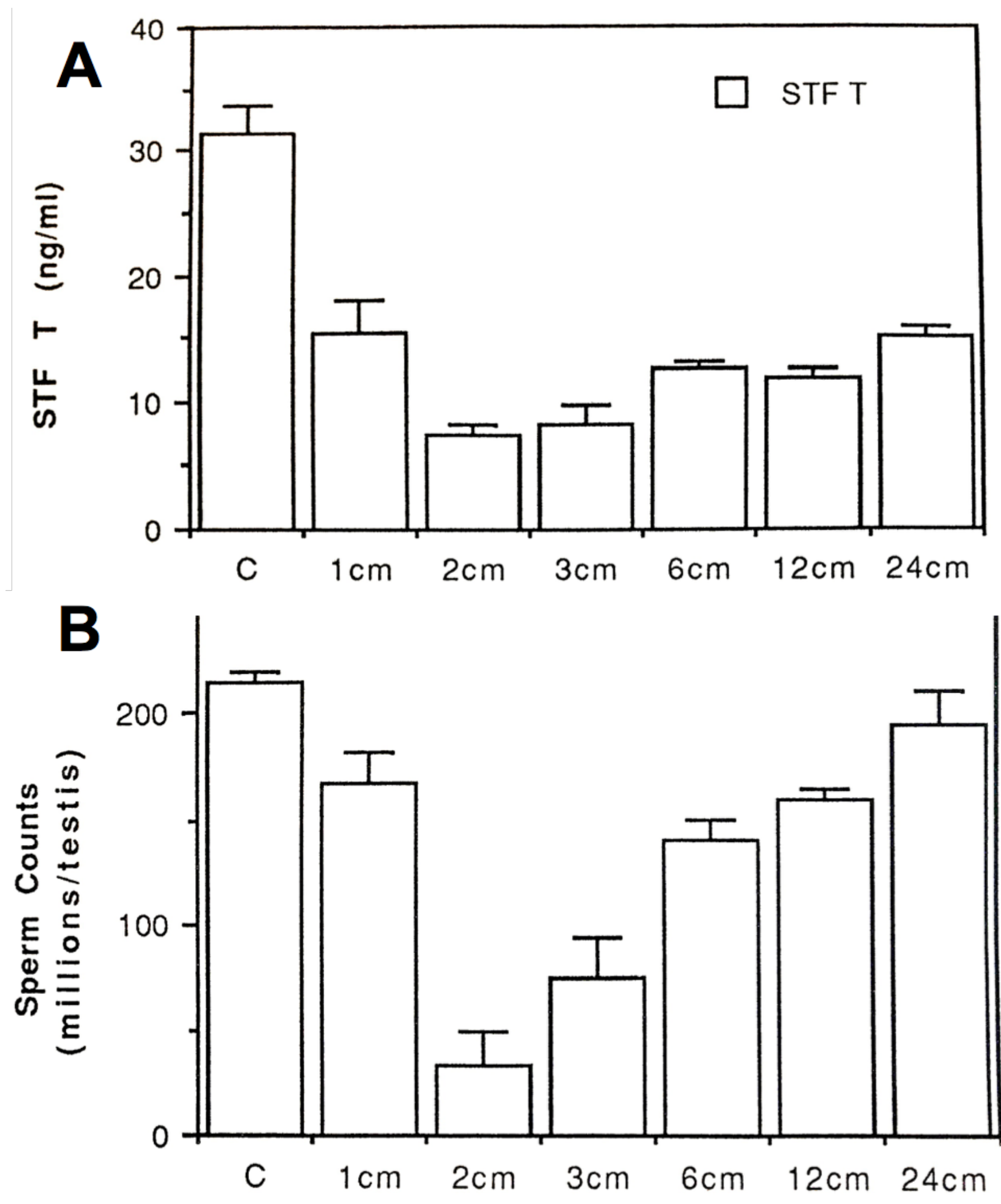
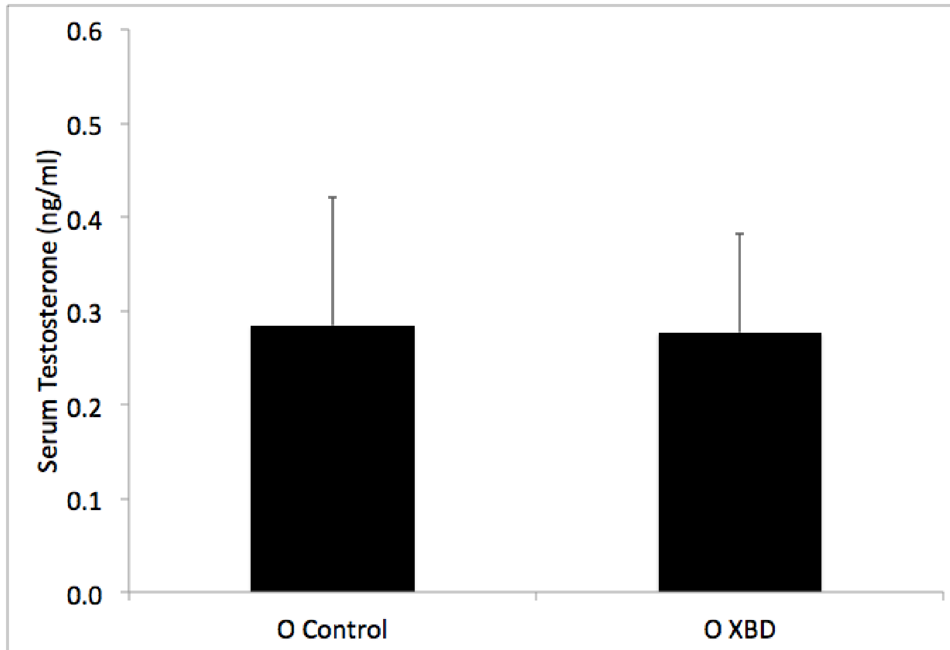


Figure 7 – Effect of T-containing silastic implants on seminiferous tubule fluid T (A) and sperm numbers per testis (sperm counts; B) (A) Seminiferous tubule fluid T levels declined in young rats administered T implants of varying sizes compared to control rats. Levels reached a minimum in response to the 2 cm T implant. (B) Sperm counts declined in young rats administered T implants of varying sizes compared to control rats. Counts reached a minimum at the 2 cm T implant, the capsule size that elevated serum T in old rats, but resulted in reduced intratesticular T.

A



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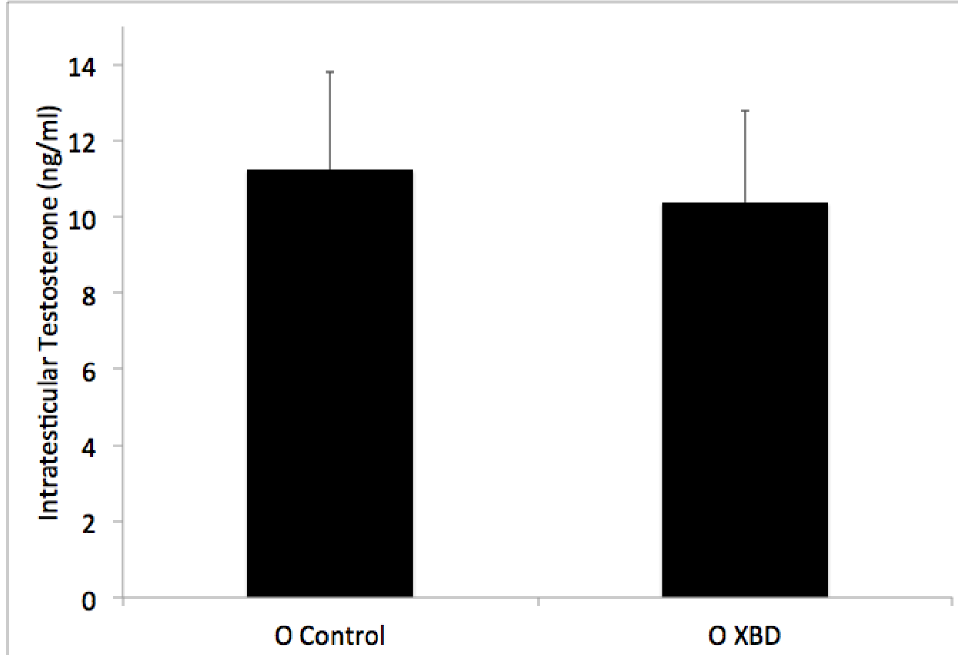

Figure 8 – Serum (A) and intratesticular (B) T in old rats administered XBD-173 via daily ip injection at a concentration of 1 mg/kg body weight for 10 days. Control rats were administered vehicle for the same time period. (A) Serum T increased significantly in old rats administered XBD compared to old control rats. (B) In contrast to the effects of exogenously administered T (Fig. 3), intratesticular T levels increased significantly in response to XBD-173 administration compared to vehicle-treated old rats (mean±SEM). Only one experiment was performed using 4 rats per group.

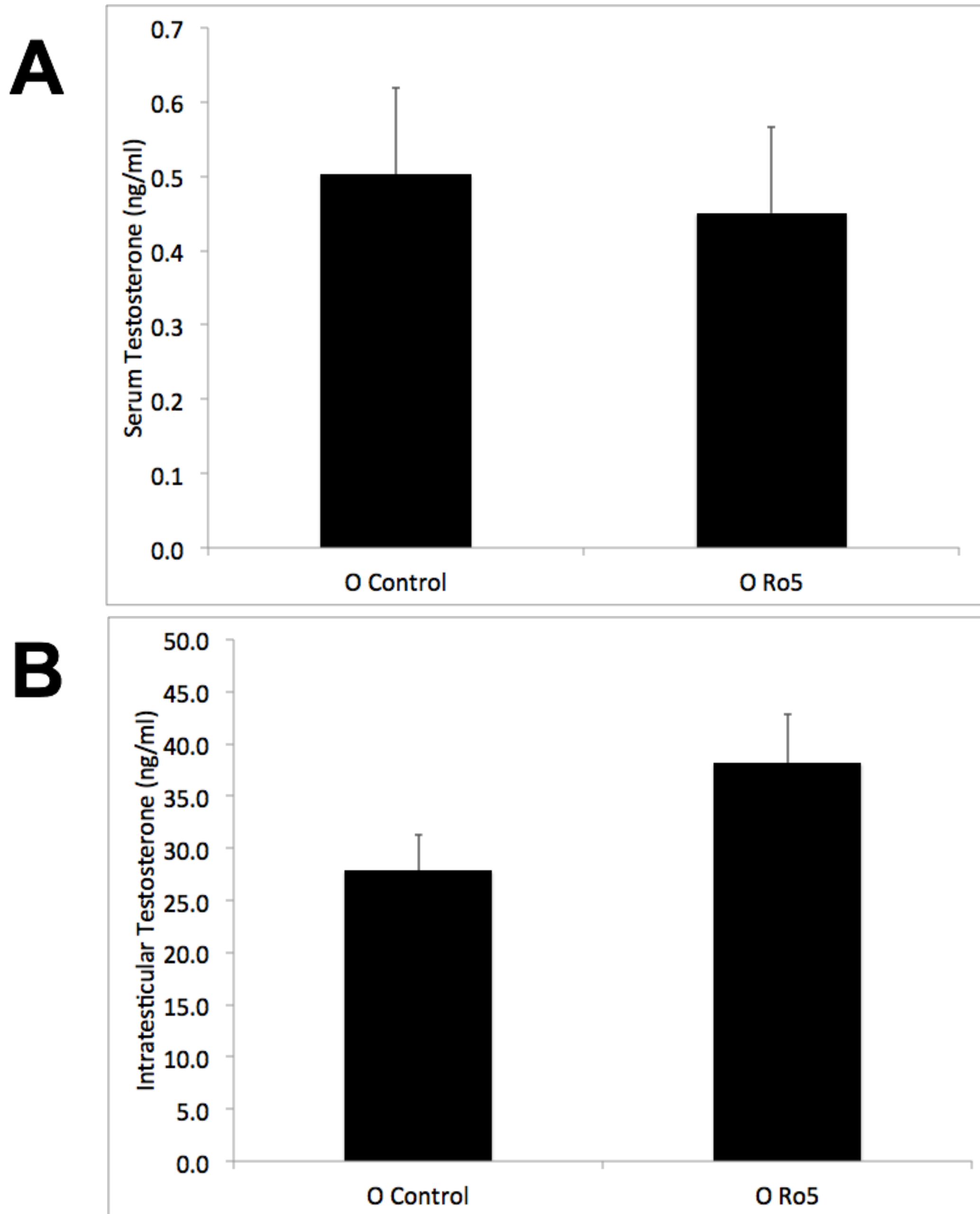

Figure 9 – Serum (A) and intratesticular (B) T in old rats administered Ro5-4864 via daily ip injection at a concentration of 3 mg/kg body weight for 10 days. Control rats were administered vehicle for the same time period. (A) Serum T increased significantly in old rats administered Ro5-4864 compared to old control rats. (B) In contrast to the effects of exogenously administered T (Fig. 3), intratesticular T levels increased significantly in response to Ro5-4864 administration compared to vehicle-treated old rats (mean±SEM). Only one experiment was performed using 4 rats per group.

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CURRICULUM VITAE

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EDUCATION

Johns Hopkins University – Class of 2016
Bachelor of Science Molecular and Cellular Biology
- **Honors:** Deans List (Fall '14, Spring '15, Fall '15)
- **Relevant Coursework:** General Chemistry I & II with Labs, General Biology I & II with Labs, Organic Chemistry I & II with Lab, Biochemistry with Lab, Physics I & II with Labs, Probability and Statistics

Johns Hopkins Bloomberg School of Public Health – Expected June 2018
Master of Science in Biochemistry and Molecular Biology Department on Reproductive and Cancer Biology track
- **GPA:** 4.0

EXPERIENCE

Teaching Assistant, Johns Hopkins University
Reproductive Physiology (Biophysics Department) and Stem Cells & the Biology of Aging & Disease (Biology Department)
(September 2015- May 2018)
- Attended various seminars and lectures by guest professors
- Helped enhance students' knowledge of the fundamentals of reproductive health
- Held sessions where I answered questions and we reviewed material discussed during lecture in preparation for upcoming exams
- Helped to construct and grade exams

Student Research Assistant, Dr. Barry Zirkin's Lab, Johns Hopkins University School of Public Health
(*September 2015-June 2018)*
- Study how the testosterone producing cells of the testes (Leydig cells) age and why as they age they begin to produce less testosterone
- Used a model cell line, MA10 cells, to examine the effect of disturbances in the redox environment (particularly reduction in protective mechanisms driven by glutathione-S-transferases) on steroidogenesis
- Studied the in vivo effects of TSPO drug ligand administration on serum and intratesticular testosterone levels and compared these effects to exogenous testosterone administration

Student Research Assistant, Dr. Xin Chen's Lab, Johns Hopkins University
(January 2015-July 2015)
- Used RNA interference (RNAi) to knockdown key metabolism enzymes to investigated the metabolic control of stem cell division in Drosophila (fruit fly) testes
- Used light and fluorescence microscopy in order to explore the cellular and morphological differences between experimental and control fly strains
- Maintained the flies by transferring them to different vials twice a week and keeping the larvae that would develop into progeny