Smith, Charity., Chronic testosterone deprivation sensitizes the middle-aged rat brain to damaging effects of testosterone replacement

Levels of the testosterone (T) fall in aging men. Recently, the number of men obtaining testosterone replacement therapy (TRT) has increased dramatically. However, other consequences of aging, such as increased oxidative stress, may result in detrimental effects when combined with TRT, including an increased stroke risk. Whether such a delay would alter the effects of TRT on stroke is not known. We hypothesized that a delay TRT following castration in middle-aged male rats would result in increased oxidative stress and a reduction in the neuroprotective effects of testosterone following stroke. We evaluated the effects of testosterone treatment after short (2 week) and long-term testosterone deprivation (10 weeks) in middle-aged male rats on cerebral ischemia, oxidative stress and cognitive function. Our data suggest testosterone treatment after long-term hypogonadism can exacerbate functional recovery after focal cerebral ischemia, however in the absence of injury improves cognition. Both effects are regulated by oxidative stress.

CHRONIC TESTOSTERONE DEPRIVATION SENSITIZES THE MIDDLE-AGED RAT BRAIN TO DAMGING EFFECTS OF TESTOSTERONE

THESIS

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CHAPTER I

INTRODUCTION TO STUDY

Testosterone, an androgen, is the dominant sex hormone in males. The primary functions of androgens are to develop reproductive tissue, for example, testes, and prostate promote secondary sexual characteristics, for instance, increased muscle mass, bone mass, and the growth of body hair, and regulate spermatogenesis and fertility (*1*). Testosterone has additional actions on bone and other tissues, including the brain, where it modulates sexual and social behaviors (*2*, *3*).

As a steroid hormone, testosterone is composed of a four-ring C18 structure derived from cholesterol that renders it lipophilic. In the blood, testosterone is primarily bound to plasma proteins (~70% sex hormone-binding globulin, ~20-30% albumin) with a small amount of free testosterone available to pass through cell membranes to have actions on target cells. In males, testosterone is produced by Leydig cells in the interstitial tissue of the testis, where it is synthesized from cholesterol via an enzymatic process (*4*). In females, only minimal amounts of androgens are produced in the ovaries (*5*), and in both sexes, the adrenal glands also produce small amounts of androgens (*6*). Testosterone production in the testes is regulated by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus into the median eminence to reach the anterior pituitary gland through the

pituitary portal system (*7*). Pulsatility is essential and critical to the function of several hormones to uphold the delicate homeostatic balance required for crucial processes of life, for instance, development and reproduction (*8*). As seen in the hypothalamic-pituitary-glandular axis, the gonadotrophs of the anterior pituitary respond to pulsatile GnRH (*9*) by secreting the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) also in a pulsatile fashion to maintain appropriate levels of bioavailable levels of testosterone (*10*).

LH and FSH peptide hormones travel through the bloodstream to act on receptors on the gonads (*1*). FSH also acts on receptors outside the gonads such as endothelial cells, umbilical vein, vessel smooth muscle cells, fallopian tube, liver, bone osteoclasts, and monocytes, promotes angiogenesis, myometrial contractility, skeletal integrity, and adipose accumulation (*11*). Whereas FSH is primarily responsible for spermatogenesis, LH stimulates steroidogenesis in Leydig cells via G protein-coupled receptors. Activation of LH receptors increases the activity of transport proteins and enzymes that result in the production and secretion of testosterone (3). The Hypogonadal-Pituitary (HPG) Axis located in the brain is composed of the hypothalamus, pituitary gland, and gonadal glands, and they work together to control processes that concern development and reproduction (*7*). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons.

Additionally, Kisspeptin may be the modulator of GnRH, an essential regulator of the HPG axis, as it contains androgen receptor (*12*). GnRH neurons do not express estrogen receptor alpha, a few have progesterone receptors, and a small amount expresses androgen receptors (*12*). The anterior portion of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (*13*). LH and FSH act on cells in the testes comprising of Leydig, Sertoli, and germ cells. Leydig cells make testosterone in the testicle, while Sertoli cells produce

inhibin B. Both testosterone and inhibin hormones feedback onto the anterior pituitary and the hypothalamus (*14*). Testosterone and inhibin limit its output through negative feedback to inhibit both the release of GnRH and LH. As a result, the anterior pituitary becomes less responsive to GnRH stimuli (*15*). Other factors, including the amount of substrate cholesterol, steroidogenic enzyme activity, cholesterol transport proteins, and plasma proteins such as albumin and sex hormone-binding globulin, can also regulate testosterone levels in the body (*6, 16-18*).

Increased risk of cardiovascular disease (CVD) associated with androgen deprivation therapy (ADT) as a result of hypogonadism has been documented in several studies (*19-21*). Interestingly, recent reports notably bring insight to the potential differences in cardiovascular risk and GnRH/LRH agonist increasing cardiovascular events and antagonist having less cardiac events (*19, 22, 23*), have resulted in some investigators to focus on ligand-specific pathways for instance t-lymphocyte, cardiac gonadotropin-releasing hormone/ luteinizing hormone-releasing hormone (GnRH/LHRH) receptor activation, and the role of FSH system in mediating cardiovascular effects (*19, 24*). Mice receiving ADT have been used as a model to understand the relationship between serum FSH and CVD (*19, 25*).

In Lui et al., they did a study with both animals and humans. For their animal model, they used eight week old C5BL/6 male mice that were immediately castrated. Animals that were given GnRH agonist received an intraperitoneal injection for four weeks to inhibit pituitary gonadotropin secretion. Another group of animals that were given GnRH agonist injection for two weeks followed up by two weeks of human recombinant FSH. Four after surgeries rats weighed, fat was measured, and 3-T MRI images were taken mice. In the animal work, they found that the effects of FSH were mediated through FSHRs coupled to Gαi due to stimulated calcium influx and phosphorylated cAMP-response-element-binding protein. In the clinical

settings, FSH levels were collected from 8736 males from January 1, 2001, to July 2011 from affiliated hospitals of Zhejiang University during routine examinations with variation in ages. Additionally, they recruited 414 males from 61 to 65 years of age. They evaluated age-dependent changes in circulating FSH levels and their correlation with body mass index. They identified the occurrence of FSHR in human adipose tissue and on adipocytes, along with a positive correlation between FSH levels as well as an increase in body mass index in men 60 and older. Circulating FSH levels were threefold higher in men 60 and older compared to men younger than 45 (*26*). In Murine 3T3-L1 preadipocytes treated with recombinant FSH in vitro demonstrated an increase in expression of lipogenic genes, comprising of fatty acid synthase and lipoprotein lipase, the principal intermediaries of lipoprotein-dependent fatty acid uptake in adipose tissue (*27*). Adipose tissue itself may have a role in cardiovascular dysfunction. Adipokines, for instance, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), are pro-inflammatory molecules emitted by adipocytes that are linked with the increase of insulin resistance and atherosclerotic disease (*28*). Taken together, the Marshall lab hypothesizes based on the data accumulated in animal and in vitro studies that the interface of FSH with FSHR on monocytes has been shown to up-regulate receptor activator of nuclear factor (RANK) expression and activate monocytic infiltration of atherosclerotic plaques (*19*). RANK is apart of the tumor necrosis factor superfamily. It is a biomarker for osteoclast differentiation (*29*) Additionally, T_H1 helper then releases RANKL, receptor activator of nuclear factor (NF)-κB ligand marker for osteoclastogenesis, and promotes RANK on monocytes resulting in osteoclast formation (*19*). Next, osteoclast reabsorb calcified areas and elicit atherosclerotic plaque instability resulting in increased risk CVD (*19*). Further studies in the clinical setting will need to be done to support this hypothesis.

In addition to testosterone itself, tissue-specific enzymes can metabolize testosterone into two bioactive metabolites, estradiol and the more potent androgen dihydrotestosterone (DHT). Estradiol, one of the primary female sex hormones, is an essential active metabolite for testosterone's actions in the brain, bone, and fat. Testosterone is metabolized to DHT in the skin, kidney, brain, and prostate, and is required for androgen actions in the prostate and hair follicles. The majority of the released testosterone is inactivated and is processed via hepatic phase I and II to inactive oxidized and conjugated products released for urinary and biliary excretion (*30*). Leydig cells secrete large amounts of testosterone in the testes as well as into the bloodstream, preserving circulating testosterone levels that apply androgenic effects on androgen-sensitive tissue (*31*).

Like other steroid hormones, the primary mechanism of testosterone action is binding to intracellular receptors that act as transcription factors in the cell nucleus, also called the "direct" pathway (*32*). The androgen receptor (AR) is a member of the steroid receptor superfamily and is encoded by a gene located on the X chromosome. Activation of the AR gives rise to specific gene expression by controlling the transcription of multiple androgen-responsive targets (*33*). The bound androgen receptor attaches to androgen response elements (AREs) to create an AR dimer in target gene promoters, leading to activation or repression of transcription and sequentially protein synthesis through the recruitment of coactivator proteins (*34, 35*). For example, in skeletal muscles, testosterone promotes the commitment of precursor cells that reside in the skeletal muscle into the myogenic lineage by increasing satellite cells and muscle fibers (*36*).

A second pathway for testosterone action is the amplification pathway, which occurs in the prostate and hair follicles. In this pathway, testosterone is converted to DHT via the actions

of the enzyme 5α reductase type II. The conversion of testosterone to DHT is required for the proper development of the prostate. Similarly, conversion to DHT is necessary for male-patterned increases in body hair, including facial and chest hair. The third pathway for testosterone action is the diversification mechanism, which permits testosterone to modify its biological effects through enzymatic conversion to estradiol by cytochrome P450 aromatase in the bone and brain. In the brain, it has been found that many of the effects of testosterone on neural cell function are facilitated by local metabolism to estradiol by aromatase (*37*). These effects of estradiol are dependent on their location in the brain. For example, estradiol in the neocortical and sensory regions play an essential role in cognition, memory, and sensory perception. Estradiol has also been shown to be neuroprotective under ischemic conditions (*38*). Additionally, the brain can also synthesize estradiol from cholesterol and utilize it in neuronal and glial signaling (*39-41*). The conversion to estradiol is also essential for the bone-sparing effects of testosterone (*42, 43*).

In addition to genomic mechanisms of action through the AR, testosterone can influence cell function through non-genomic mechanisms, such as rapid membrane-mediated non-transcriptional processes (*34, 44*). For example, androgen receptors in the cytoplasm or associated with the plasma membrane can stimulate intracellular signaling molecules such as mitogen-activated protein kinases (MAPK) ERK-1 and ERK-/2 (*45*) or rapidly increase intracellular calcium concentrations as observed in osteoblasts (*46*). These membrane-mediated androgen effects provide quick and transient activation of MAP-kinases ERK-1 and ERK-2 to promote increased levels of GTP-bound $p21^{ras}$, a growth-stimulating signal (*45*).

Male Hypogonadism

Maintaining normal physiological testosterone levels is essential for male health, and as men age, testosterone levels begin to fall. The decline of testosterone levels in males begins at age thirty with a 1% annual gradual decline as they age (*47*). When this decline in testosterone is accelerated and dramatic, it may be clinically classified as hypogonadism. The threshold of total serum testosterone levels that are correlated with an increased prevalence of hypogonadal symptoms ranges from 320-375ng/dL (*48*). Current clinical guidelines define male hypogonadism as morning total testosterone concentrations <300 ng/dL on two occasions at least two weeks apart, and the presence of at least one additional symptom (*49*). However, the symptoms of hypogonadism are relatively indistinct and associated with several other conditions. These include fatigue, loss of libido, weight gain, and depression. Age-related hypogonadism occurs because of defects in the testis, pituitary, and hypothalamus of older men (*50*). By 2020, approximately 20% of the United States population will be older than 65 years, and the size of the population older than 85 years is expected to double to 7 million, likely leading to increases in hypogonadism (*47*).

Male hypogonadism indicates a decrease in one or both of the two primary functions of the testes: sperm and testosterone production. These aberrations may come from diseases of the testes (primary hypogonadism) or disease of the pituitary or hypothalamus (secondary and tertiary hypogonadism). Genetic causes of hypogonadism include Klinefelter's syndrome (XXY genotype), which results in small testes, gynecomastia, and low testosterone production (*51*). Cognitive and other neurological developmental delays may also be present in Klinefelter's syndrome. Klinefelter syndrome is associated with high-risk venous thromboembolism (*52*). Additional causes of primary hypogonadism include chemotherapy, radiation therapy, bilateral

torsion, trauma, and swelling associated with mumps (*53-56*). Secondary hypogonadism can result from pituitary hormone deficits that can indicate a mass lesion in the pituitary or hypothalamus (*57*). Secondary hypogonadism can also be a result of developmental diseases such as Kallman syndrome, in which GnRH neurons fail to stimulate pituitary gonadotropin secretion (58) correctly. Kallman syndrome is a condition that is also associated with an impaired sense of smell. Kallman syndrome is associated with hemorrhagic telangiectasia in which a stoke had occurred. Hemorrhagic telangiectasia is a rare autosomal dominant genetic disorder that results in abnormal blood vessel formation in the skin, mucous membranes, lungs, liver, and brain (*59*). Still, it has not been associated with the risk of stroke. Certain inflammatory diseases, obesity, sleep apnea, certain medications, and acute alcohol ingestion can also lead to secondary hypogonadism (*60*). In addition to the non-specific symptoms noted above, consequences of hypogonadism can include infertility, osteoporosis, hyperlipidemia, an increased risk of cardiovascular and cerebrovascular disease (stroke), and an increased risk of dementia (*61-63*).

Cross-sectional studies demonstrate a decrease in serum total testosterone concentration with an increase in age (*64, 65*). For example, in the European Male Aging Study (EMAS) of 3,220 men aged 40-79, total testosterone concentration in serum dropped 0.4 percent each year (*64*). Another example demonstrated a slight decrease in testosterone levels following 35 years of age, along with dramatically more decrease later than 80 years of age (*65*). Longitudinal studies support the findings found in cross-sectional studies, although reductions are more significant than in cross-sectional as men as age (*66-68*). According to the New England Research Institute study on lifestyle factors affecting testosterone levels in men ages 40-70, testosterone concentration in serum declined at a rate of -4.0%, regardless of further clinical

variables (*66*). Additionally, the Massachusetts Male Aging Study found a decline in total testosterone concentration at 1.6% per year in serum with advanced age but an even more significant drop in free testosterone at 2-3% per year (*67, 68*).

Hypogonadism and Neurological Diseases

Hypogonadism is also associated with neurological diseases such as dementia and Alzheimer's disease (*69, 70*). Individuals with Alzheimer's disease that have the apolipoprotein E4 (APOE4) allele have an increased incidence of hypogonadism compared to those who do not have low testosterone (*69*). Additionally, in a cohort of 3650 men ages 65 years and older, men with low bioavailable testosterone are linked with a higher risk of dementia. Also, it was shown that the risk of dementia is seen in men with low bioavailable testosterone was more significant in men 80 years and older than in men younger than (*71*).

Also, a meta-analysis that used the random effect model and included several prospective cohort studies of older men concluded that low plasma testosterone level was significantly related to an increased risk of Alzheimer's disease in older men (*72*). Moreover, in a cohort of 56 men ages, 21-84 years, age-related decreases in bioavailable testosterone projected age-related decline in visual and verbal memory (*73*). Lastly, in the Baltimore Longitudinal Study of Aging (BLSA), an age-related decline in endogenous testosterone concentrations was associated with decreased neuro-psychological performance in 407 men ages 50-91 years (*74*). The BLSA also showed that eugonadal men had higher spatial cognition, verbal memory, visual memory, and a reduced rate of decline in these areas compared to hypogonadal men. As a result, there is a possibility that low T/free T levels could serve as a marker for age-related decrease in cognition even though the data cannot show a causal impact (*75*).

Although cross-sectional studies show that stroke is also associated, not a predictor of low testosterone (*76-79*), in older men, lower total testosterone levels were shown to predict increased incidence of stroke or transient ischemic attack after adjusting for conventional risk factors for cardiovascular disease (*80*). Additionally, in a cohort of 7892 that included both (3876) men and women with ages ranging between 25-74 years old, demonstrated

that low testosterone is linked with increased risk of potential atrial fibrillation and ischemic stroke in men (*81*). Furthermore, in a prospective observational study with a total of 3443 men, 70 years and older indicate that in older men with lower testosterone levels are projected to have an increased risk of stroke or transient ischemic attack, and men with low-normal testosterone levels also had an increased risk of stroke (*82*). Also, low male testosterone was found cross-sectionally related to crucial cardiovascular disease risk factors and a marker for other cardiovascular risk factors (*83*). Furthermore, in a cohort of 802 men from 40-80 years old, those with testosterone levels less than 300 ng/dl were found to have a significantly increased incidence of cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, obesity, endothelial function score, myocardial infarction, death after myocardial infarction and stroke (*84*).

Treatment of Male Hypogonadism -Testosterone Replacement Therapy

Male hypogonadism is currently classified as idiopathic, regardless of cause, and men diagnosed with hypogonadism may be treated with Testosterone Replacement Therapy (TRT). The goal of TRT is to treat hypogonadal men to bring their testosterone levels back to normal physiological levels to reduce the effect of hypogonadism in the body. There is inconsistency in the literature on whether TRT is detrimental in hypogonadal men, particularly concerning cardiovascular disease and stroke. Some studies have shown that hypogonadal men receiving TRT have improved stroke outcomes (*7, 27-32*). Others have shown that men accepting TRT do not affect stroke incidence (*8, 30, 33, 34*).

Conversely, some have reported that they could not rule out a risk of stroke in men receiving TRT (*9, 35-37*). Other laboratories have found that there is a risk of stroke in older hypogonadal men receiving TRT (*5, 10, 14, 38, 39*).

Additionally, in a recent study with men ages 45-59 that compared men with low testosterone receiving TRT to those that did not found that out of a total of 15,401 men, 850 patients had undergone a transient stroke, ischemic attack, or myocardial infarction with the highest risk within the first six months to 2 years of regular use of TRT (*85*). The variation in data amongst laboratories for hypogonadism and stroke in TRT patients is concerning. It provides a need for further understanding of the mechanism in which TRT is most effective in hypogonadal men that have co-morbidities such as underlying cardiovascular or cerebrovascular disease.

Testosterone Replacement Therapy- Testosterone Formulation

Lack of consistency in which testosterone treatment is given amongst men receiving treatment is a contributing factor to varied results in men receiving testosterone treatment (*86-92*). Specifically, in testosterone measurement and method of supplementation (*93*). The method in which testosterone is administered has a direct effect on how the testosterone is absorbed in the body and therefore affects the subject's response to testosterone treatment. Testosterone can be given in several ways such as oral, intramuscular injection, oral or buccal application, intranasal spray, implantable pellet, and transdermal patches or gels (*93*). Each of these methods of testosterone administration can influence how much testosterone is found in serum. The most common method testosterone is administered in the United States is either by intramuscular injection or transdermal gel or patch. The transdermal method is the preferred method because of the way its formulated. The transdermal formulation allows for more stable physiological levels of testosterone that closely mimics the circadian fluctuations of endogenous testosterone (*94*). Testosterone levels are also influenced by dosage, formulation, timing in which testosterone is measured, and duration of treatment (*93*). As a result, any inconsistency in these areas amongst studies can lead to inconsistency in literature. Studies focused on understanding the effects of testosterone on cognition and stroke studies have had varied and conflicting data (*95-98*). The inconsistency in the literature testosterone relationship with cognition or stroke could be due to several reasons, for instance, the subject-specific characteristics like age, gonadal state, or neurological disorder such as dementia in terms of cognition (*93*). Also, for cognition studies, the type of assessment used to measure cognition in subjects is varied amongst studies, and that also has affected the outcome of the study (*93*). Understanding the gonadal state of subjects in the study is important. The Endocrinology Society classifies hypogonadism in men as a disorder

that comes about from the testis inability to yield functional levels of testosterone and an endogenous number of spermatozoa as a result of one or more irregularities of the hypothalamic-pituitary-testicular axis (*99*). The diagnosis of hypogonadism must be consistent and explicitly low levels of testosterone and the symptoms must directly cause by having low levels of testosterone. This is an issue when measuring testosterone levels in older men (*93*). Testosterone levels in older men range from slightly below normal testosterone or between lower to normal testosterone levels (*93*). Also, many of the symptoms that are associated with hypogonadism are common in older men and are also associated with other causes such as fatigue and depressed mood. Additionally, the method used to assess T levels in model whether they measured free testosterone, bioavailable testosterone, or total testosterone.

Like the various was testosterone can be administered in males, there can also be many ways in which testosterone can be evaluated and explained. Total testosterone (TT) is comprised of bound and unbound testosterone, which can be measured in serum. The most accurate way of measuring the physiological levels of testosterone is by measuring free testosterone (FT) and bioavailable testosterone (BT), not by measuring TT. This because when testosterone is released into the bloodstream, a small portion of testosterone remains bound as FT, whereas the rest reacts weakly with albumin or is inactivated by sex hormone-binding globulin (SHBG). BT is composed of FT and testosterone bound to albumin (*100*). The intervention used testosterone supplementation, or no supplementation affects the data received from testosterone studies. Additionally, assays such as radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) are used to measure testosterone levels for endocrine disorders have been filled with inaccuracy and precision problems for over ten years due to the arrival of high throughput and direct assays placed on automated analyzers (*101*). RIAs are immunoassays that use

radiolabeled molecules in a sequential formation of immune complexes. Due to their sensitivity, they are used very commonly to measure hormones in the blood (*102*). An ELISA is a plate-based assay technique created to detect and quantify substances, for instance, peptides, proteins, antibodies, and hormones. ELISAs can detect by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product (*103*). The transition from manual radioimmunoassay (RIA) in the early days to the current automated RIAs. Rapid demand h b as resulted in the production of high volume and high quantity handling of several specimens in a short time for measurement of testosterone levels. This rapid change in RIA has to lead to far less sensitivity than the original RIA method and lack sufficient precision to permit their routine use on specimens that contain low levels of steroids such as seen in hypogonadal men (*101*). The inconsistency has made it hard for endocrinologists to reproduce correlations found with testosterone in laboratory settings utilizing automated RIAs and ELISAs to patient testing (*101*). The inconsistency amongst automated RIAs and ELISAs leads to the development of the "gold standard" for analyzing testosterone levels, which is liquid chromatography-tandem mass spectrometry (LC/MSMS) (*101*). LC/MSMS is a potent analytical method that merges the separating power of Liquid chromatography with the extremely sensitive and selective mass analysis capacity (*104*). LC/MSMS has higher specificity, sensitivity and accuracy that has been able to overcome the limitations observed RIAs and ELISAs. An Interlaboratory comparison study of serum TT measurements performed by mass spectrometry compared to reported immunassays observe that the variability of TT measurements results among MS assays are significantly reduced than those reported for immunoassays (*105*).

Role of Testosterone in Cognition

The literature on the role of testosterone on cognition in males is inconsistent. Some studies have shown that low testosterone is associated with a decline in cognition. For example, a prospective longitudinal study of 4,069 men with ages ranging from 71-88 at the beginning of the study found that over an average of 10.5 years, 499 men in the study developed dementia. Furthermore, men with the lowest quartiles of total and calculated free testosterone were found to have a higher risk of developing dementia in comparison with those who were in the highest quartiles (*106*). Additionally, another study of 547 men ranging in ages from 59-89 years showed that bioavailable testosterone decreased with age and was associated with significantly worse cognitive function test scores (*107*). A clinical study of 40 men with prostate cancer treated with an androgen blockade therapy (flutamide and leuprolide) for 36 weeks examined the effects of reversible chemical castration on mood and cognitive performance. The result of chemical castration was a rapid decrease in testosterone levels and an increase in plasma amyloid-beta levels (*108*).(*107*). A clinical study of 40 men with prostate cancer treated with an androgen blockade therapy (flutamide and leuprolide) for 36 weeks examined the effects of reversible chemical castration on mood and cognitive performance. The result of chemical castration was a rapid decrease in testosterone levels and an increase in plasma amyloid-beta levels (*108*). Conversely, other studies have shown that low testosterone is not connected to a decline in cognition. In a cross-sectional study that included 70 men and 70 women with ages of 60 and older, no significant correlation between testosterone levels and primary cognitive measures were observed in either sex (*109*). Additionally, in a population-based study that included the Massachusetts Male Aging Study (MMAS), no significant effects of hormones on cognition were observed (*110*). (*109*). Additionally, in a population-based study that included the

Massachusetts Male Aging Study (MMAS), no significant effects of hormones on cognition were observed (*110*).

Additionally, there is a similar pattern of variation in the literature in the treatment for hypogonadism testosterone replacement therapy (TRT). Some studies provide evidence that TRT is beneficial in improving cognition. A randomized, double-blind, placebo-controlled study that included 17 men with Alzheimer's disease and and 13 men with mild cognitive impairment (MCI). 19 subjects took weekly intramuscular (IM) injections of 100 mg T enanthate and 13 subjects received weekly injections of placebo (saline) for 6 weeks. Men receiving testosterone treatment had significantly improved spatial memory, constructional abilities, and verbal memory compared to those who did not (*111*). Another study of 25 healthy men ages 50 to 80 years old found that short-term administration of TRT for 6 weeks improved cognitive function (*112*).

Other clinical studies failed to show any effect of TRT on cognition. In a randomized, double-blind, placebo-controlled, and parallel-group study with 16 male patients with Alzheimer's disease (AD) and 22 healthy male control patients, TRT improved overall quality but had minimal effects on improving cognition in AD patients (*113*). Additionally, in a study that included 22 MCI and low testosterone patients, TRT treatment minimally improved verbal memory and depression symptoms in both MCI and low testosterone patients (*114*). Another study in healthy men ages 65 to 83 years old with baseline testosterone levels of 350ng/dL and no cognitive impairment found that TRT gave alone or with finasteride, which inhibits the conversion of testosterone to DHT, has no effect on cognitive function in healthy older men (*115*). Also, a study with 76 healthy men 60 years or older had free testosterone index (FTI) of 0.3-0.5, which was defined by the study as the value below the normal lower limit for young men

ages 19-30 demonstrated that testosterone undecanoate (TU) treatment for a year did not affect the visuospatial test, mood, or quality of life of healthy older men with low to normal gonadal status (*116*). Another pilot study of men aged 73-87 years old with very early cognitive decline and a baseline testosterone below 128ng/dL, found that TRT treatment did not affect behavior, function, depression, or cognition compared to a placebo group (*62*). Finally, a large clinical trial that included 12 US academic medical centers with 788 men 65 years or older and testosterone levels <275ng/dL. A subgroup of 493 met the criteria for age-associated memory impairment (AAMI). They concluded that older men with low testosterone and AAMI that received TRT for a year had no improvement in memory and cognition (*117*).

Hypogonadism and TRT in Rodent Model

Animal studies provide further evidence of the role of hypogonadism on cognition. Studies show that low testosterone worsens cognition and impairs neurons in the hippocampal region. A study done in male gonadectomized Charles River Sprague Dawley rats showed that there was a 50% decrease in CA1 spine synapse density compared to intact rats (*90*). The study suggest that testosterone is essential for the maintenance or natural spine synapse density in CA1 region in the hippocampus of male rats. These results were also confirmed in male St. Kitts Vervet monkeys, where one month of gonadectomy led to a 40% decrease in spine density of the CA1 region of the hippocampus (*91*). In C57BL/6J transgenic male mice expressing apolipoprotein E4 allele, the androgen receptor antagonist flutamide impairs memory (*92*). In male Sprague Dawley rats that were gonadectomized for 60 days, chronic low levels of testosterone impair hippocampus-dependent measures in T-maze (*118*). Sprague-Dawley gonadectomized male rats performed poorly in the novel object recognition test, which examines working memory, compared to control rats. This study provides further evidence that low testosterone negatively influences memory (*119*). Also, in male Sprague Dawley rats, after two weeks of gonadectomy resulted in significantly increased levels of amyloid β compared to control (*98*). These results were also confirmed in male St. Kitts Vervet monkeys, where one month of gonadectomy led to a 40% decrease in spine density of the CA1 region of the hippocampus (*91*). In C57BL/6J transgenic male mice expressing apolipoprotein E4 allele, the androgen receptor antagonist flutamide impairs memory (*92*). In male Sprague Dawley rats that were gonadectomized for 60 days, chronic low levels of testosterone impair hippocampus-dependent measures in T-maze (*118*). Sprague-Dawley gonadectomized male rats performed poorly in the novel object recognition test, which examines working memory, compared to

control rats. This study provides further evidence that low testosterone negatively influences memory (*119*). Also, in male Sprague Dawley rats, after two weeks of gonadectomy resulted in significantly increased levels of amyloid β compared to control (*98*).

Similar to studies in men, the effects of TRT on cognition in animal studies reveal that under some conditions TRT improves cognition while in other conditions TRT does not affect cognition. TRT treatment after gonadectomy in animals improves cognition. In gonadectomized male Sprague Dawley rats treated with testosterone one week later showed an increase in spine density levels compared to intact rats (*90*). The senescence accelerated model of SAMP8, mice there is an age-related decrease in serum testosterone 71% from 4-12 months, and exogenous testosterone improves learning and memory (*120*). Gonadectomized male rats given exogenous testosterone via Silastic capsules or intrahippocampal infusions also show improved learning compared to controls (*90*). The senescence accelerated model of SAMP8, mice there is an age-related decrease in serum testosterone 71% from 4-12 months, and exogenous testosterone improves learning and memory (*120*). Gonadectomized male rats given exogenous testosterone via Silastic capsules or intrahippocampal infusions also show improved learning compared to controls (*71*). Additionally, in Sprague-Dawley, gonadectomized male rats performed poorly in the novel object recognition test, which examines the working memory, compared to control, but when given TRT, working memory significantly improved (*119*).

Some studies show that TRT does not affect cognition. In Rhesus monkeys treated with a gonadotropin-releasing hormone agonist to suppress endogenous testosterone for three days and subsequently given testosterone injections for four weeks, multiple cognitive tests showed that exogenous testosterone did not affect cognition (*121*). In summary, previous studies in animal

models have shown that low testosterone impairs cognition and neuron structure in the hippocampal area.

On the other hand, TRT, in some instances, reverses the effects of low testosterone and improves cognition. As a caveat, most of the experimental animal literature is derived from studies in young animal models and not in middle or older animal models. The data collected from animal models and clinical trials have yet to make clear on how testosterone effects cognition and more studies need to be done to elucidate its role on cognition.

Role of Testosterone in Stroke

Stroke is the fifth leading cause of death and the number one cause of long-term disability in the United States (*122*). According, to the American Stroke Association, a stroke is caused by blockage from a clot or a rupture within a blood vessel that carries oxygen and nutrients to the brain. When this occurs, a part of the brain that is affected doesn't receive the oxygen or nutrients delivered by the blood vessels. As a result, brain cells die. Additionally, since the brain controls many functions of the body when a stroke occurs, whichever region of the brain is affected results in associated loss of full function of that body part. There are three types of stroke ischemic, hemorrhagic, and transient ischemic attack (TIA) also known as a mini stroke. In the United States, 87% of strokes are ischemic and 13% are hemorrhagic. Worldwide 68% of strokes ischemic, and 32% of stroke is hemorrhagic (*123*).

There is inconsistency in the literature on whether TRT is detrimental in hypogonadal men and stroke risk. Some studies have shown that hypogonadal men receiving TRT have improved stroke outcomes (*86, 87, 95, 124-127*) . Others have shown that men receiving TRT have no different outcome of stroke from the rest of the population (*87, 89, 96, 128*). Conversely, some have reported that they could not rule out a risk of stroke in men receiving TRT (*88, 129-131*). Lastly, some laboratories have found that there is a risk of stroke outcome in older hypogonadal men receiving TRT (*97, 132-135*). The variation in data amongst laboratories for hypogonadism and stroke in TRT patients is concerning. It provides a need for further understanding of the mechanism in which TRT is most effective in hypogonadal men that have co-morbidities such as stroke.

There is also inconsistency in the literature in animal ischemia models on the role of testosterone in stroke. Some studies provide evidence that low testosterone is beneficial, and the

addition of TRT reverses it. In male Charles River Sprague-Dawley rats undergoing middle cerebral artery occlusion (MCAO) for 40 minutes one week after gonadectomy and treated with either testosterone alone, estrogen, or testosterone plus estrogen, gonadectomy reduced lesion size. However, when testosterone was added, the ischemic brain damage was exacerbated compared to intact animals (*136*). Another study done in male rats showed that after 22 hours of reperfusion, gonadectomized rats had decreased infarct size, and when testosterone was given infarcts were larger (*95*). Similarly, in young Wistar rats, castrated rats had a smaller infarct size compared to testosterone replaced and intact groups (*95*). Furthermore, in a study with gonadectomized male C57BL/6 mice age 9 to 10 weeks were treated with testosterone at 24hrs following 90 minutes of MCAO, showed exacerbated injury (*137*). Moreover, in male Charles River Sprague-Dawley rats that were castrated and given testosterone for 6 hours before MCAO, the lesion was worse compared to control (*138*).

Conversely, other animal studies provided evidence that under low testosterone conditions, testosterone replacement is beneficial. A study was done in male Wistar castrated rats that underwent MCAO followed by treatment with testosterone; they found that in this group, there was a significant decrease in infarct size, less oxidative stress, and increased neurogenesis by day 10 (*127*). In another study that focused on understanding the role of testosterone on recovery from neurological damage, 8 to 9 week old adult male Sprague-Dawley rats were castrated and underwent 90 minutes of MCAO. After seven days, these rats were given testosterone replacement, and rats that received testosterone, they had improved neurological deficits compared to placebo (*139*).

Critical Window of Hormone Replacement

The Women's Health Initiative was the largest randomized clinical trial of postmenopausal women for hormone therapy (HT). Among the finding of the WHI, it was reported that there was an increase in dementia in women taking a conjugated equine estrogen in combination with synthetic progestin (*40*). However, the average age of women receiving treatment in this study was >65 years of age, which is more ten years after menopause. Conversely, many other studies found benefits to postmenopausal HT initiated closer to the menopausal transition (*140*). For this reason, researchers have proposed the concept of a "critical window" in which estrogen is beneficial, but outside this window, the benefits are lost (*11, 15, 41-45*). Simarly, like estrogen studies their could exist a analogous therapeutic "critical window" for testosterone replacement in men.

In agreement with the "critical window" hypothesis, studies with experimental ischemia in mice and rats (*46*) demonstrate that a delay of as little as ten weeks in estrogen HT abolishes neuroprotection observed with estrogen replacement within two weeks of ovariectomy. In the 4-vessel occlusion model of global ischemia estrogen loses its protective effects after long term estrogen deprivation (LTED) (*11*). LTED has been shown to weaken estrogen-dependent support immune function and antioxidant activity (*11*). Furthermore, estrogen HT given during this window has been shown beneficial in reducing Alzheimer's disease. Additionally, immediate treatment with estrogen after ovariectomy protects the brain from ischemia, but LTED increases amyloidogenesis in the hippocampus, and this effect cannot be inhibited by estrogen (*49*). This would suggest that an indirect effect of testosterone through conversion to estradiol might similarly lose protective effects (*13*). Castration also impairs spatial recognition memory in rats, an effect corrected by replacement of T (*50-52*) or E2 (*52*), reinforcing a likely

role for T metabolism to E2. Thus, the beneficial effects of TRT could also occur through a similar "critical window." Estrogen replacement therapy (ERT), a treatment for post-menopausal women, is similar to TRT. Studies on ERT provide evidence for a "critical window" for administering estrogen to post-menopausal women, as estrogen replacement loses its beneficial effects when given outside this window (*6*).

Testosterone Links to Oxidative Stress

Oxidative stress is caused by an overproduction of reactive oxygen species (ROS). There several types of ROS, such as superoxide anion, hydroxyl radicals, and hydrogen peroxide. Under physiological conditions, ROS is beneficial (*141*). It regulates the function of the immune system, maintains the redox homeostasis, participates in several processes, and plays a role as a second messenger in some pathways such as oxidation of thiols (*142*). The brain has a well-equipped antioxidant system to regulate ROS accumulation, that is composed of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, and several antioxidant enzymes to help defend the brain against excess ROS (*143*). Drugs, such as Tempol, were developed to replicate these beneficial effects of antioxidants in the brain. Tempol is a free radical scavenger and a superoxide dismutase mimetic. Tempol has been used in diabetic models and has been shown to improve endothelial dysfunction. In Zucker rats, a diabetic model for Type 2 diabetes, tempol improved insulin sensitivity and decreased oxidative stress (*144*).

Additionally, under conditions of increased oxidative stress testosterone has been shown to have low cognitive performance in males with low testosterone (*145*). In the rodent model, low testosterone for long periods results in elevated levels of oxidative stress, and the addition TRT reverses the detrimental actions. Gonadectomized male rats had a significant increase in oxidative stress and reduced mitochondrial function administration of testosterone reverse these effects (*146*). Additionally, in orchiectomized rats, there are increases in oxidative stress and morphological changes in the hippocampus, and these changes were inhibited when testosterone was given (*147*). Moreover, in gonadectomized Sprague Dawley male rats they found an increase in hippocampal oxidative stress and cognitive decline, and TRT improved their

conditions (*148*). Conversely, administering testosterone has been shown to increase oxidative stress under chronic low testosterone conditions in the hippocampus in rats (*149*).

The timing of TRT is also important. *In vitro* studies provided evidence that pretreating neuronal cells with testosterone provide neuronal protection. On the other hand, giving testosterone after oxidative stress has occurred is detrimental to neuronal cells (*150*). Additionally, castration is a mild oxidative stressor and stroke is a severe oxidative stress that might influence the ability of T to be protective. Thus, the interaction of castration with testosterone treatment during stroke may influence the ability of testosterone to provide neuroprotection. Furthermore, tempol has shown to decrease arterial pressure in salt-sensitive stroke-prone spontaneously hypertensive rats (*144*). Oxidative stress can create havoc under pathological conditions. As seen under ischemic conditions, during the reperfusion phase, there is an overproduction of free radicals that overpowers the brain's natural antioxidant process and leads to ischemic injury (*141*). Further, the amount of oxidative stress and the timing in which testosterone is given could also diminish the beneficial effects of TRT. Under conditions of high oxidative stress and inflammation such as obesity, diabetes, and asthma, TRT has been shown to lose its neuroprotective effect and even exhibit detrimental effects in men with co-morbidities (*14*).

Summary

The overall objective of this research proposal is to determine the role of long-term testosterone deprivation and replacement in a middle-aged comorbidity model. The rationale for the proposed project is that: a) Testosterone given outside of "critical window" will worsen stroke outcome (*15*); b) Testosterone given after prolonged oxidative stress will lead to the detrimental outcome (*16*). *We hypothesize* that in middle-aged rats, long-term testosterone deprivation (LTTD) will inhibit the neuroprotective effects of testosterone, increase oxidative stress, and worsen outcomes after stroke.

The *overall aim* of the study is to use stroke as a co-morbidity model to determine if LTTD will inhibit the neuroprotective effects of testosterone: Our *working hypothesis* is that LTTD will worsen subsequent stroke outcome even after testosterone replacement. Our objectives for this aim are to:

1. Assess functional recovery after stroke using a co-morbid LTTD-stroke model.
2. Determine the effects of LTTD in the co-morbidity stroke model on oxidative stress and androgen receptor levels.

Collectively, *we expect* that the neuroprotective effects of testosterone replacement will be diminished after long-term testosterone deprivation, worsen stroke outcome, and increase oxidative stress in the middle-aged model. The findings from this study will provide further insight for physicians and middle-aged hypogonadal men considering TRT.

CHAPTER II

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Chronic testosterone deprivation sensitizes the middle-aged rat brain to damaging effects of testosterone replacement

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Abstract

Introduction: An increasing number of middle-aged men are being screened for low testosterone levels and the number of prescriptions for various forms of testosterone replacement therapy (TRT) has increased dramatically over the last 10 years. However, the safety of TRT has come into question with some studies suggesting increased morbidity and mortality. Objective: Because the benefits of estrogen replacement in postmenopausal women and ovariectomized rodents are lost if there is an extended delay between estrogen loss and replacement, we hypothesized that TRT may also be sensitive to delayed replacement. Methods: We compared the effects of testosterone replacement after short (2 week) and long-term testosterone deprivation (10 weeks) in middle-aged male rats on cerebral ischemia, oxidative stress and cognitive function. We hypothesized that long-term testosterone deprivation would increase oxidative stress levels and abrogate the beneficial effects of TRT. Results: Hypogonadism itself and TRT after short-term castration did not affect stroke outcome compared to intact rats. However, after long-term hypogonadism in middle-aged male Fischer 344 rats TRT exacerbated the detrimental behavioral effects of experimental focal cerebral ischemia, whereas this detrimental effect was prevented by administration of the free-radical scavenger tempol, suggesting that TRT exacerbates oxidative stress. In contrast, TRT improved cognitive performance in non-stroked rats regardless of the length of hypogonadism. In the Morris water maze peripheral high oxidative stress was associated with decreased cognitive ability. Conclusions: Taken together, these data suggest that TRT after long-term hypogonadism can exacerbate functional recovery after focal cerebral ischemia, but in the absence of injury can enhance cognition. Both of these effects are modulated by oxidative stress levels.

Introduction

The interest in testosterone replacement therapy (TRT) has grown rapidly in the last decade (*151, 152*) . In the absence of known organic causes of hypogonadism such as pituitary or testicular disease, safety and prescribing practices for younger men remains in question (*153*). Because symptoms of "low T" are indistinct and T levels do not fall precipitously in men as they do in women after menopause, the duration, extent, and causes of "low T" in middle-aged men are rarely clear (*154*). Currently, contraindications for TRT prescribing include polycythemia (HCT>54%), breast cancer, and prostate cancer (*154*), untreated severe sleep apnea, and uncontrolled congestive heart failure (*155*). Other than these indications, TRT is considered relatively safe, particularly in older men (>65), although the benefits may be limited to mood, bone health, and sexual function (*156*).

Although it is clear that low T levels are associated with cardiovascular disease, including stroke (*79, 81*), the health effects of TRT are less clear. Most recent longitudinal studies support the benefits of TRT for cardiovascular and cerebrovascular disease risk (*86, 124, 132*). However, the duration of subclinical hypogonadism for most men is not known, and despite relatively constant levels of low T in laboratory testing, sales for TRT in the US quadrupled between 2000 and 2011 (*157*). Moreover, in the US more than 90% of prescriptions for TRT are for men under 65 years of age (*158*), and the number of prescriptions for TRT far exceeds the prevalence of strictly diagnosed hypogonadism (two low fasted morning T levels, at least one additional symptom) (*151*).

In women, hormone replacement therapy (HT) with estrogens or estrogens + progestins after menopause can have both beneficial and detrimental effects. In addition to predicted adverse effects such as breast cancer, the detrimental effects of HT observed in the Women's

Health Initiative trials included an increased risk of cerebrovascular events, including stroke, thromboembolism and cognitive impairment (*159, 160*). One of the primary hypotheses for the cerebrovascular effects is that the average age of women entering the study (>65 years) meant that treatment was initiated after a significant period of hypogonadism after menopause and, thus, women missed a "critical window" for benefit (*161, 162*). This critical window is recapitulated in several laboratory reports regarding the beneficial effects of estrogen in experimental stroke in mice (*163*) and rats (*164*) wherein a delay of as little as 10 weeks in estrogen replacement abolishes neuroprotection. Similarly, estrogen is no longer protective in global cerebral ischemia after long-term estrogen deprivation (*165*). Long-term estrogen deprivation (LTED) also impairs estrogen-dependent support of immune function (*163*) and antioxidant activity (*166*). Furthermore, although immediate treatment with estrogen after ovariectomy protects the brain from ischemia, LTED increases amyloidogenesis in the hippocampus, and this effect cannot be inhibited by estrogen (*165*).

We hypothesized that there may be a similar "critical window" for the beneficial effects of TRT. Low T is associated with several comorbid states that impact the response to cerebral ischemia including obesity, hypertension, dyslipidemia, and type 2 diabetes mellitus (*167, 168*). One common feature of these low T associated states is elevated oxidative stress levels, and the underlying increase in oxidative stress may increase susceptibility to the detrimental effects of TRT. Although T can have antioxidant actions in experimental stroke (*127, 169, 170*), T can also induce oxidative stress in the brain (*146*). In addition, preexisting oxidative stress can result in toxic effects of T treatment in dopaminergic neurons (*150*) and male rat brains (*171*).

The effects of T on stroke outcome in males appear to be age-dependent with studies of short-term T deprivation and replacement revealing beneficial effects in middle age, but

detrimental effects in young animals (*95, 136, 137, 172, 173*). Supraphysiological T or DHT leads to worse ischemic injury (*95, 137, 172, 174*), but low doses of either T or DHT have also been shown to improve stroke outcome in young animals (*137, 174*).

As with experimental stroke, several studies support a correlation between T levels and cognition. Longitudinal studies show that men with the greatest decline in T levels with aging have more cognitive decline and greater Alzheimer's Disease risk (*74, 175*). Furthermore, reductions in T to treat prostate cancer increases stroke risk in humans (*131, 176*). Supraphysiological TRT can enhance visuospatial cognition in older men in some studies (*177, 178*). However, the data in human studies are inconsistent, with several studies showing no benefits (*115, 116, 179, 180*), although TRT effects may be dose-dependent (*177, 181*).

In this study we used physiological TRT in middle-aged rats to determine whether a critical window exists for the effects of testosterone on stroke and cognition and whether oxidative stress plays a role in the beneficial and/or detrimental effects of TRT.

Materials and Methods

Animals:

All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Middle-aged (12 mo) male Fischer 344 rats were obtained from the National Institutes on Aging colony. Rats were maintained on a 12:12 light: dark cycle (lights on 7:00 AM) with ad libitum rat chow and water in an AAALAC approved centralized animal facility. Animals were weighed weekly.

Gonadectomy and hormone treatment:

Rats were gonadectomized or sham gonadectomized under isoflurane anesthesia (2-2.5%). Sham surgery consisted of an incision in the scrotum and visualization of the testes. Testosterone or cholesterol treatments were performed with subcutaneous silastic capsules. Crystalized testosterone and cholesterol were obtained from Steroids (Newport, RI) and packed into silastic tubing (1.47 mm i.d. × 1.96 mm o.d. × 10 mm length, Dow Corning, Midland, MI) sealed on the ends with silastic adhesive. Capsules were placed in 10x PBS overnight to ensure patency (floating capsules were used). Two capsules were placed in a subcutaneous pocket in the chest under brief isoflurane anesthesia.

Focal Cerebral Ischemia:

Rats were randomly assigned to 5 experimental groups (Figure 1). Intact (Intact) rats were sham gonadectomized and otherwise untreated throughout the protocol. Long-term testosterone deprived rats were castrated ten weeks before implantation of cholesterol (LTTD) or testosterone containing implants (LTTD+T). The third group of LTTD rats was treated with testosterone and the antioxidant Tempol (1 mg/ml) in their drinking water starting eight weeks

after castration and continuing until the conclusion of the study. Short-term testosterone deprived with testosterone (STTD+T) were castrated and treated two weeks later with T capsules. Three weeks after treatments began, rats underwent middle cerebral artery occlusion, as described below. All treatments were continued throughout behavioral studies and until rats were humanely euthanized.

Middle cerebral artery occlusion:

Rats underwent middle cerebral artery occlusion (MCAO) via intraparenchymal injection of endothelin 1 (1.5 mg in 3 ml PBS; Sigma) adjacent to the left middle cerebral artery, as previously described (*182*). Briefly, rats were anesthetized with isoflurane and placed in a stereotax with Bregma and Lambda level. Body temperature was maintained with a servo-controlled heating pad connected to a rectal thermometer. After opening the scalp through a midline incision, a laser Doppler flow probe (Perimed) was attached to the left side of the skull 3 mm caudal and 5 mm lateral to Bregma to assess parietal cortex blood perfusion. A burr hole was drilled in the skull 0.9 mm rostral and 3.4 mm lateral to Bregma on the left side. A 26-gauge Hamilton syringe attached to a stereotaxically mounted Micro4 syringe pump (World Precision Instruments) was lowered 8.5 mm from Bregma to inject endothelin 1 (ET-1) over a period of 6 minutes. The syringe was left in place 3 minutes before removal. Bone wax was used to close the skull. The scalp was infiltrated with 0.25% bupivacaine (Hospira, Lake Forest, IL), and the scalp was closed with surgical staples and treated with triple antibiotic ointment. A group of intact sham-stroke rats was injected with PBS vehicle instead of ET-1. The surgeon was blinded to treatment.

Seven rats were euthanized after tumors were detected. Four rats died at 4, 5, 6, and 7 weeks following gonadectomy, and three rats died within three days of stroke surgery.

Behavioral assessment of rats following ischemia:

Three, 7, and 14 days after MCAO rats underwent neurological testing, including an overall neurological score, the cylinder test, rotarod, and ladder walking. All tests were carried out in a room lit with red lights between 2:00 and 5:00 PM. The neurological score used an 11-point scale similar to a 14-point scale used previously (*183*). The composite score is derived from the following: spontaneous circling (0=no circling to 3=continuous ipsilateral circling), contralateral hindlimb retraction (0=immediate replacement to 3=no replacement of >2 minutes), bilateral forelimb grasp (0=grabs with all digits on both forelimbs to 3=cannot grasp with either forelimb), and contralateral forelimb flexion (0=both limbs extend when lifted to 2=shoulder adduction with forelimb flexion).

The cylinder test for forelimb placement was performed as previously described (*183*) and scored by video analysis by a blinded observer. Assessments were performed on the day prior to MCAO (day -1) and post-stroke days 3, 7, and 14. Rats were placed in a clear plexiglass cylinder for 10 minutes and forelimb touches on the cylinder side when rearing was recorded. Data are expressed as a ratio of contralateral to ipsilateral touches.

An accelerating Rotarod (Omnitech Electronics, Columbus, OH) was used to assess balance and motor learning. Rats were trained in 4 trials/day for 3 days prior to MCAO as the Rotarod accelerated from 0 to 75 RPM in 150 s. The time to fall on the final day of training, one day before MCAO, was recorded as the baseline ability. Trials were repeated on days 3, 7, and 14 following MCAO. Data are reported as the average of 4 daily trials.

Three days prior to stroke, rats were acclimated to a ladder walking test on a 4-foot long automated horizontal plexiglass ladder with rungs spaced 0.5 inches apart (San Diego Instruments, CA). The ladder was suspended 2 feet above a table. Rats were placed on one end

of the ladder and encourage to traverse the ladder to a dark box by air puffs and a bright spotlight. An infrared beam activated timer automatically started when rats moved to the first rung and turned off when rats reached the box. A separate infrared beam detected foot faults that were verified by video recording. To account for differences in motivation and speed, foot faults are reported as a function of total time to cross the ladder.

Cognitive Experimental Protocol:

A separate cohort of animals was randomly assigned to 4 groups. Intact (Intact) rats were untreated throughout the protocol. Long-term testosterone deprived rats were castrated 10 weeks prior implantation of cholesterol (LTTD) or testosterone containing implants (LTTD+T). Short-term testosterone deprived with testosterone (STTD+T) were castrated and treated immediately with T capsules. All treatments were continued throughout behavioral studies and until rats were humanely euthanized.

Cognitive Assessment:

Rats were assessed for cognitive function using a Y-maze, an object location memory test (OLMT), and Morris water maze (MWM) starting 12 weeks after randomization. The Y maze was used to assess hippocampal-dependent memory (*184*) in a black plexiglass maze with three identical arms (50cm L x 13cm W x 35cm H). Rats were placed in one arm of the Y maze facing the closed end. A poster on the wall adjacent to the arms of the maze was used as an external spatial cue. Rats were allowed to explore the maze stem and a single open arm for 15 minutes during an informational trial. Twenty-four hours later both arms were open, and rats were allowed to explore the maze for 5 minutes. The number of entries and amount of time spent in each arm was determined using video recording with AnyMaze with a camera positioned

above the maze. Total exploration time and percent time in the familiar and novel arms were calculated.

The Object Location Memory Test (OLMT) was performed as described by McConnell et al. (*185*) . Rats underwent 4 days of habituation to an 80 x 80 x 30 cm open field with a black floor and white plexiglass walls. Days 1 and 2 consisted of 20 minutes habituation in pairs. On day 1 the apparatus contained bedding material that was removed on day 2. On days 3 and 4 habituation occurred individually, again with bedding present on day 3, but not on day 4. The following day rats underwent an exposure trial with two identical objects (plastic juice containers) placed 20 cm from the walls. A poster was place on the wall as an external maze cue. Rats were allowed to explore for 5 minutes and were recorded with AnyMaze software (Stoelting). The field and objects were washed with 70% ethanol. One object was then moved to one of two new locations (counterbalanced) and the test was repeated after a 30-minute delay. The same procedure was repeated 4 days later, and data from the two days was averaged. Exploration was defined as the rat nose falling within 2 cm of the object.

The MWM was performed as previously described (*186*). Briefly, after training in a straight maze with visible platform (3 trials x 2 sessions), rats underwent 7 MWM sessions beginning the following day. Four days of acquisition trials (3 trials, 10 min inter-trial interval), were followed by a two-day rest. A retention session was performed on the day following the rest period (3 trials, 10 min inter-trial interval). On the following two days rats underwent reversal trials in which the hidden platform was moved to a new location. Probe trials without the platform were run prior to acquisition session on day 4 and 10 min after the final reversal trial on day 7. Data was collected by a digital camera and AnyMaze software. Path length,

latency to find the platform, and swim speed were automatically calculated. A group of young (3 mo) intact rats was added to the MWM protocol for comparison.

Tissue Collection:

Within 5 days of the conclusion of behavioral testing, rats were deeply anesthetized with isoflurane and rapidly decapitated with a guillotine. Rats were euthanized between 10:00 am and 12:00 pm. Trunk blood was collected into EDTA coated tubes, inverted multiple times, and placed on ice for less than 60 min. Blood was centrifuged for 15 minutes at 2000 x g and plasma was collected and frozen at -80° C until assay. Brains were removed, cooled in ice-cold saline for two minutes, and placed in a brain matrix. A 2 mm coronal section was made between -0.5 and -2.5 mm relative to Bregma. The remaining forebrain and hindbrain were fixed for 48 hours in 4% formaldehyde at 4° C before being moved to PBS for storage before cutting.

Plasma Measurements:

Total testosterone concentrations were measured in duplicate from plasma following euthanasia using a commercial ELISA from BioVendor (RTC001R, Ashville, NC). Peripheral oxidative stress was measured in plasma with a commercial kit for Advanced Oxidation Protein Products (AOPP; OxiSelect, Cell Biolabs, Inc., San Diego, CA) using manufacturer's instructions. Samples were diluted 1:6 and assayed in duplicate. AOPP results from the interaction of reactive oxygen species with proteins to yield modifications including dityrosine, pentosidine, and carbonyls (*187*). Increase AOPP levels are associated with many pro-inflammatory diseases and are used as a biomarker for oxidative stress (*188*).

Statistical Analysis:

All data were analyzed with GraphPad Prism v8. For biochemical data (T, AOPP, western blotting), results were analyzed by one-way analysis of variance (ANOVA) with

treatment group as the independent variable and P set as <0.05. Pair-wise comparisons were made with Tukey-Kramer tests. For cognitive testing, two-way ANOVAs were used with treatment group and time as independent variables. In some cases, repeated measure ANOVA's used a mixed effect model due to missing values. Pair-wise comparisons were made with Tukey-Kramer tests. In some cases with pretreatment measures, a Dunnett's test was also used to compare baseline to post stroke results. Covariance was determined with Pearson's correlation coefficient. For post MCAO behavioral assessments, data were analyzed by 2-way ANOVA with repeated measures with treatment group and time as the independent variables and P set at 0.05. Pair-wise comparisons were made with Tukey-Kramer tests and comparisons to baseline measures were made with Dunnett's tests. All data are presented as mean ± SD, and animal numbers are stated in the figure legends. Rats that did not complete all days of testing for a particular behavioral assessment due to incapacitation, death, failure to meet pretest criteria, or failure to perform test were excluded from repeated measures analysis resulting in different n's across experiments.

Results

Intact and sham stroke rats gained significant weight over the initial twelve weeks prior to stroke (11.3 and 40.1 g, respectively, $P<0.05$). Although other treatment groups gained weight, the changes were not significant (data not shown). In non-stroked rats from the cognition study cohort, long-term testosterone deprivation (LTTD) significantly ($F_{3,35} = 7.65$; $P < 0.001$) reduced circulating total testosterone to near undetectable, and silastic implants of crystalline testosterone restored levels to those observed in age-matched testes-intact rats (Figure 2A). Also, in non-stroke rats, peripheral oxidative stress, as measured with advanced oxidized protein products (AOPP), did not differ in intact and LTTD rats (Figure 2B). Testosterone treatment after LTTD did not significantly alter AOPP, but T significantly reduced AOPP levels in STTD+T rats (ANOVA $F_{3,34} = 5.08$, $P < 0.006$, Tukey, $P >0.05$; Figure 2B).

Injection of ET-1 significantly reduced parietal laser-Doppler flow, indicating successful constriction of the middle cerebral artery (Figure 3). In response to vehicle injection, laser-Doppler flow (LDF) in the parietal cortex increased by 19% (Figure 3). In contrast ET-1 injection (1.5 mg) resulted in significantly decreased LDF ($F_{5,83} = 74.3$; $P < 0.001$). In Intact rats, the drop averaged 42%, but LDF fell significantly (Tukey df 83 $P<0.05$) further in all castrate experimental groups (Figure 3). However, the drop was not different among the treatment groups, indicating that differences among these groups are not a result of differential ischemia.

Neurological deficits were present in all stroke groups 3, 7, and 14 days after stroke (Figure 4). Two-way repeated-measures ANOVA revealed main effects of time ($F_{1.859, 158} = 18.63$; $P < 0.001$), group ($F_{15, 85} = 18.28$; $P < 0.001$), and a time x group interaction ($F_{10, 170} = 3.91$; $P < 0.001$). LTTD rats treated with testosterone showed no

improvement over time and exhibited a significantly worse neuroscore than all the other treatment groups (Tukey, $P < 0.01$; Figure 4). Multiple comparisons revealed that testosterone treatment was detrimental when treatment was delayed 10 weeks (LTTD+T), but not 2 weeks (STTD+T; Figure 4). The detrimental effect in the LTTD+T rats was abrogated by treatment with the antioxidant Tempol (Figure 4).

Analysis of the cylinder test was complicated by the number of animals that failed to rear at all following stroke (Figure 5A) with fewer animals rearing in all but the Sham group by day 14. Because not all rats reared on each day, a mixed-effect analysis 2-way ANOVA was used for analysis. A significant effect of time ($F_{2.698, 158.3} = 5.92$; $P = 0.002$) and time x treatment interaction ($F_{15, 176} = 2.26$; $P < 0.007$) was observed, but no significant treatment effect was observed. Dunnett's multiple comparisons showed that, compared to pre-stroke (Day -1) results, STTD+T was significantly biased toward the ipsilateral forelimb on day 3 (Dunnett, $P = 0.002$) and LTTD+T was significantly biased on day 14 (Dunnett, $P = 0.001$) after MCAO (Figure 5B).

The aged Fisher 344 rats performed poorly on the rotarod, falling within seconds of the start of rotation. However, all groups reached the same competency by the day prior to stroke (Figure 6). Similar to neurological deficit scores, ANOVA revealed significant main effects of time ($F_{2.248, 69.67} = 224.7$; $P < 0.001$), treatment ($F_{4, 31} = 217$; $P < 0.001$), and time x treatment interaction ($F_{12, 93} = 2.26$; $P < 0.001$). The LTTD+T group performed significantly worse than all other groups (Tukey $P<0.05$). On day 3, STTD+T performed significantly better than other stroked groups (Tukey $P < 0.001$; Figure 6). By day 7, all groups began to improve except the LTTD+T group, whose performance continued to decline through day 14 (Figure 6). One squad of Tempol-treated animals (n=4) was later added to assess the effect of reducing oxidative

stress. Tempol significantly reversed the detrimental effects of T in the LTTD+T group on days 7 and 14 (Tukey $P < 0.01$; Figure 6).

In the ladder walking test, there was a significant effect of treatment ($F_{6, 60} = 8.37$, $P < 0.001$) and a treatment by time interaction ($F_{10, 120} = 2.81$, $P < 0.004$), but no significant effect of time alone. All stroke groups performed significantly worse than sham stroke rats (Tukey, $P < 0.05$; Figure 7). There was also a significant difference between the LTTD and the LTTD+T group (Tukey, $P = 0.016$), with testosterone treatment leading to progressively worse performance (Figure 7). This effect was significant on day 14, when the LTTD+T group had significantly more foot faults than the LTTD group (Tukey, $P < 0.003$). As with other behavioral assessments, Tempol reversed the detrimental effect of testosterone in the LTTD+T group (Tukey, $P < 0.04$).

Three different tasks were used to assess the effects of hormone deprivation and T replacement on cognitive function. In the Y-maze there were no significant differences among the groups in novel arm exploration (Figure 8A). In the OLMT, there was a significant overall effect of object location, with all groups showing increased exploration of the moved object ($F_{1, 39} = 15.94$, $P = 0.003$), but no treatment effects were observed (Figure 8B). To ensure that rats were cognitively aged, a group of four 3-month old males was added for the MWM. The MWM revealed that exogenous testosterone improved performance compared to intact rats regardless of the timing of T (Figure 9). Data in Figures 9A, C, and E are expressed as a mean of acquisition sessions 2-4. One-way ANOVA for path length showed a significant effect of treatment ($F_{4,39} = 8.15$, $P < 0.001$). Young rats had a shorter path length than both Intact and LTTD rats (Tukey $P < 0.05$), and treatment of castrated old rats with exogenous T improved performance compared to intact rats regardless of treatment timing (Tukey $P < 0.01$; Figure 9A and 9B). A

similar main effect of treatment was observed for latency to find the platform ($F_{4,39} = 4.49$, $P < 0.005$; Figure 9C and 9D), and both T treated groups showing significant improvement from intact rats (Tukey $P < 0.05$). Interestingly, LTTD was not different from intact or T-treated groups despite the observation that T levels were equivalent in the Intact and T-treated groups (Figure 2). Young rats swam significantly faster than aged rats ($F_{4, 39} = 11.67$, $P < 0.001$; Figure 9E and 9F), but no differences were observed in the treatment groups. Pearson correlations demonstrated a strong relationship between AOPP levels and increased MWM path length ($R^2=0.337$, $P < 0.002$), and a small, but significant relationship between T and AOPP, with higher AOPP associated with low T levels (Figure 10). No similar relationship between T and path length was observed.

Discussion

Both hypogonadism and testosterone treatment in men are associated with cardiovascular, cerebrovascular, and cognitive dysfunction (*187*). Because the beneficial neuroprotective effects of estrogen replacement are sensitive to the length of pretreatment hypogonadism in women and animal models (*188, 189*), we hypothesized that the effects of testosterone on the male brain would be lost following long-term hypogonadism. The present study revealed a significantly worse behavioral stroke outcomes when TRT was delayed 10, but not 2-weeks, after castration in early middle-aged male rats. This detrimental effect occurred at physiological levels of T that were similar to testes-intact rats of the same age. After stroke, long-term hypogonadism itself had little to no effect compared to gonad-intact rats but sensitized the brain to the detrimental effects of TRT. In contrast, TRT after short-term hypogonadism was not different than intact rats with similar circulating T levels. Thus, it appears that the combination of LTTD with T, rather than T itself resulted in poor outcomes. The detrimental effect of TRT could be completely abrogated with the free radical scavenger tempol, implicating increased oxidative stress as a mechanism for increasing brain sensitivity to TRT. This sensitizing effect was not reflected in AOPP as a measure of peripheral oxidative stress. AOPP was not elevated by LTTD and was only reduced by T after short-term castration.

In contrast to stroke, TRT improved performance in the Morris water maze, but not two other cognitive tasks, compared to gonadally intact rats regardless of the timing of TRT. Water maze performance was significantly correlated with peripheral oxidative stress, further implicating oxidative stress as a correlate of cognitive decline. Thus, like estrogen replacement in chronically hypogonadal females, chronic hypogonadism in males enhances the detrimental effects of TRT, and this effect may involve the enhancement of oxidative

stress. In contrast, exogenous T may provide cognitive benefits beyond those observed with endogenous T in middle-aged males. These chronic effects of castration and T on cognition do not appear to be reflected under stroke conditions in the brain itself where oxidative stress levels are expected to be exacerbated.

Hypogonadism in men is associated with several morbidities, including cerebrovascular disease, cardiovascular disease, and cognitive decline (*76*). Many recent studies of TRT support benefits in aged men (>60) including for cardiovascular and cerebrovascular disease (*86, 124, 132, 156*). Low T, even within the reference range, is associated with an increased risk of ischemic stroke (*76, 81, 135, 190*), and in some studies TRT reduced risk (*86, 124*). Nevertheless, others found that initiation of TRT could increase the risk of major cardiovascular events, including stroke (*190, 191*). These studies point to a complex relationship between T levels and cardiovascular disease that may be both time and dose-dependent (*78, 79*) resulting in uncertainty in meta-analyses (*85*) similar to that resulting from reviews of hormone therapy in postmenopausal women (*188*).

The effects of T and hypogonadism on stroke have been studied in rodents with mixed results. In young rats one week of castration reduced infarct size and T reversed this effect (*77, 136*). Cheng et al. also reported beneficial effects of castration, and detrimental effects of supraphysiological doses of T or DHT (*172*). Dose effects were confirmed in mice undergoing cardiac arrest/global ischemia (*174*) or middle cerebral artery occlusion (*137*) with lower T doses showing some protective effects and higher doses worsening outcomes. Interestingly, both beneficial and detrimental effects are inhibited by the androgen receptor antagonist flutamide (*137*). Only one other study by Cheng et al. (*95*) confirmed previous studies in 3-month old rats showing that castration is neuroprotective, and T reverses

this effect. In contrast, T reduced infarct size in 14-month old rats and 12-month old mice (*95*). However, free T levels were 1.7 to 40 times the levels in intact animals, suggesting supraphysiological replacement (*95*). These results support the notion that high T is detrimental to young animals, but beneficial to older animals. In the present study, total T levels were maintained at the physiological level of the intact middle-aged males which represents a low physiological dose of T. In addition, the Cheng study used commercial dissolvable pellets (Innovative Research of America) whereas we used silastic capsules. Previous studies demonstrate significant kinetic and supraphysiological dose release from commercial pellets, albeit for estradiol, that could affect outcomes (*129, 138, 192*). Importantly, in our study, intact, STTD+T, and LTTD+T all achieved similar circulating T, suggesting that the duration of deprivation rather than the dose of T contributed to the detrimental effects in LTTD+T rats.

Contrary to the beneficial effect of T in middle-aged rats and mice (*95*), we saw neither a detrimental effect of castration nor a beneficial effect of T after short term castration. Whereas previous studies used 1 to 2-week castration protocols and short-term outcome measures (24-72 hours), our study examined functional outcomes for two weeks following stroke. Thus, it is possible that the detrimental effects of T in chronically hypogonadal rats are a reflection of the presence of T during the chronic, rather than acute, phase of stroke. However, Fanaei et al. observed beneficial effects of T in young castrate rats when initiated 24 hours *after* focal ischemia (*127*) and Pan et al. observed mild behavioral improvements when T treatment began one week after stroke (*193*). These results would argue for a beneficial effect of T when absent in the acute phase of stroke and present in the chronic phase. Notably, both of these studies used young animals after short-term castration, and we did observe a small benefit in STTD+T rats in rotarod behavior.

The ability of tempol to reverse the detrimental effects of T in long-term castrate rats strongly implicates an interaction between oxidative stress status and T in stroke outcomes. Oxidative stress long has been recognized as an important contributor to ischemic injury (*194*), and antioxidants and free radical scavengers can reduce experimental stroke injury (*139*). The ability of tempol to reduce the detrimental effects of T in LTTD rats supports the idea that T exacerbates oxidative stress injury. However, in the absence of ischemic insult, there was no exacerbation of peripheral oxidative stress with LTTD as determined by AOPP and the relationship between T and AOPP, though significant was mild. T moderately reduced AOPP, but only after short-term deprivation, whereas T after long-term deprivation showed an intermediate effect. This difference persisted in spite of similar circulating T levels suggesting that the level of T alone is not the primary determinant of peripheral oxidative stress. Based on our previous study (*195*), we hypothesize that this may be the result of a ceiling effect of age on AOPP levels in otherwise healthy animals. Other measures of oxidative stress might better reflect the effects of T. In the rodent brain, long-term castration leads to an increase in oxidative stress and antioxidant defenses in several regions including the hippocampus and TRT can reverse this effect (*146-148*). However, T itself can also act as a mild oxidative stressor (*195*) and this effect appears to follow an inverted U dose-response, with both low and high doses increasing oxidative stress (*146*). Synthetic androgens also increase oxidative stress in the rat brain (*196*) and *in vitro* the effects of T on oxidative stress are also time-dependent wherein pretreatment with T can protect neuronal cells from oxidative stress, but treatment after oxidative stress exacerbates cell death (*150*). Dopaminergic neurons appear particularly sensitive to this effect as T enhances the neurotoxic effects of methamphetamine (*197*) and 6-OHDA (*198*) *in vivo*. The specific pathways mediating

the interaction between T and ROS in the ischemic brain remains to be determined, but our previous studies suggest that NADPH oxidase may play a key role in the detrimental effects of T (*171, 199*).

The effects of T on water maze performance in non-stroked rats support beneficial actions on hippocampal function that persist even in the context of chronic hypogonadism. Although we observed no detrimental effects of hypogonadism or T in OLMT or the Y maze in the present study, other cognitive tests can reveal steroid-dependent differences. In young rats, 12 weeks of hypogonadism leads to impaired water maze and novel object recognition performance that can be improved with T (*200, 201*). Four weeks of hypogonadism can lead to deficits in Barnes maze and Y-maze performance in young male rats that also can be reversed with T (*184, 200*). In contrast, Frye, et al. (*202*) showed that short-term gonadectomy impaired water maze performance in 4-month old Fischer 344 rats but not 13-month old animals that already had reduced cognitive ability. Moreover, T improved performance in the young, but not middle-aged animals (*202*) . In our study, we observed a similar increase in path length as rats aged and no significant effect of gonadectomy in middle-aged animals. However, exogenous T improved cognition in both short and long-term hypogonadal animals. The fact that total T levels in the treated animals were the same as intact rats further suggests that the benefits of T may be due to differences in the metabolism of T in castrate rats or the expression of steroid receptors. Since both short- and long-term castrated rats showed similar benefits after T treatment, the cognitive response, unlike the stroke response, is not refractory to T even after chronic hypogonadism. Another potential reason for this difference is that other testicular factors may inhibit the response of the brain to endogenous T, but this inhibition is removed by castration. For example, castration reduces not only T, but

other testicular hormones such as inhibin B that can result in increased follicle-stimulating hormone and may have independent effects on cognition (*19*). Additionally, follicle stimulating hormone receptors (FSHRs) are found on neovascular endothelium (*203*) and could play a role in testicular hormones independent effects on cognition. Future studies to specifically examine the role of other testicular factors on cognition will be needed to address these apparent inconsistencies. Regardless of mechanism, the results of the present study support clinical findings that show modest cognitive benefits of TRT in men under 60 (*204, 205*), but not older cohorts (*117, 206*).

Androgens can have actions on many cells in the brain and may influence ischemic injury through one or many members of the neurovascular unit. Castration can lead to reductions in neuronal expression of choline acetyl transferase, tyrosine hydroxylase, and neurotrophins in certain neuronal populations that influence cognitive function and provide neuroprotection (*207*). Castration of adult male rats reduces astrocytic glial fibrillary acidic protein (GFAP) in the substantia nigra (*208*), interpeduncular nucleus (*209*) , and hypothalamus (*210*), but increases expression in the hippocampus (*210*). The site of detrimental effects after ischemia is not known, but in male mice, 9 weeks of hypogonadism reduces the integrity of the BBB and endothelial tight junctions and increases the inflammatory response of astrocytes and microglia (*211*). Castration similarly enhances oxidative stress leading to endothelial and neuronal senescence in senescence accelerated mice, in part through enhancing vascular inflammation (*212*). T reverses this effect and enhances endothelial nitric oxide production (*212*) . Interestingly, all of the castrate groups in the present study showed enhanced vasoconstrictor responses to ET-1, even in the context of TRT. Such a result suggests

that cerebrovascular responses to castration may be refractory to T, since several studies support a vasorelaxant effect of T (*213*).

Several mechanisms have been proposed to account for the loss of estrogen-dependent neuroprotection following long-term ovariectomy, including changes in receptor expression, micro RNAs, inflammation, and vascular reactivity (*190*). Similar mechanisms may be in play for the detrimental effect of TRT observed in the present study. We chose to treat with T rather than a non-aromatizable androgen, because testosterone is used clinically in the treatment of hypogonadism. However, both the protective effects on cognition and the detrimental effects in LTTD rats after stroke may be dependent on the conversion of T to estrogen in the brain. Cheng et al. observed increased aromatase activity in the striatum of young, but not middle-aged rats following experimental stroke, but aromatase knockout mice had similar injury as wild type animals (*95*). Furthermore, the androgen receptor antagonist flutamide blocked the detrimental effects of T in young animals and the beneficial effects in middle-aged animals, suggesting that conversion to estrogen was not responsible for the effects of T (*95*). Nevertheless, future studies are needed to determine which steroid metabolites and receptors are responsible for the detrimental effects observed after LTTD.

The use of TRT, especially in young men (<50), without diagnosed organic hypogonadism remains controversial, and it is likely that the balance of effects of TRT will be dependent on comorbidities. Results of the present study suggest that factors that lead to increased oxidative stress, including long-term hypogonadism, may predispose the ischemic brain to exaggerated injury. Indeed, even in the uninjured state, peripheral oxidative stress was highly correlated with poor cognitive performance, similar to the effects observed in men with low T (*95*). Although the castrate rat model may not fully reflect men with idiopathic low T,

regardless of the reason for hypogonadism, our results support early rather than delayed TRT. Future studies will be required to assess which comorbidities put men at high risk for adverse outcomes and whether the beneficial effects of TRT on cognition, libido, and mood outweigh potential risks.

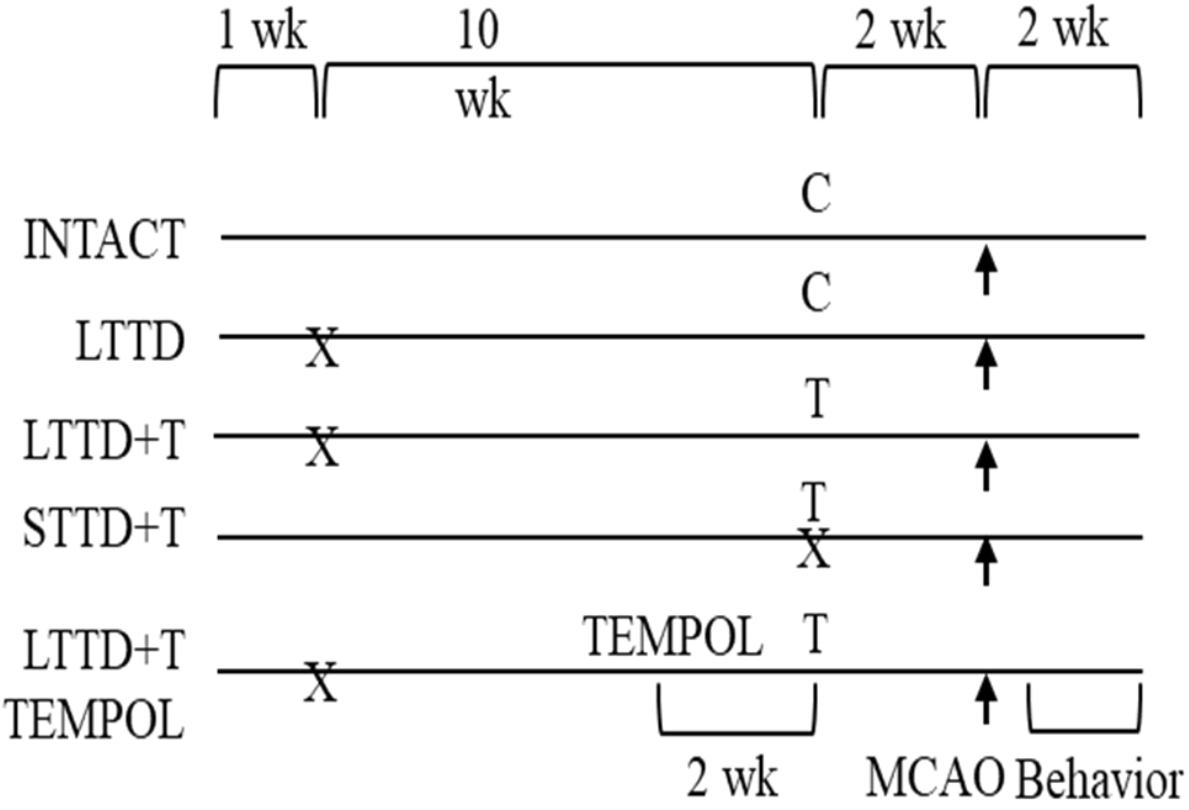

1 wk
10 wk
2 wk
2 wk
INTACT
C
LTTD
C
X
LTTD+T
T
X
STTD+T
T
X
LTTD+T TEMPOL
TEMPOL
T
X
2 wk
MCAO
Behavior

Figure 1

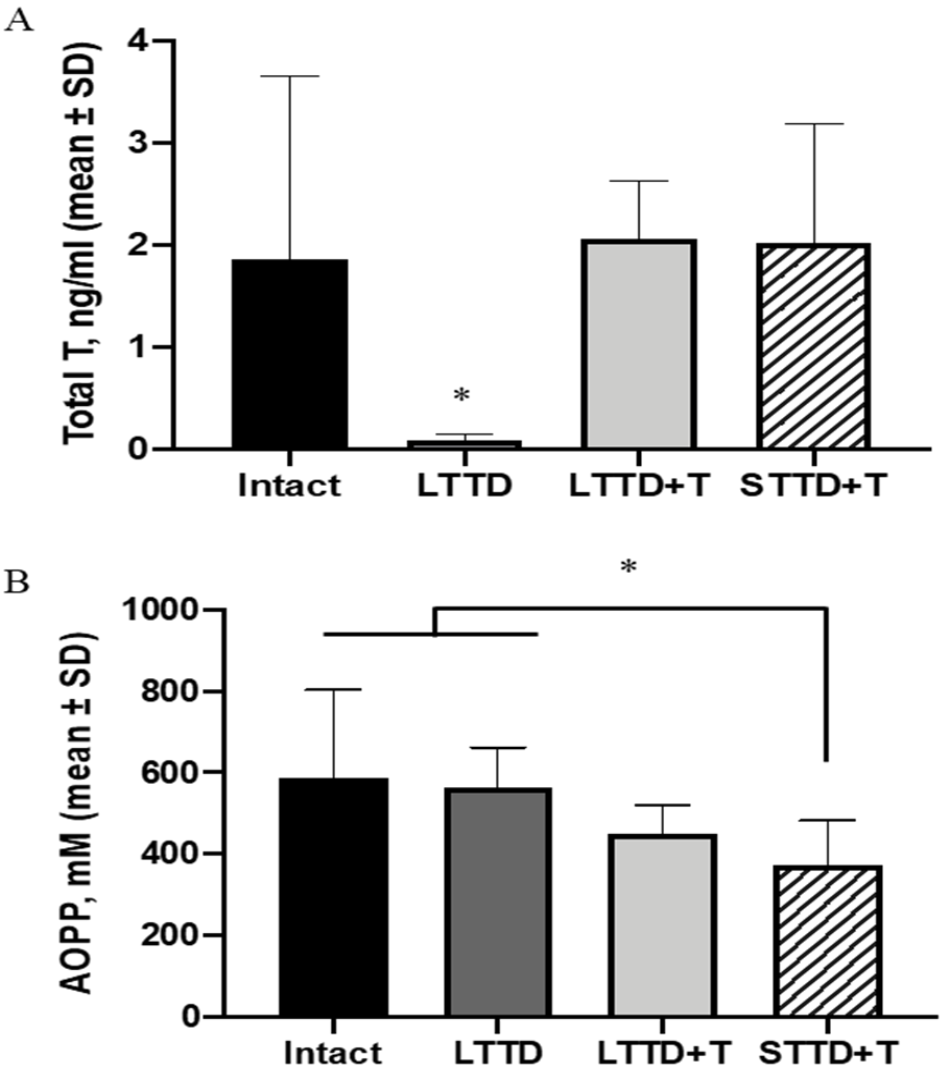

A
Total T, ng/ml (mean ± SD)
4
3
2
1
0
*
Intact
LTTD
LTTD+T
STTD+T
B
*
AOPP, mM (mean ± SD)
1000
800
600
400
200
0
Intact
LTTD
LTTD+T
STTD+T

Figure 2

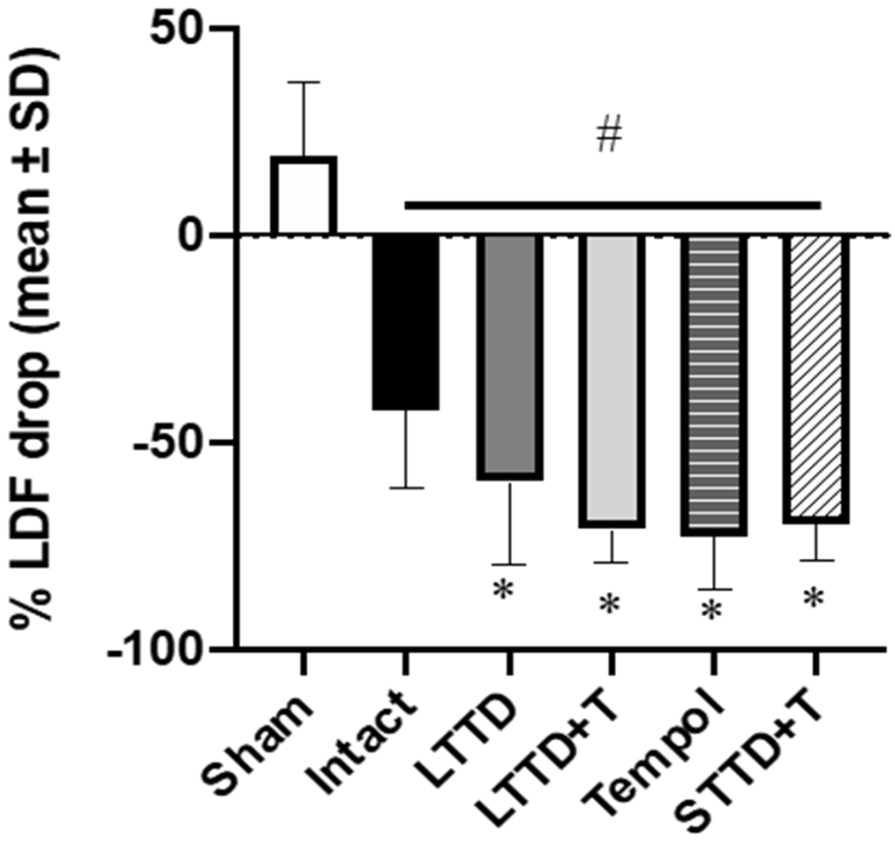

% LDF drop (mean ± SD)
50
0
-50
-100
#
*
*
*
*
Sham
Intact
LTTD
LTTD+T
Tempol
STTD+T

Figure 3

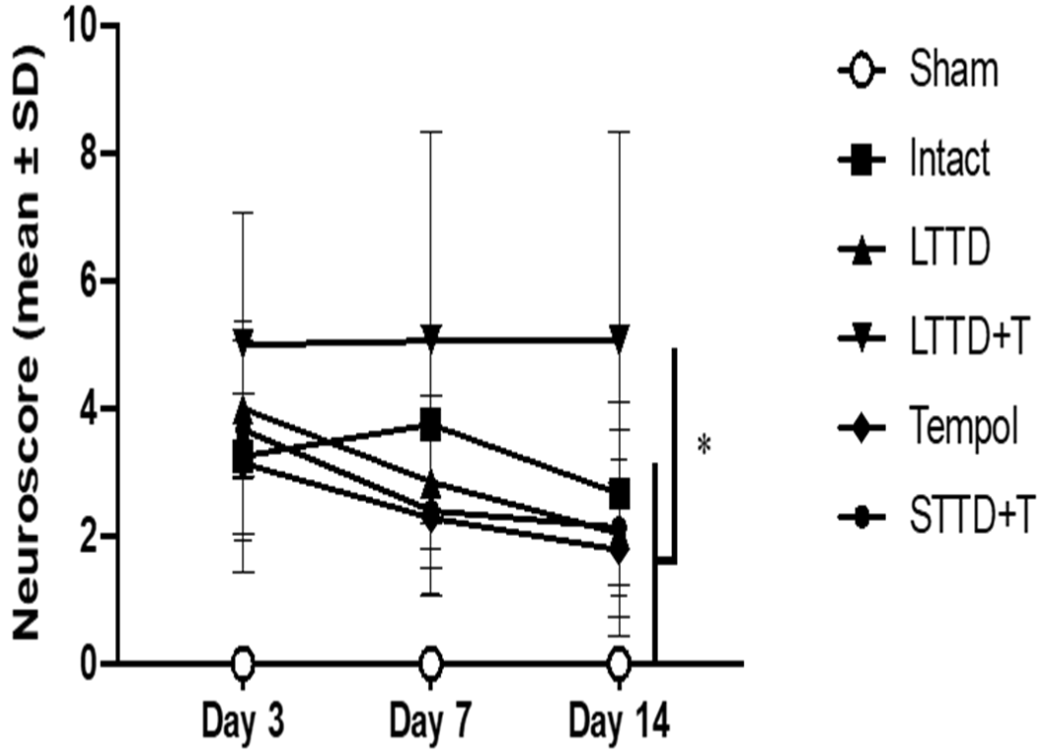
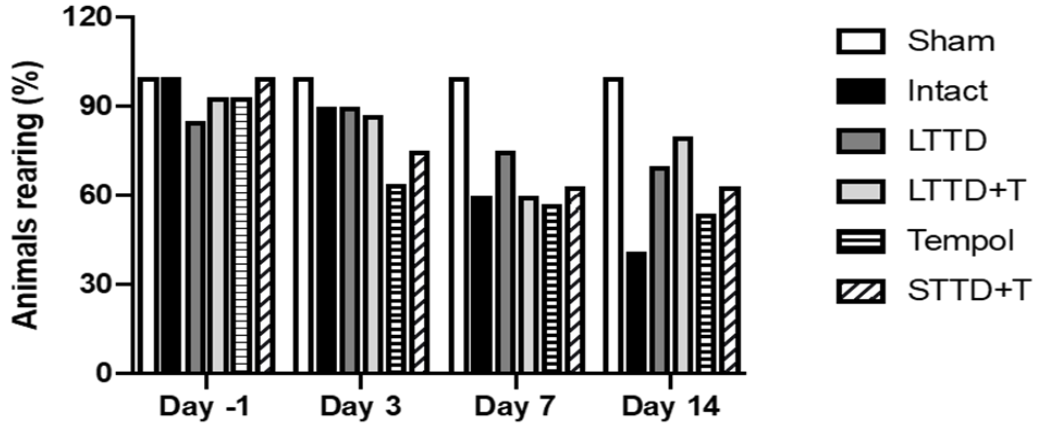

Neuroscore (mean ± SD)
10
8
6
4
2
0
Day 3
Day 7
Day 14
*
Sham
Intact
LTTD
LTTD+T
Tempol
STTD+T

Figure 4

A


120
90
60
30
0
Animals rearing (%)
Day -1
Day 3
Day 7
Day 14
Sham
Intact
LTTD
LTTD+T
Tempol
STTD+T

B

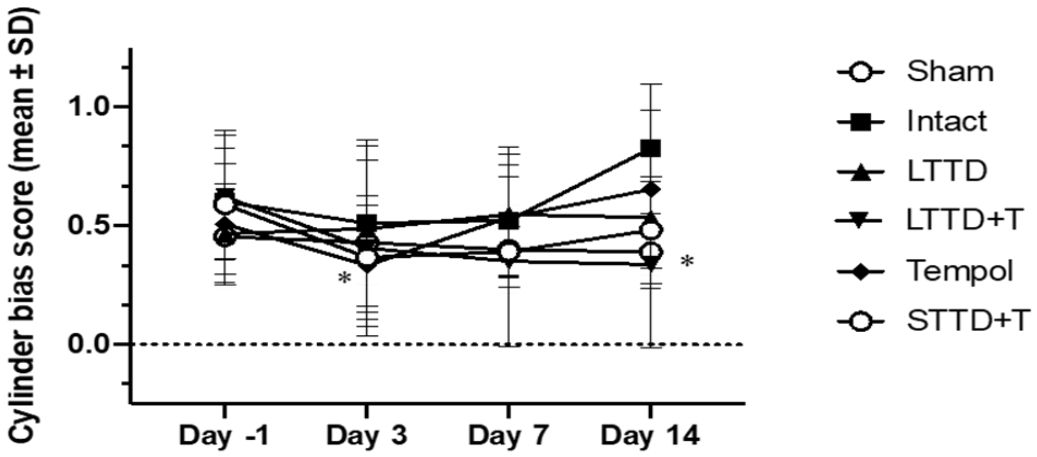

Cylinder bias score (mean ± SD)
1.0
0.5
0.0
*
*
Day -1
Day 3
Day 7
Day 14
Sham
Intact
LTTD
LTTD+T
Tempol
STTD+T

Figure 5

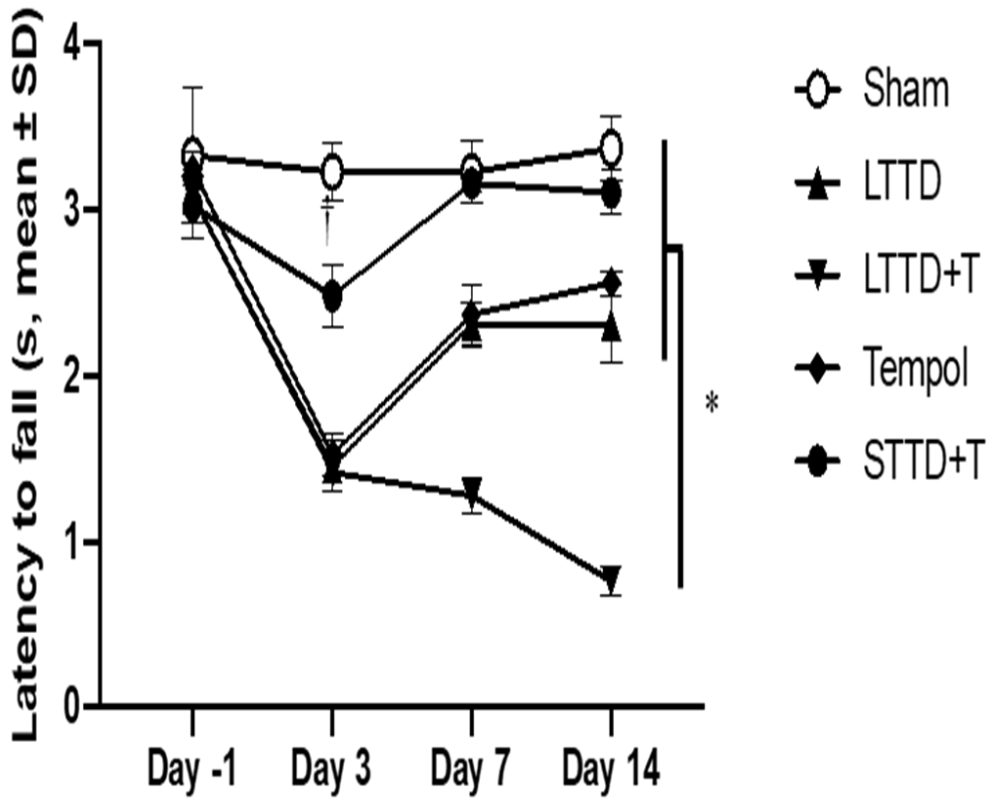

Latency to fall (s, mean ± SD)
4
3
2
1
0
Day -1
Day 3
Day 7
Day 14
*
Sham
LTTD
LTTD+T
Tempol
STTD+T

Figure 6

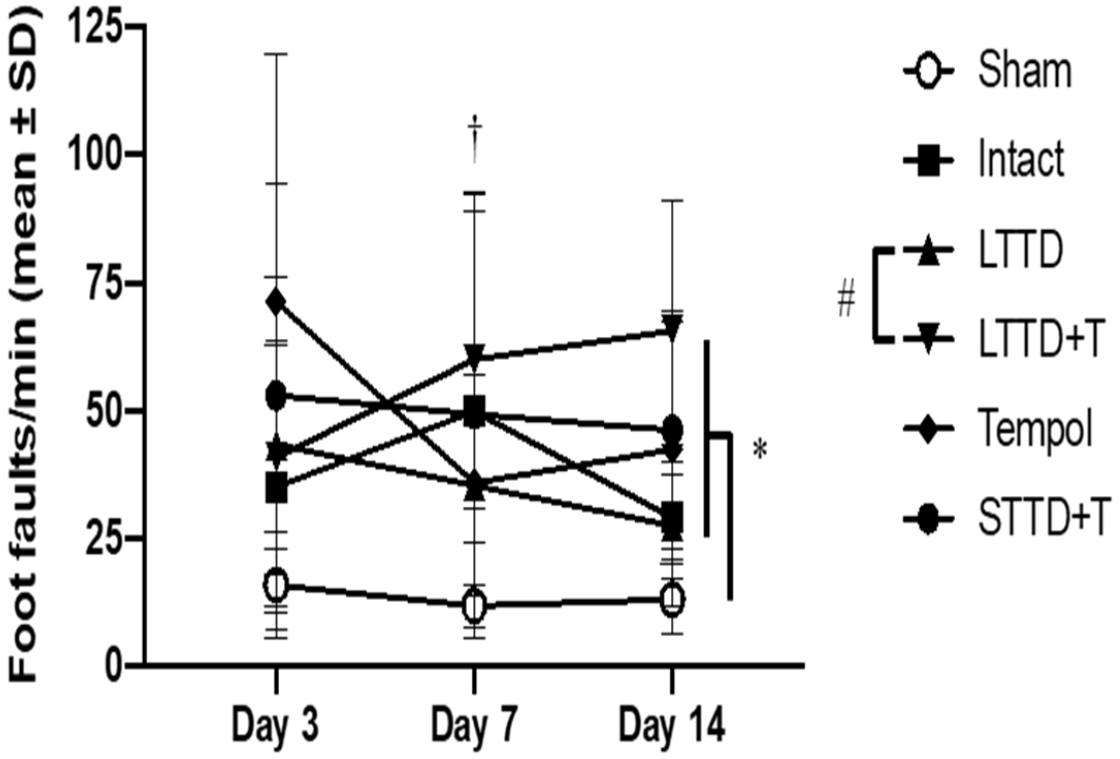

Foot faults/min (mean ± SD)
125
100
75
50
25
0
Day 3
Day 7
Day 14
†
*
#
Sham
Intact
LTTD
LTTD+T
Tempol
STTD+T

Figure 7

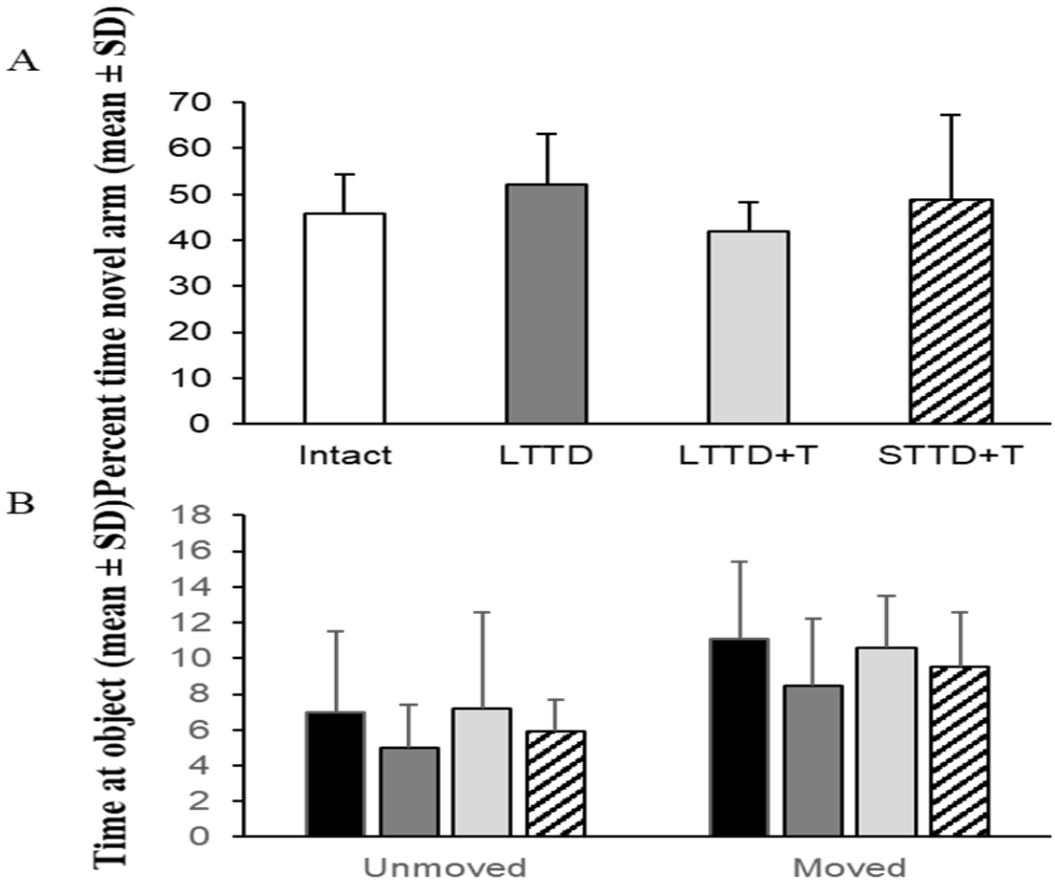

A
Percent time novel arm (mean ± SD)
70
60
50
40
30
20
10
0
Intact
LTTD
LTTD+T
STTD+T
B
Time at object (mean ± SD)
18
16
14
12
10
8
6
4
2
0
Unmoved
Moved

Figure 8

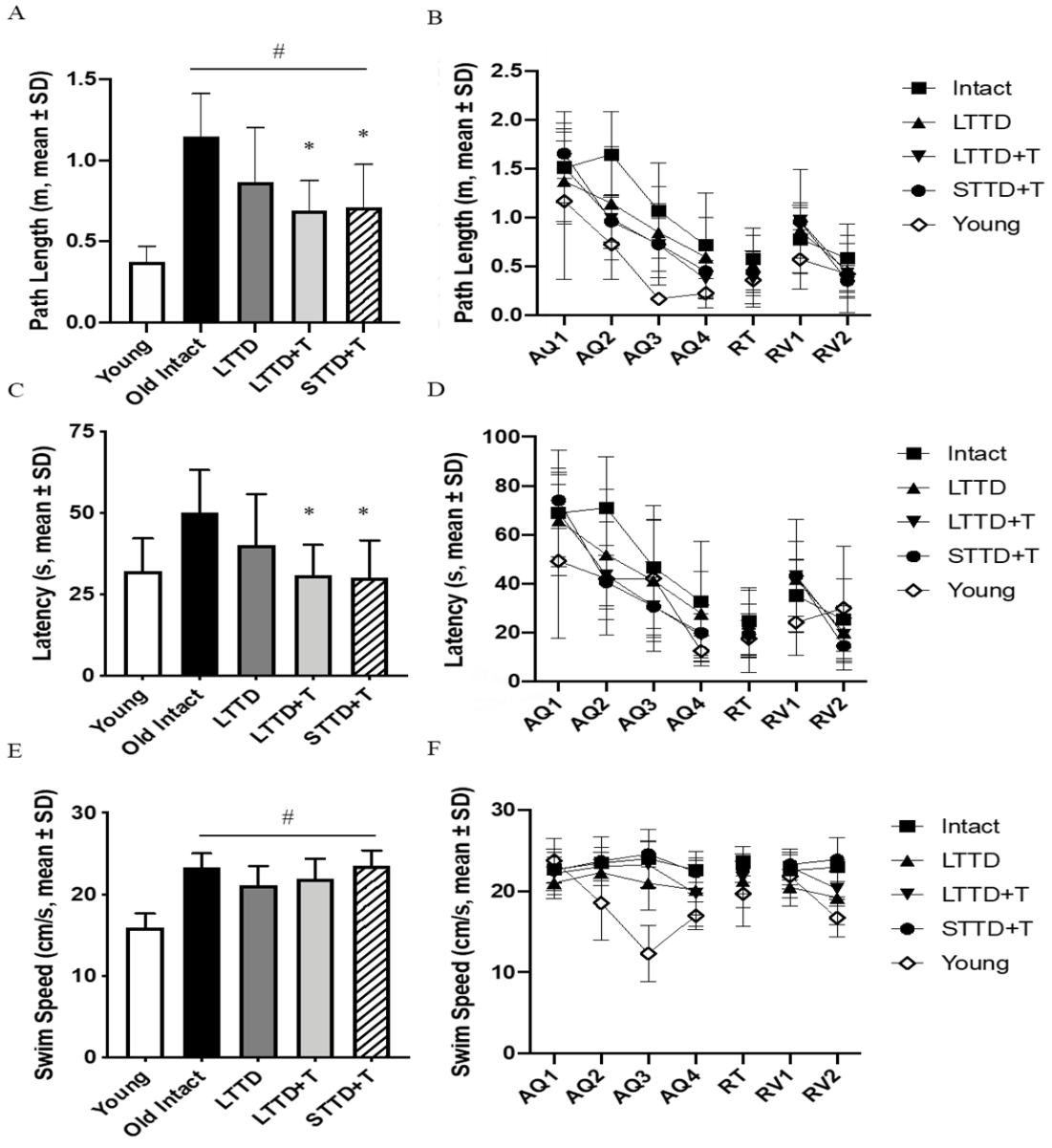
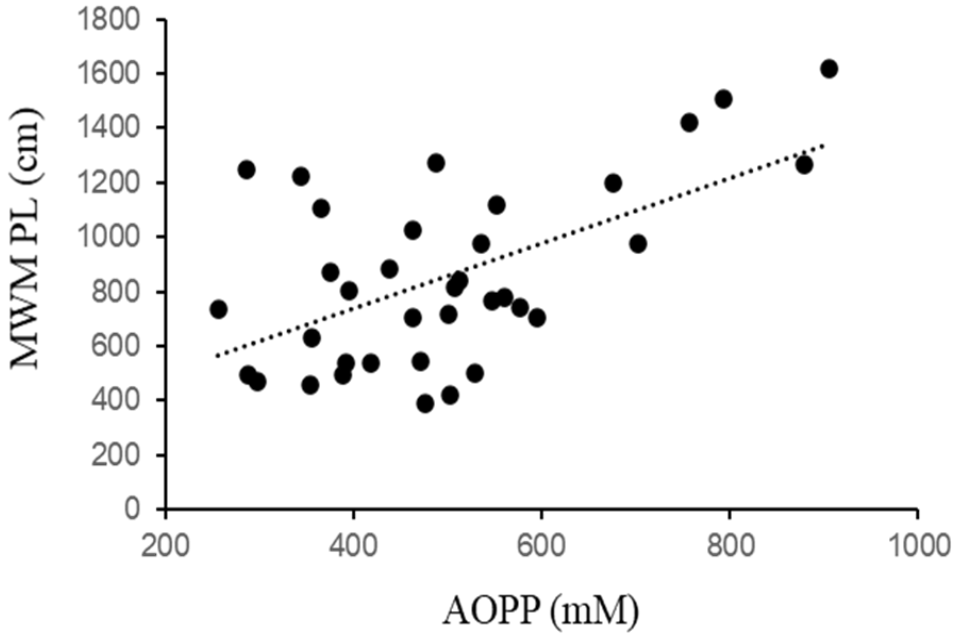

A
B
Path Length (m, mean ± SD)
1.5
1.0
0.5
0.0
#
*
*
Young
Old Intact
LTTD
LTTD+T
STTD+T
2.5
2.0
1.5
1.0
0.5
0.0
AQ1
AQ2
AQ3
AQ4
RT
RV1
RV2
Intact
LTTD
LTTD+T
STTD+T
Young
C
D
Latency (s, mean ± SD)
75
50
25
0
100
80
60
40
20
E
F
Swim Speed (cm/s, mean ± SD)
30
20
10

Figure 9



Comparison	R^2	P
T:AOPP	0.115	0.043
T:PL	0.067	0.127
AOPP:PL	0.337	0.0002

Figure 10

CHAPTER III

DISCUSSION

TRT and hypogonadism have both been shown to be linked to stroke and cognitive impairment (*189*). Our study seeks to bring understanding to the point at which this occurs. Previously, studies done in women and animals have provided evidence that there are neuroprotective benefits of estrogen treatment. Estrogen treatment given early in menopause or as a pretreatment is beneficial under these conditions (*190*, *214*). Our lab hypothesized that the beneficial effects of testosterone replacement would be reduced following long-term testosterone deprivation. We found that TRT in middle-aged rats ten weeks, but not two weeks after gonadectomy exacerbated neurological deficits resulting from experimental stroke. The testosterone replacement given was physiologically relevant to levels in healthy intact rats to serve as a replacement rather than supraphysiological or very young male levels. Furthermore, long-term testosterone deprivation alone did not affect stroke outcome compared to intact rats, and TRT two weeks after gonadectomy did not affect stroke outcome. Importantly, it is the addition of TRT to long-term testosterone deprivation that exacerbated stroke outcomes. Conversely, TRT given after short-term testosterone deprivation was seen to have similar effects to healthy intact rats. The negative effects of TRT were reversed by the SOD mimetic tempol, linking elevated oxidative stress as a plausible process for the detrimental effects of TRT. AOPP, a marker for measuring peripheral oxidative stress, was not significantly increased in long-term testosterone deprivation. Also, AOPP was decreased in short-term testosterone deprivation after

testosterone replacement. These results suggest that basal levels of oxidative stress in the periphery are not reflective of the brain's response to ischemia.

On the contrary, TRT enhanced overall Morris water maze performance but not in other cognitive assessments compared to control rats. This effect was evident irrespective of the timing of testosterone replacement. Data from the Morris water maze also provided support that performance was significantly correlated with peripheral oxidative stress. Furthermore, like long-term hypogonadal females receiving estrogen treatment, long-term hypogonadal men receiving testosterone replacement exacerbates the negative effects of testosterone treatment and is possible due to elevated oxidative stress. Conversely, in our study, testosterone replacement has been shown to improve cognition compared to intact rats with the same circulating levels. Long-term testosterone deprivation plus testosterone replacement is exacerbated stroke injury, but this effect isn't exhibited on cognition.

Stroke, cardiovascular disease, and cognitive dysfunction are just a few illnesses that have been associated with male hypogonadism (*76*). Several studies have shown that TRT is beneficial in men 60 and older for both cardiovascular and cerebrovascular events (*86, 124, 132, 156*). Low levels of testosterone have been shown in several studies to be linked with a high risk of stroke (*78, 81, 84, 135, 191*), and TRT can reverse those outcomes (*86, 124*). Conversely, some studies have shown TRT increases the risk the stroke (*85, 135*). These studies put together to contribute to the multifaceted relationship between testosterone levels and stroke that include in cooperation of time- and dose-dependent factors (*77, 79*). The complexity of the association between testosterone levels and stroke impacts the ambiguity in clinical studies like what is seen in estrogen studies in women (*214*).

In rodent models, the actions of testosterone and hypogonadism on stroke are varied. In young rodent models, gonadectomy after a week decreased infarct size, and testosterone treatment reversed the outcome (*136, 138*). Additionally, another study provided evidence that castration in a rodent model was beneficial. Still, when testosterone was given at high doses, it was detrimental (*172*) A study done in mice demonstrated a dose-dependent effect on stroke or cardiac outcome. In this study, low testosterone doses were protective, and high testosterone doses resulted in exacerbated stroke or cardiac events (*137, 174*). Intriguingly, both neuroprotective and damaging of testosterone were prevented when flutamide, an androgen receptor antagonist was added (*137*). In contrast to young animals, supraphysiological levels of testosterone treatment decreased infarct size in older animals at levels that were detrimental in young animals (*95*). In our study, testosterone levels kept at the same testosterone levels in uncastrated middle-aged males represent a low dose of testosterone treatment (*215*). Also, we used silastic capsules to deliver testosterone treatment in our animal model, whereas other studies used biodegradable pellets (*95, 215*). Thus, the route and dose in which testosterone treatment is given could influence results (*192-194*). Notably, intact, short term testosterone deprived plus testosterone treatment, and long term testosterone deprived plus testosterone treatment all had comparable testosterone levels, indicating the length of testosterone deprivation instead of the amount testosterone resulted in the negative effects found in long term testosterone deprived plus testosterone treatment in rats (*215*).

Even though previous studies have shown positive effects of testosterone treatment in rodent models (*95*)in our study, the positive effects of testosterone treatment were neither seen after gonadectomy nor after short term testosterone deprivation. Furthermore, previous studies used procedures that included 1-2 weeks gonadectomy and measured outcomes within 24-72

hours, while our study measured outcomes two weeks post-stroke. Therefore, the negative effects of testosterone treatment could be a result of testosterone deprivation in a long-term hypogonadal rodent model, rather than the occurrence of stroke (*215*). Nevertheless, the beneficial effects of testosterone treatment were seen in young gonadectomized rats 24 hours after stroke and one week after stroke (*127, 139*). Conversely, these results would suggest that the positive effects of testosterone treatment are present in the absence of testosterone in the short-term after stroke and also in long-term testosterone deprivation. Interestingly, both of these studies were done young rodent models, and we also saw small beneficial effects of testosterone treatment in the short-term testosterone deprived middle-aged rats on the rotarod (*215*).

In our study, tempol was shown to reverse the detrimental effects of testosterone treatment linking the relationship between oxidative stress and testosterone in stroke (*215*). Previous studies have provided evidence that oxidative stress exacerbates stroke outcomes (*141*), and the detrimental effects of oxidative stress on stroke outcomes are reversed by antioxidants (*216*). Since Tempol was able to reduce the negative effects of testosterone treatment in chronic hypogonadal middle-aged rats, this data provides further support for the evidence that testosterone worsens oxidative stress damage. Yet, under the presence of just long-term testosterone deprivation, there was no worsening of peripheral oxidative stress. Additionally, there was a minor significant link between peripheral oxidative and testosterone. Testosterone treatment mildly reduced peripheral oxidative stress in short-term testosterone deprivation; however, testosterone treatment is given after long-term testosterone deprivation revealed an intermediary effect. Since the difference continues even though testosterone treatment was like circulating testosterone levels, this reinforces the notion that testosterone isn't the only contributor to peripheral oxidative stress. Based on previous studies, we hypothesize that in

healthy animals without insult that this could be due to a maximum effect of age on peripheral oxidative stress. Also, another measure should be used for oxidative stress analysis of its relationship with testosterone. In rodent models, chronic hypogonadism has led to significant oxidative stress, and antioxidant levels in several areas of the brain, such as hippocampus and testosterone treatment, reverse the outcome (*146-148*). Yet, studies have shown that testosterone alone can act as a moderate oxidative stressor, and this action has been shown to pursue an inverted U dose-response, leading to both high and low levels of testosterone increasing oxidative stress (*146, 149*). Additionally, testosterone treatment has been shown to increase oxidative stress in a rodent model (*196*), *in vitro* studies show that the link between testosterone and oxidative stress is time-dependent. Therefore, pre-treatment with testosterone was neuroprotective, and giving testosterone after oxidative stress was occurring was detrimental in neuronal cells (*150*). Additionally, dopaminergic cells have undergone negative effects in response to testosterone by exacerbating the damaging impacts of methamphetamine and 6-OHDA (*197, 198*). The mechanism by which testosterone and oxidative occurs in the brain is unknown, but a study provides evidence that NADPH oxidase may play an essential part in this pathway (*171, 199*).

The beneficial effects of testosterone treatment were also seen in chronic hypogonadal rodents without stroke (*215*). Testosterone treatment revealed a positive hippocampal function in the water maze. Additionally, in our study, we did not see an effect of testosterone treatment in our non-stroke rats in the OMLT or the Y maze (*215*). However, other studies that included cognitive testing provided evidence of steroid-dependent distinctions. In young rats that underwent hypogonadism for 12 weeks, performance in the water maze and novel object recognition diminished, yet when testosterone treatment was performed improved (*200, 201*). In

another young study, rats underwent four weeks of hypogonadism, and this resulted in impaired performance in Barnes maze and Y maze that was also improved with testosterone treatment (*184*, *217*). Conversely, short-term castration diminished performance in young 4-month-old rats but not in 13-month-old rats (*202*). Also, testosterone treatment was beneficial in young rats' performance but not in middle-aged rats (*202*). In our study, we saw no significant difference castrated middle-aged rats, but we did see as rats aged a comparable increase in path length. Since the exogenous testosterone treatment led to similar circulating testosterone levels as in intact rats, this suggests the positive effects of testosterone treatment could be due to variations in the metabolism of testosterone in gonadectomized rats.

Sex hormone-binding globulin (SHBG), also known as the carrier protein, plays an essential role in many physiological and pathological pathways. Locally synthesized SHBG in the brain (2 and 6 months of age) and also circulating SHBG (6 and 12 months of age) of Sprague-Dawley male rats was shown to have a positive association with age (*218*). Even though testosterone treatment provides similar benefits in short and long-term hypogonadal rats in cognition, which is different from stroke, there aren't damaging effects of testosterone even in the chronic hypogonadal stage in cognition. Furthermore, since testosterone isn't the only hormone released from the testes, other factors could contribute to the brain's response to circulating testosterone. For example, castration also reduces, Inhibin B is and leads to increased follicle stimulating hormone, luteinizing hormone from the pituitary and gonadotropin-releasing hormone. Kisspeptin in the hypothalamus can have other important individual effects on cognition (*19*). More studies will need to done to understand the role of the other testicular and testosterone-regulated factors on cognition. Irrespective of the pathway, the data from our study provides further evidence to support the clinical findings that testosterone treatment provides

beneficial effects in men younger than 60 (*204, 205*), however not in men older than 60 (*117, 219*).

Testosterone and other androgens can have an important impact on brain cells and can affect stroke insult through associated nerves and blood vessels. Essential neuronal factors such as choline acetyltransferase, tyrosine hydroxylase, and neurotrophins provide neuroprotection and impact cognitive function are decreased during castration. Additionally, in male mice after nine weeks of hypogonadism, blood-brain barrier integrity. Tight junction proteins were reduced, and inflammatory reaction from astrocytes and microglia was increased (*211*). Castration has also been shown to increase oxidative stress resulting in endothelial and neuronal senescence in mice by increasing inflammation in the vasculature (*211*). A plausible link between oxidative stress and inflammation has been associated with NADPH oxidases (NOX). The main role of NOX is to produce ROS. NOX has also been shown to be involved in the development and progression of cardiovascular disease (*220*). Also, ROS produced by NOX stimulates the expression of pro-inflammatory cytokines such as TNF-α and IL-β (*220*). In 12-week-old SAMP8 mice, a model for hypogonadism and cognitive impairment, after two weeks of testosterone treatment, lead to increase endothelial nitric oxide assembly and reduction of oxidative stress compared to wildtype (*212*). Notably, in our study, all gonadectomized groups provided evidence of increased vasoconstrictor reaction to ET-1, also when testosterone treatment was given. As a result, the data implies that cerebrovascular reaction to castration leads to detrimental effects of testosterone treatment because testosterone has been well studied as vasorelaxant (*213*).

Many studies of hypogonadism in women have suggested that fluctuations in estrogen receptor expression, microRNAs, inflammation, and vascular reactivity could be the reason for

reduced estrogen-dependent protection in the brain (*190*). Such changes could also be occurring in our study and hypogonadal men. Since testosterone, rather than non-aromatizable androgens, are used in the clinical setting to treat hypogonadal men, we used it as well in our study. Though the beneficial effects of testosterone treatment on cognition and the damaging effects of testosterone treatment in hypogonadal rats after stroke insult could be due to a mechanism by which exogenous testosterone is converted to estrogen in the brain. Previous studies have provided evidence that there is enhanced aromatase action in the young striatum; on the contrary, middle-aged rats after stroke increased expression of aromatase was not seen (*95*). Also, flutamide, an androgen receptor agonist, has been shown to block the damaging effects of testosterone in a young rodent model and the neuroprotective effects of testosterone in middle-aged rats, suggesting that the transition to estrogen was not necessarily responsible for the actions of testosterone (*95*). Regardless, more studies are needed to understand which metabolites and receptors are essential for the beneficial and detrimental actions of testosterone observed in the hypogonadal male model.

In young men, the treatment of testosterone without a clear diagnosis of hypogonadism is still debatable, and the effectiveness of testosterone treatment could be based on comorbidities. The data from our study proposes that there could be other components that could influence the increase in oxidative stress, including chronic hypogonadism. Chronic hypogonadism could influence the stroke-affected brain to worsen the injury. Certainly, under conditions of no brain insult, oxidative stress in the periphery is significantly associated with impaired cognitive performance, like actions seen in hypogonadal men (*145*). Even though gonadectomized male rats might not completely mirror men with unknown causes of hypogonadism, irrespective of the cause of hypogonadism, our findings provide evidence to support early testosterone treatment

rather than later. More studies are needed to understand which comorbidities place men at increased risk for damaging effects of testosterone treatment and whether the positive actions of testosterone treatment on cognition and mood offset the possible dangers.

It is well established in the literature that as men age, testosterone levels decline gradually. In a recent study by NHANES in healthy non-smoking men (20-80 years old) with no comorbidities showed a steady decline in total testosterone levels and free testosterone as men aged (*221*)**.** A total of 4,045 adolescents and young adult men ages 15-40 years of age had total testosterone (TT) measured from 1999-2016. After controlling for body mass index, comorbidity status, alcohol use, smoking status, level of physical activity. Interestingly, TT in men ages 15-40 was found to have lower levels in later years (2011-2016) than in the early years (1999-2000) (*222*). Elevated BMI was associated with lower TT in men ages 15-40, notably even among men with normal BMI who had a decline in testosterone levels (*222*). This observation is also seen in older men without comorbidities and non-smokers (*221*) In the European Male Ageing Study (EMAS), they compared 3369 community-dwelling lean weight to overweight (BMI 25-30kg/m^2) was associated with the reduction of TT and FT in middle-aged and older men 40-79 years old (*223*). Additionally, according to the CDC, the occurrence of obesity was 39.6% among young adults (age $\geq$20yr), and 18.5% youth ages 2-19 in 2015-2016 (*222*), and there was also shown a significant linear increase in obesity from 1999-2016 for adults and youth (*224*). Furthermore, in men aged 40-79, obesity is the strongest risk factor associated with low testosterone levels (*223*). Three different assays biotin-streptavidin (1999-2004), isotope dilution liquid chromatography (2011-2012), and high-performance liquid chromatography-tandem mass spectrometry (2013-2016) were used to analyze the TT levels in adolescents and young adults from (1999-2016) (*222*). Since three different assays were used from 1999-2016, the assays pose a limitation to the

study by increasing variation in how the data was analyzed. Over the last decades, there has been a decrease in smoking in the United States due to the harmful side effects of increased risk of cardiovascular disease and cancer. Interesting, there is a link between smoking and testosterone levels in men. Cotinine, metabolite nicotine found cigarettes, has been shown to inhibit androgen breakdown competitively and therefore increase androgens in the blood. As a result, smoking is associated with higher testosterone in men (*225*).

In the United States, intramuscular injection and transdermal patch or gels are the main methods testosterone replacement is administered in men (*93*). The disadvantage in intramuscular injection testosterone administration is that it produces strong fluctuations in testosterone levels that includes a spike immediately after injection and drops to its lowest point right before the next injection (*93*). The benefit of a transdermal application is that it promotes more stable physiologic testosterone levels that mimic fluctuations of endogenous testosterone (*93*). Similarly, in our rodent model, rats received testosterone replacement via silastic capsules implanted subcutaneously. The slow release of testosterone into the bloodstream throughout treatment is similar to the benefits of the transdermal application (*215*). Conversely, as men age, the fluctuations changes to and even when the transdermal application is made absorption correctly is highly variable (*94*). Oral preparations are not available in the United States due to first past metabolism by the liver worsens the side effects of testosterone replacement and does very little to raise the testosterone levels. Oral testosterone undecanoate avoids the issues that occur oral testosterone. It presents other challenges, such as high Intra and inter-individual variability, which can result in reduced absorption and testosterone delivery (*93*).

Some of the contributing factors behind the inconsistent data surrounding middle-aged receiving TRT are aging itself (*221*), low testosterone in the presence of comorbidities such as

cardiovascular disease (*215*), and the various formulations in which TRT is given (*93*). Hypogonadism in the presents of other diseases, diabetes, obesity, and cardiovascular disease produces an environment in which oxidative is exacerbated, resulting in adverse effects of testosterone treatment. The method in which testosterone treatment is has a direct effect on how middle-aged men metabolize testosterone in their body (*93*). How testosterone is measured varies from lab to lab (*93*). All of the factors play a part in the underlying inconsistency in the current TRT data on middle-aged men.

Limitations and Future Directions

Some of the limitations of the study were the purpose of the study. The study aimed to develop a model to understand if there is a similar therapeutic "critical window" in testosterone replacement in males, as seen in previous estrogen studies (*163, 226*). To adequately evaluate testosterone-related research, the ideal conditions would be to monitor testosterone precursors, byproducts, and interacting proteins to infer causality and fully understand the direct versus indirect influences of testosterone and testosterone replacement (*93*) on stroke or cognition. Still, for this study, the primary goal was to evaluate the effects of testosterone replacement under chronic hypogonadal conditions and its impact on stroke outcome and cognition. As the next step for future studies to elucidate the direct effects versus indirect effects of testosterone replacement. Testosterone replacement may be complicated by the fact that testosterone is generally metabolized to estrogens and other androgens in the periphery and brain. Furthermore, some of the effects of testosterone on cognition in men may be dependent on aromatization to estrogen. Future studies would include using aromatase inhibitors to determine if the protective effects of T are due to aromatase function, or dihydrotestosterone (DHT).

Additionally, the type of stroke used in the study endothelin 1 (ET-1) injection adjacent to the MCA is a limitation. Many studies use the transient or permanent MCAO model and evaluate the infarct volume or size to determine if the treatment works by the increase or decrease infarct size (*95, 173*). Still, for this study, our model was developed based on what is observed in clinical settings. So we evaluated the effects of testosterone replacement under long-term low levels of testosterone and measured stroke outcome by behavior assessments such as cylinder test and rotarod. Also, since our rodent model was 12-month-old male Fisher 344 rats, which is representative middle-aged human male population 40 to 50 years of age. Middle-aged

Fisher 344 rats' blood vessels are weaker and using traditional transient or permanent MCAO model would cause these vessels to bleed out.

SUMMARY

The data indicate that TRT after long-term hypogonadism can worsen functional recovery following focal cerebral ischemia, but the lack of injury can heighten cognition. Oxidative stress levels regulate both effects. Additionally. The timing in which hormone therapy is given has been shown to play an essential role in the effectiveness and therapeutic actions. So, it's necessary to understand if there is a "critical window" for testosterone treatment.

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