THE ROLE OF THE SEX STEROIDS IN REGULATION OF PHYSICAL ACTIVITY LEVELS IN MICE

by

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ABSTRACT

ROBERT STEPHEN BOWEN. The role of the sex steroids in regulation of physical activity levels in mice. (Under the direction of DR. J. TIMOTHY LIGHTFOOT)

Low physical activity (PA) levels are associated with many chronic diseases and place a massive burden on the health care system. The extrinsic environment has been suggested to regulate activity levels, but an increasing prevalence of physical inactivity in developed societies suggests the presence of other regulating factors. The sex steroids affect PA levels, but the regulating mechanisms are not known. The purpose of this study was to evaluate the interrelationship of sex steroids and PA in mice and the involvement of dopamine 1 receptors (*Drd1*) in sex steroid regulation of PA. First, sex steroid levels were manipulated in male and female mice by gonadectomy and replacement via silastic implants. Wheel running decreased in both sexes after removal of the gonads. Testosterone administration to gonadectomized mice completely reversed the PA deficit while administration of 17β-estradiol recovered 50% of the deficit. Next, the aromatase complex was pharmacologically inhibited. Wheel running levels remained unchanged in normal, orchidectomized, and steroid replaced male mice suggesting the presence of an androgen responsive PA regulating mechanism. Finally, PA levels were observed after orchidectomy in mice receiving either testosterone or a *Drd1* antagonist. PA levels were elevated by testosterone, but were unaffected by the antagonist. The levels of *Drd1* mRNA in the nucleus acumbens and striatum were evaluated after administration of these two compounds. *Drd1* mRNA expression was not different between treatments or compared to control mice. These results suggest that PA is regulated by the estrogens and androgens, but the influence is not regulated by *Drd1*.

DEDICATION

This dissertation is dedicated to my amazing and loving wife Julia. You continue to recognize that I am here to "publish or perish."

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INTRODUCTION

Physical inactivity is associated with decreased health and excessive health care burdens in developed societies. Increasing physical activity can have profound effects on quality of life and certain diseases including obesity (150), cardiovascular disease (33), certain types of cancers (132), and diabetes (148). Physical activity levels are significantly regulated by genetic and biological (non-genetic) mechanisms (83). The sex steroids represent potential biological modulators of activity and have been shown to alter the activity patterns of rodents (81). After surgical removal of the gonads, rodents exhibit very low levels of activity (6, 7, 15, 22, 43, 54-56, 107, 108, 110, 111, 114, 141, 143, 144, 151). The surgically induced activity deficit is reversed by administration of both testosterone and 17β-estradiol (6, 7, 15, 43, 55, 107, 108, 110, 114, 144, 151).

Compounds released from ovarian and testicular tissue are known to influence behavioral, physiological, and anatomical characteristics. Physical activity, as quantified by wheel running in rodents, has been studied as a behavioral characteristic for over a century (129). As early as the 1920s, it was known that physical activity was dramatically affected by the sex steroids. In 1923, Wang (141) first observed a cyclical relationship between activity levels and the vaginal content of female rats. In 1924, Slonaker (121) confirm the involvement of the reproductive tract through the study of activity patterns during periods of significant reproductive changes in female rats. Then in 1925, Hitchcock (52) observed significant differences in the wheel running activity between the sexes noting a higher propensity for locomotion in female rats. These observations suggested the involvement of a compound(s) that was related to sexual gland physiology in females. In order to isolate the tissues and/or compound(s) involved

in the phenomenon several research efforts were undertaken using various isolation and preparation techniques (15, 22, 107, 108, 120, 142, 143). In brief, removing the ovaries reduced the activity drive. The drive was recoverable; however, with the administration of whole ovarian tissue extracts (22), reproductive related fluids including urine from pregnant rodents (107), and crude extracts of amniotin and oestrin, early estrogen containing compounds (108). Interestingly, changes in ovarian steroid concentrations were not limited to females. Wang et al. (144) found increased wheel running in male rats surgically grafted with the ovaries of female littermates.

More recent studies have confirmed previous research with more precise conditions, delivery techniques, and steroidal preparations (43, 72). Using specific estrogen receptor knockout mice, Ogawa et al. (92) demonstrated a clear involvement of the alpha isoform of the estrogen receptor (ERα) in activity regulation. Testosterone was suggested to act on activity in rats through estrogen dependent means (114); however observations (results presented in Chapter 2) indicate the presence of multi-faceted (androgenic and estrogenic) effects regulating activity. The remaining mechanistic steps, from the molecular and cellular pathways initiated by the ERα to the outward expression of the physical activity phenotype, are yet to be understood.

Despite the differences in activity patterns noted between the sexes by Hitchcock (52), several studies investigated the effects of the male sexual glands on activity levels. In 1925, Hoskins (54) noted elevated activity levels in male rats with normal functioning sex organs compared to castrated littermates. In attempts to recover the induced activity deficit, Hoskins (55) grafted testicular tissue into subcutaneous pockets over the abdominal muscles. The grafting technique did not lead to noticeable deviations in

activity levels in the castrated rat. The affinity of the grafting techniques used to reintroduce testicular tissue were subsequently reanalyzed (110). Richter and Wislocki (110), in 1928, found a correlation between successful testicular grafting surgeries (notable remnants of implanted tissue) and the activity levels of castrated male and female rats.

As previously discussed, the androgenic effects on activity are hypothesized to be estrogen dependent. Roy and Wade (114) observed increased activity when estradiol benzoate or aromatizable testosterone propionate were administered to castrated male rats. A similar response was not noted with the administration of non-aromatizable dihydrotestosterone propionate. The stimulatory effects of estradiol benzoate and testosterone propionate were notably inhibited via MER-25 administration, an estrogen antagonist (114).

The regulation of physical activity may involve not only estrogenic and/or androgenic compounds. These compounds likely interact with sex steroid receptors leading to activation of downstream factors (81). The specific compound(s) involved in the downstream actions of the sex steroids are yet to be fully determined. Of noteworthiness, however, are dopamine and the dopaminergic system. Previous studies have not only shown interaction between the sex steroids and the brain (48, 50), but recent research (70) has also shown differential expression of dopamine receptors between high and low active mice. Specifically, the dopamine 1 receptor expression was shown to be lower in high active mice (C57L/J) compared to low activity mice (C3H/HeJ). Further, in pharmacological studies using a D1-like antagonist in our lab, activity levels in C57BL/6J mice were significantly increased above baseline (A. M.

Knab, personal communication). The interaction between the sex steroids and dopaminergic system within the striatum and nucleus acumbens has previously been examined (9). Becker (9) found changes in dopamine activity following ovariectomy and during the cyclical pattern of the estrous cycle in female rats. The interaction is suggested to influence addiction susceptibility to psychomotor stimulants and increases pacing behavior in rats (9).

Currently, there are no studies investigating how the aromatase complex, sex steroids, and downstream factors interact to regulate activity. In the following chapters, these facets will be investigated. Chapter 1 contains an extensive review of both rodent and human literature focused on physical activity regulation via the sex steroids. Currently, the human portion of this chapter has been conditionally accepted to the journal *Sports Medicine*. In Chapter 2, the effects of gonadectomy and steroid replacement were evaluated in both male and female C57BL/6J mice. It was observed that activity was greatly reduced in both sexes after gonadectomy and testosterone recovered activity more efficiently than 17β-estradiol in both sexes despite the steroids being reintroduced in equal amounts. This chapter is in preparation for review in *Physiology and Behavior*. Chapter 3 evaluated the involvement of the aromatase complex in regulating activity levels in male mice using pharmacological inhibition. Activity levels were not altered in intact or orchidectomized mice nor enhanced by steroid supplementation or replacement strategies. This chapter is in preparation for review in *Endocrinology*. Lastly, Chapter 4 investigated the interplay between the sex steroids and the dopaminergic system. Wheel running was inhibited via orchidectomy and recovery from the induced deficit was attempted through administration of

testosterone and a dopamine 1 receptor antagonist (SCH23390). Testosterone significantly increased wheel running activity, however, the antagonist failed to resolve the deficit. Striatum and nucleus acumbens dopamine 1 receptor mRNA and protein levels were not found to vary in mice receiving testosterone treatment suggesting that the sex steroids' effects are not transmitted through dopamine 1 receptors in these brain regions. This chapter is also being prepared for review in *Physiology and Behavior*.

This dissertation represents a concrete basis for future studies involving rodents and human subjects. The evidence gathered by this study suggests that activity is regulated by testosterone and other androgenic molecules in mature male mice. Furthermore, although the brain is a likely physical activity center, the dopaminergic system does not appear to relay testosterone's effects on activity levels. In the future, research will need to focus on other factors that are potentially involved in relaying the effects of the sex steroids on physical activity levels.

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ARE THE SEX STEROIDS INVOLVED IN THE REGULATION OF PHYSICAL ACTIVITY LEVELS IN RODENTS OR HUMANS?

Introduction

It has become axiomatic that the daily accumulation of physical activity will offset many different health conditions and will lead to increased quality and quantity of life (138). However, there are stark differences in the amount of regular physical activity that human males and females complete with little scientific explanation for these differences. While the regulation of physical activity has long been thought of as voluntary and/or influenced solely by environmental factors, there is a growing body of evidence that suggests that physical activity, as defined as any movement requiring the expenditure of energy including tasks of daily living and planned exercise, is at least partially regulated by biological factors (28, 63, 64, 76, 79, 82, 83, 97, 130, 131, 136). These biological regulating factors may take many forms including an increase or decrease of various physiological substance or structures, and/or genetics which may fundamentally alter receptor/protein interaction in the intact organism (82, 83, 130). The extensive animal literature has shown that sex of the individual significantly influences physical activity patterns by working through various sex-related hormonal pathways (81). Biologically, sex steroids play a large role in regulating various physiological parameters; thus, they have naturally been the subjects of investigations trying to elucidate the possible roles they play in regulating physical activity in animals. At this

point, the role that sex steroids play in regulating human activity has been largely unexplored, probably due to the unappreciated role that biological factors play in regulating 'voluntary' activity. The increasing rate of cardiovascular and other hypokinetic diseases in women (31) and the strong association between low physical activity levels and all cause mortality in the developed world (87) make understanding the mechanisms regulating physical activity critical in the context of the health-related goals of our society. The focus of this chapter is to review the literature investigating the effect of and the possible physiological mechanisms through which the various sex steroids regulate physical activity. Both the rodent and human literature will be considered in this review.

For the purpose of this review, physical activity level in rodents will be based primarily on wheel running indices (revolutions, distance, duration, speed) or in a few cases breakage of infrared beams in in-home cage type studies. In the human literature, physical activity will be considered the total amount of activity an individual accomplishes in the course of a day which not only includes formal exercise, but movement that is associated with activities of daily living (e.g. stair climbing, yard work, gardening, etc.). This operational definition includes estimates of physical activity level based on energy expenditure levels that are corrected for body mass. Whereas males of most species generally have larger masses than females, presenting energy expenditure data as physical activity levels without correcting for mass biases the estimates toward males regardless of total daily activity.

Brief Overview of the Biochemistry of Sex Steroids

The biochemistry of sex steroid steroids is well understood and summarized in

Figure 1. Sex steroids primarily consist of androgens (testosterone) and estrogens (17β-estradiol). Both are primarily derived from progestins (progesterone) via dehydroepiandrosterone, androstenedione, and androstenediol, which are formed from cholesterol and pregnenolone upon stimulation by adrenocorticotropic hormone (ACTH).

While the primary sex steroids differ between males (testosterone) and females (estrogen) as do the primary sites of synthesis (testes – male; ovaries – female), quantities of testosterone and estrogen occur in both sexes. Testosterone is also an intermediate substance in the formation of estrogen in both males and females. Through an aromatization process using the aromatase (*Cyp19*) enzyme complex, testosterone is converted to 17β-estradiol; this conversion is not reversible. In males, while some testosterone is converted to estrogen, the majority of testosterone is converted into dihydrotestosterone, which cannot be aromatized into estrogen. While testosterone concentration exhibits minor variations on a daily basis in healthy, adult males (≈6-10 $ng \cdot ml^{-1}$), estrogen and progesterone exhibit cyclical peaks in females, with estrogen peaking at approximately 200-300 $pg \cdot ml^{-1}$ at day 12 of the menstrual cycle (during the *follicular* phase) then drops to approximately 100-150 $pg \cdot ml^{-1}$ during the early *luteal*/late *follicular* transition. A progesterone peak of 8-10 $ng \cdot ml^{-1}$ occurs at approximately day 20 of the cycle and is coupled with a concurrent rise in estrogen to approximately 150-200 $pg \cdot ml^{-1}$ (during the *luteal* phase). The hormonal fluctuations evident in females are characteristic of healthy, adult females and are not typically seen in pre-pubescent girls or menopausal women.

Are Physical Activity Patterns Regulated by the Estrogens in Rodents?

The sex steroids have long been known to influence behavioral and physiological

characteristics of animals. Berthold (10) found extensive differences in adult roosters' comb and wattle morphology after the removal of their testicles while still chicks. The researchers observed behavioral and interactive differences; the individuals with the smallest combs and wattles were suppressed to the lowest ranks in the pecking order and were overly subordinate. The presence of certain chemicals emitted from the testes of the roosters was important in the listed behavioral and morphological characteristics. The presence of such chemicals originating in the sexual organs of animals could be related to a large number of behavioral and morphological characteristics given the proper ability to measure and quantify a behavior of interest.

Sexually Dimorphic Wheel Running: In the first part of the 20th century an apparatus to measure and record wheel running behaviors in small rodents was devised (119, 129) and used initially to evaluate activity in response to environmental variables (129) and during aging (122, 123). It was observed during these studies that activity differences existed between the sexes. Female rats tended to be more active than male rats (122). The presence of sexually dimorphic activity patterns were also observed by Hitchcock (52) using running wheels. Females ran consistently more than males and interestingly both sexes displayed seasonal variation in activity (52). The observations of a sexually dimorphic activity pattern made by Slonaker (122) and Hitchcock (52) supported the idea that activity was regulated, in part, by the sexual glands of rodents.

Wheel Running across the Estrous Cycle: Comprehensive descriptions of the estrous cycle in the white rat by Long and Evans (84) and the activity pattern—originally suggested to be hunger induced—by Richter (105) allowed for more formal study of activity regulation via the sexual glands in females. Wang (141, 142) under the direct

mentorship of Richter evaluated the vaginal secretions of female white rats using the techniques conveyed by Long and Evans (84) and correlated the smear content of the secretions with the activity measured during daily exposure to a wheel running cage. Wang observed well-defined and consistent peaks in activity when cornified epithelial cells (estrous phase) were present alone in the vaginal smear. During this phase, the female rat ran nearly 4,000 revolutions on the running wheel, nearly twice as many revolutions compared to the other phases of the cycle. Based on these seminal studies, it was concluded that the activity pattern in female rats was inherently associated with the function of the ovaries and was stimulated at the onset of the estrous cycle.

Effects of Reproductive Condition on Physical Activity: The female rat undergoes several life changes in which the estrous cycle is altered. Prior to puberty, the estrous cycle is not present. At puberty estrous begins and develops throughout adolescence. In the middle of life, this cycle is consistent and remains naturally uninterrupted until menopause. The presence of the estrous cycle during middle life can be artificially altered by several interactions including copulation, pregnancy, and pseudo-pregnancy induced via cervical stimulation. These interactions and at the onset of menopause reduce the variation of the estrous cycle considerably. The relationship between activity levels and these natural and artificial life conditions provides a unique opportunity to investigate how the two factors are related.

The activity of rats prior to puberty is very low and vigorously changes at the onset of puberty (121, 141, 142). Slonaker (121) observed several female rats during the puberty transition and observed an increase in wheel running from zero to greater than 20,000 revolutions in the middle of life. Wang (141) showed similar results at the onset

of puberty; however, the development of a cyclical pattern took a longer period of time, an effect potentially associated with lower levels of interactions (lack of vaginal smear collections) compared to Slonaker's (121) study. The additional stress induced by the collection technique may stimulate the process, thereby forcing accelerated development of a consistent estrous cycle.

After the onset of puberty, the activity pattern of the rats was elevated but remained variable (120, 121, 141, 142). Activity peaks typically developed every fourth day in a manner consistent with the presence of cornified epithelial cells during the estrous phase of the estrous cycle. Wang (141, 142) and Slonaker (120, 121) consistently observed wheel running levels between 4,000 and 40,000 revolutions, or about 6 to 50 km per 24 hour time period when the wheel's circumference is taken into account.

The elevated levels of wheel running observed throughout midlife remain consistent unless the estrous cycle is disrupted. Slonaker (120) found distinct alterations to the running pattern throughout the processes of copulation and pregnancy. Upon successful mating, the female rat reduced wheel running activity from about 8,000 revolutions per day to about 3,000 revolutions per day (120). This effect was consistent throughout the entire gestational period. At arrival of the litter, the wheel activity further reduced to about 1,500 revolutions per day, remaining low throughout the lactation period. After weaning the pups, Slonaker (120) noted a substantial increase in wheel running activity within five to seven days. The decrease in activity observed after copulation could be mimicked by manual stimulation of the cervix, inducing pseudo-pregnancy. The activity levels during pseudo-pregnancy were similar to the levels observed during gestation (120). In most cases, the activity pattern returned to normal

within fifteen days and coincided with the reoccurrence of a normal, fluctuating estrous pattern (120). Interestingly, in 2002, Girard et al. (40) observed similar levels of wheel running between control mice and mice artificially selected for high wheel running activity immediately following parturition. The effect was concluded to be genetically independent of the wheel running phenotype (40). Speculatively, the decreased amount of estrogen during pregnancy and following parturition may be overriding the genetic drive, established via artificial selection, for activity in these animals.

The end of life induces another series of changes to the estrous cycle in the female rat. Slonaker (121) evaluated the estrous cycle and activity pattern of aged rats and noted a lengthening of the time between estrous peaks that coincided with a lengthening of the time between activity peaks. Furthermore, as menopause progressed to an absence of cyclicity, Slonaker (121) noted a reversion of activity to very low levels which persisted to the end of natural life. The use of natural breaks in the estrous cycle from pregnancy, onset of puberty, and development of menopause provided beneficial insight into the interaction between the estrous patterns and activity patterns of female rats, however, a more stringent control of the estrous pattern could be used to better define the relationship between the two patterns.

Effects of Ovariectomy on Physical Activity: The added level of estrous cycle control was achieved experimentally through surgical removal of the ovaries (2, 6, 7, 15, 22, 23, 43, 107-109, 128, 141, 142, 151), surgical traumatization (143), or chemical blockade of ovarian function (57). Upon manipulation of the ovaries, activity has consistently been shown to decrease in both rats and mice. The effects of ovariectomy, traumatization, and chemical gonadectomy are displayed in Table 1.

The effect of gonadectomy is relative consistent across published studies, with the exception of Asdell et al. (6). The researchers observed an increase in activity after gonadectomy; however, this data is problematic on two accounts. First, Asdell et al. (7) corrected the previously published paper suggesting an inability to repeat all aspects of the original project. Second, the increase in wheel running was observed between a single control and a single ovariectomized animal. The general observations made by Asdell et al. (6) could be skewed due to this extremely low and non-statistically computable sample. If these data are considered outliers, it becomes very apparent that the surgical removal of the ovaries from either rats or mice results in a great reduction in activity vigor.

The other strategies used, ovarian traumatization (143) and chemical gonadectomy (57), elicited similar responses to full surgical ovariectomy, but wheel running in these animals was not permanently reduced. After a matter of weeks, wheel running returned to normal, pre-interventional levels. This return of function indicates that unless complete removal of the tissue is preformed, the ovaries recover the ability to produce activity-regulating substances. As research progressed, several chemicals originating in the ovarian tissue were discovered and purified and researchers adopted replacement strategies to evaluate the effects of different compounds on recovering general activity in both normal and ovariectomized rodents.

Effects of Replacement Strategies on Physical Activity: During the early parts of the 20th century, clinicians utilized ovarian extracts to alleviate reproductive and sexual disorders in humans. Durrant (22) fed ovariectomized rats glycerine extracts containing ground remnants of porcine ovaries, but did not observe a change in activity levels. It

was speculated that oral administration of extracts was an inadequate delivery method because the digestive process altered the function of the not yet discovered hormone that was expected to cause the observed effects on activity (22). The digestive issues were circumvented by Bugbee and Simon (15) by subcutaneously injecting ovarian follicular fluid to ovariectomized female rats. This technique elicited positive results. Wheel running was reinvigorated in the ovariectomized rat to levels observed prior to the ovariectomy surgery (15). When injections were ceased, activity reverted to the lower levels observed after the ovariectomy surgery (15). In contradiction to the digestive issues suggested by Durrant (22), Richter (107) found large and maintainable changes in activity when ovariectomized rats were fed urine from pregnant human females. The extracts and solutions administered in these initial investigations supported the existence of an ovarian hormone regulating the activity patterns of rodents, but lacked the necessary specificity to identify the compound(s) responsible of this effect.

In 1933 and 1934, E.R. Squibbs and Sons developed a technique to produce substantial quantities of an impure form of estrogens (oestrin, amniotin), allowing for a higher level of identification specificity (108). Richter and Hartman (108) injected ovariectomized rats with this compound and observed wheel running levels equal to the activity levels of control animals. As purification technology advanced and based on the previous research involving administration of ovarian extracts, urines, and amniotin, it became clear that the compounds affecting physical activity patterns were estrogens and estrogenic metabolites (151). Several other studies have administered pure forms of estrogens to confirm this assertion (2, 6, 19, 42, 43, 89, 90, 127, 151).

These effects are also existent in males. Wang et al. (144) transplanted ovaries

from mature female rats to castrated male rats and observed a reinvigoration of activity in the inactive castrated rats. The effect was not completely consistent, but was observed to be primarily related to a propensity of some rats to reabsorb the implants; the presence of an ovarian implant in an unabsorbed form was essential to observe changes to the activity pattern of the male rat (144). The estrogens elicited changes to the activity pattern in rodents, but the mechanisms by which such effects were propagated remained enigmatic.

Potential Mechanism of Effect: The majority of mechanistic studies regarding the estrogenic control of activity have focused on central factors. Some general effects of estrogen on brain physiology and neuron morphology have been documented (9, 37, 48, 50, 51, 58, 59, 75) and provide ample evidence that estrogens may be modulating their effects via the brain. Becker (9) found sex differences in dopaminergic neurons in the nucleus acumbens and striatum of rats, an area in the brain that has been suggested to influence wheel running activity via alteration to the dopamine system (70).

The anterior hypothalamic and medial pre-optic areas of the brain are also highly responsive to estradiol treatment. Hill et al. (50) observed apoptotic degradation of dopaminergic neurons in these regions of the brain during ovariectomy-induced estrogen deficiency. More recently, the medial pre-optic area in mice has been shown to contain estrogen responsive, sexually dimorphic cells (51). These brain regions, thus, are potential regulators of physical activity. Fahrbach et al. (26) cannulated several rats and delivered a mixture of estradiol and cholesterol at various concentrations to these brain regions and noted four to seven fold increases in wheel running relative to rats treated with cholesterol alone.

The estrogens' abilities to induce physiological changes in an organism are

directly relayed via anti-oxidant properties (88) or indirectly relayed through several receptors (73). Gorzek et al. (43) evaluated the effects of tamoxifen, an estrogen analogue that lacks the anti-oxidant properties of natural estrogens, on physical activity in mice. Tamoxifen's ability to reinvigorate wheel running activity in ovariectomized mice was equal to 17β-estradiol's ability (43); therefore, the authors concluded that the physiological changes in wheel running were likely due to estrogen's interaction with either isoform of the estrogen receptor. Estrogen receptor α (ERα) and β (ERβ) have similar distributions in the brain and near identical affinities for 17β-estradiol, estrone, and estriol, the three main physiologically relevant estrogens (73, 74).

The direct manipulation of the estrogen receptors is difficult. To circumvent this barrier, phytoestrogens with substantially different receptor binding affinities and receptor specific knockout mice have been used to evaluate the ability of each receptor isoform to modulate the activity response. Genistein, a phytoestrogen found in soy-based products, has a seven-fold higher affinity for ERβ than ERα (73). Flynn et al. (30) provided reproductively intact male and female Sprague-Dawley rats (63-77 days old) genistein (0, 25, 250, or 1250 ppm) via standard rodent chow and noted no significant difference in wheel running activity. Slikker et al. (118) observed similar results in 140 day old Sprague-Dawley rats. The presence of normal functioning gonads in the rats used by Flynn et al. (30) and Slikker et al. (118), could have provided a substantial source of estrogens and masked any effect the genistein had on the wheel running response. Garey et al. (36) evaluated the effects of the phytoestrogen coumestrol (affinity: ERβ > ERα, ref. 73) on wheel running in ovariectomized Swiss-Webster mice. The mice provided coumestrol alone did not run significantly more than control mice, but ran far

less than the mice that received estradiol benzoate (36). Hertrampf et al. (45) provided an isoflavone rich diet (genistein and daidzein) to ovariectomized rats and found no effect on wheel running. The binding affinity differences of phytoestrogens (higher propensity for ERβ than ERα) suggest that wheel running is not directed via the ERβ pathway.

Ogawa et al. (92) gonadectomized ERα and ERβ knockout mice and administered estradiol benzoate pellets. The mice that retained the gene for ERα (the ERβ knockout mice) responded to the estradiol treatment in a similar fashion to gonadectomized wild-type mice. The mice lacking the ERα gene (the ERα knockout mice) exhibited low physical activity levels (92) suggesting that ERα is a primary regulator of activity.

Despite a long legacy of research regarding estrogen-induced activity regulation, the physiological pathways responsible for this regulation, from synthesis of estrogens to the alteration of brain regions, remains incomplete.

Are Physical Activity Patterns Regulated by the Androgens in Rodents?

The number of studies devoted to the effect of testosterone and other androgenic molecules on physical activity are several magnitudes less than the literature focusing on estrogenic mechanisms. Despite these vast differences in quantity of available sources, the role of androgens in regulating physical activity also present a long and varied history of scientific discourse. In addition, the presence of sexual dimorphisms in physical activity patterns in adult humans, with males being more active than females (134), requires further discussion of the subject.

Effects of Orchidectomy and Replacement on Physical Activity Patterns: In rodents, females of a mouse or rat strain tend to run farther than male counterparts (see *Sexually Dimorphic Wheel Running* section); however, the removal of the testes of male

rodents leads to significant degradations in the wheel running response (20, 34, 35, 54, 56, 106). The effects of castration and orchidectomy are shown in Table 2. Much like the literature with female rodents, several androgenic replacement strategies have been utilized in orchidectomized rodents in attempts to reinstate the deficient wheel running behavior.

Hoskins (55) provided both castrated and senile rats grafts of testicular tissue from donor animals. Neither group of rats had altered levels of activity. Upon further investigation of the grafts, a high level of necrosis was noted rendering the grafts non-productive. Richter and Wislocki (110) formulated a second study using testicular grafts under more stringent surgical controls. In this experiment, several grafting surgeries were successful and these animals—deemed "takers"—were evaluated for wheel running. Both male and female rats were successfully transplanted with grafts and displayed increased wheel running activity, but rats that received ovarian tissue responded more vigorously compared to testicular graft recipients (110). The successful implantation, even under stringent conditions was still very limited and resulted in a low percentage (10 to 30%) of the total attempted surgeries being successful (110).

Asdell et al. (6) and Jakubcazak (60) administered testosterone in a purified form and successfully avoided the issues associated with the grafting techniques used previously. Asdell et al. (6) initially suggested that testosterone elevated activity in gonadectomized female rats, but not in males. Unfortunately, this project was unrepeatable and was later withdrawn (7). Jakubcazak (60) was more successful and noted significantly elevated wheel running in castrated rats treated with testosterone. Daan et al. (20) observed a similar response in rats housed in 24 hours of darkness;

testosterone effectively abolished the orchidectomy-induced wheel running deficit.

Broida and Svare (12) evaluated the effects of testosterone in two inbred strain of mice (C57BL/6J and DBA/2J) and one outbred strain (Rockland-Swiss). Interestingly, the response to castration in the three strains of mice was different. The Rockland-Swiss were unaffected by castration (wheel running remained normal) and implantation of testosterone-containing silastic capsules prevented the development of a running deficiency in the inbred mice (12). Unfortunately, the circulating levels of steroids were not quantified and it is possible that the differences were due to poor or inadequate laboratory techniques. The differential response observed between species (rats versus mice) and amongst genetically distinct rodent strains (inbred versus outbred) suggests a higher level of complexity regarding steroid responsive wheel running effects.

Effects of Androgen Receptors and Aromatase Complexes on Activity:

Correlation studies of testosterone levels and wheel running activity allow the genetic and differential complexity to be evaluated in a more systematic fashion in inbred mice. A single correlation study (24) was completed in the 1970s and graphically summarized in Figure 2. Eleftheriou et al. (24) evaluated the levels of testosterone and wheel running in eleven recombinant inbred strains, progenitors, and F_1 hybrid crosses. There was no correlative association between wheel running and testosterone levels across these genetically related mouse strains. Eleftheriou et al. (24) concluded that the variation between strains was related to a genetic predisposition and not because of different levels of plasma testosterone; changes to testosterone levels (due to selective changes in genetic background) were not required for activity regulation.

Roy and Wade (114) further evaluated the androgens' abilities to alter physical

activity patterns in rodents. Using two sources of androgens, testosterone propionate and dihydrotestosterone propionate, Roy and Wade (114) were able to distinguish between the estrogen independent and estrogen dependent (androgens converted to estrogens prior to eliciting effects) effects of the androgens. Testosterone propionate readily converts to 17β-estradiol in the orchidectomized rat, while dihydrotestosterone propionate remains in an androgenic and non-aromatizable form (114). Wheel running was increased by administration of testosterone propionate, but remained depressed after administration of the non-aromatizable androgen. Taken together, the data from Eleftheriou et al. (24) and Roy and Wade (114) provide substantial evidence that any androgenic effects on activity pattern are dependent upon estrogens.

The assertion that androgenic effects on activity are dependent upon estrogens has been inadvertently and non-scientifically substantiated by the relative paucity of androgen/activity research from the 1970s to the late 1990s. With a single article (12) published in the area and more than 15 articles published regarding estrogens effects, it was clear that activity was assumed to be dependent upon estrogens and that in regards to physical activity, the androgens were simply precursory sources of this activity regulating sex steroid. However, several discoveries in the past 12 years have challenged the previously accepted estrogen-dependent hypothesis of androgenic activity regulation.

Initiating the reinvestigation of the androgenic role in activity regulation was a study in 1998 by Perret-Sinol and coauthors (96) that observed a significant correlation (r=0.546, p<0.05) between activity levels and testosterone levels in wild-caught male meadow voles. Supporting these correlational findings, Flynn et al. (29) used vinclozolin, a environmental hormone disrupter with anti-androgenic properties to

evaluate wheel running activity in rats. Both male and female rats were exposed to the chemical during gestation (mothers exposed to chemical in their food) and continued exposure after weaning via food intake. The rats with the highest dosage of vinclozolin (750 ppm) exhibited lower activity compared to controls. Flynn and coauthors concluded that the behavioral changes observed in these animals were due to an inhibitory interaction with the androgen receptor (29).

Inhibition or elimination of the aromatase complex in a living organism would allow for valuable assessment of the need for estrogens in order to regulate activity levels. Hill et al. (49) generated mice that did not express the aromatase complex (*Cyp19)* gene which resulted in mice that lack estrogens. The *Cyp19*-knockout males exhibited high levels of wheel running, exceeding wild type mice by a factor of two (49). Interestingly, the administration of 17β-estradiol reverted wheel running to the levels observed in the wild type mice (49). It was suggested that the lack of estrogens allowed artificial depression of catechol-O-methyl transferase, an enzyme involved in the degradation of dopamine and other catecholamines (49). Conversely, Watai and others (145) observed decreased wheel running in aromatase knockout mice as compared to the wild type mice. Administration of estrogens altered wheel running in the male knockouts, but not in the female knockouts. The differences in these studies is difficult to reconcile and although the use of knockout animals has been invaluable to science and the discovery of biological mechanism, limitations exist (16). These differences and limitations require further study utilizing alternative techniques including administration of pharmacological agents and other potent anti-androgens to manipulate components of the androgenic system.

Are Physical Activity Patterns Defined during Neonatal Development?

Running wheel activity appears to be a relatively stable phenotype in inbred strains of mice (71). Speculatively this stability may be the product of development during the prenatal stages of life. The individual sex effects resulting from exposure to the sex steroids during this stage of life may carry developmental consequences and provide a priming stimulus that program female rodents to be more active than male rodents. To investigate this suggestion, several studies have been completed in which activity was measured in rodents exposed to high levels of estrogens or androgens during natal development (38, 39, 65, 67, 126).

Kennedy (67) compared the wheel running activity of normal male and female rats to androgenized female rats. The androgenized animals received an androgen bolus containing 1.25 mg of testosterone propionate either 5 days prior to birth (pre-natal), 2 days after birth, or 6 days after birth. Kennedy (67) did not observe significant degradations in activity in any of the groups despite serious alterations to mating and sexual behaviors. Gerall (38) provided estrogen and androgen boluses to 5 days old intact or spayed female rats and compared wheel running to intact and spayed controls. The spayed animals lacked running vigor regardless of injected substrate. The estrogenized rodents with functional ovaries ran similar distances to the intact control rats. Based on the data from Gerall (38), it appeared that the presence of estrogens during infancy and an estrogen source beyond infancy were required to stimulate wheel running activity, an effect not necessarily caused by androgens.

Kawashima and Shinoda (65) further evaluated the necessity for the persistent presence of estrogens after neonatal development. These coauthors (65) estrogenized

female rats which lead to a group of rats that were persistently in estrous (elevated estrogens throughout development) and a group that were persistently in diestrus (deflated estrogens levels). The persistent estrous group ran as much as controls; the persistent diestrus group ran significantly less than controls. After ovariectomy, both groups had greatly reduced wheel running, an effect that was reversible with estrogen administration in the persistent estrous group, but only partially recoverable in the diestrus group (65).

Gerall (39) further investigated the effects of androgenization on wheel running in female rats using multiple concentrations of androgens and observed low wheel running levels only in animals with the largest dosage of androgens. Administration of estrogens had no effect on wheel running in these rats and suggested an ability for neonatal androgen administration to prevent estrogen function during the adult portion of life (39).

The development of running drive during the neonatal stages of life has also been shown to persist into adulthood even if estrogens are not present (126). Stern and Jankowiak ovariectomized rats at birth and administered estrogens on days 2 and 4. These animals aged to adulthood and then were measured for wheel running. Wheel running was habitually low, but could be reinvigorated upon estrogen administration (126). Currently, there are no available estrogenization or androgenization studies utilizing male rodents. The current literature suggests that the development of wheel running vigor is established very early in the rodent life cycle, persists throughout life regardless of hormone status, and requires an estrogenic source for development and promulgation during adulthood. The effects of androgens in the development and perpetuation of wheel running in neonatal and adult rodents remain unclear.

Are Physical Activity Patterns Regulated by the Progestins in Rodents?

The progestins have rarely been the focus of activity regulation studies. The studies that have included progestins, mostly progesterone, in their study design have usually focused on the steroid as a secondary variable. Young and Fish (151) evaluated the effects of estradiol benzoate and estrone on activity and noted no effect during a side experiment in which progesterone was administered to animals receiving estrogen treatments. In 1964, Hervey (47) injected 5 mg of progesterone per day for 12 days to normal, intact female rats. Wheel running was assessed in these animals during estrous and non-estrous portions of the cycle before and immediately after injections (47). Wheel running was highest prior to injections and during estrous (Figure 3) and decreased during the non-estrous phases of the cycle, but the cyclical behavior observed in the normal rat was abolished in the presence of progesterone (47).

Rodier (111) expanded the experiments of Hervey (47) and controlled estrogen levels via ovariectomy. Again, the normal, intact rats had reduced wheel running during progesterone administration, but after ovariectomy, progesterone's effects were obscured. Progesterone required the presence of estrogen for its inhibitory effects to be observed, but under the conditions of ovariectomy, activity reached a nadir (≈15,000 revolutions → ≈1,000 revolutions). This apparent nadir may mask the effects progesterone has on activity by preventing the large and significant fluctuations observed in the reproductively intact animal (111). Rodier and Segal (112) evaluated activity during normal and low estrogen conditions in rats chronically deprived of food. Under these conditions the effects of progesterone administration on activity remained depressive, but the element of food deprivation added additional variability. The coauthors (112) found

that food deprivation eliminated the inhibitory effects of progesterone in the normal cycling rodent.

Unfortunately, the effects of progesterone on activity regulation remains highly understudied. Based on the currently available literature, progesterone's effects appear to be inhibitory. Furthermore, in order for progesterone to modulate activity, a rat's estrogen levels (leading to elevated activity) must be pronounced, as is typical during estrous. Further, the physiological and behavioral changes associated with food deprivation block progesterone's effects.

Similar to the progestins, the androgens and estrogens appear to elicit substantial activity regulation effects in rodents (see Figure 4). The value of basic animal literature is defined by its ability to translate into useful human biology related knowledge. Compared to the available basic scientific literature, literary sources detailing sex steroid regulation of human activity levels remain primarily unavailable. The remainder of this review is devoted to the literature investigating activity patterns in humans across various sex steroid conditions.

Are Physical Activity Patterns Regulated by the Sex Steroids in Humans?

The available animal literature (see ref. 81 for a current review) strongly suggests the presence of a physical activity regulating mechanism centered around sex steroid physiology. In rodents, wheel running is reduced after surgical or pharmacological gonadectomy and is increased after hormones are reintroduced via capsules or injections. The necessary ethical limitations of human subjects research have limited the use of such experimental manipulations in humans leading to a reduced understanding of the biological mechanisms present in humans contributing to physical activity regulation.

Through use of natural (due to sex, aging and the menstrual cycle) and artificial changes in clinical populations (pharmacological and hormone replacement therapies), limited research has been compiled to evaluate the extent to which sex steroids regulate activity in the human population.

Are females and males differentially active: If sex steroids play a role in the regulation of daily activity patterns, it is appropriate to hypothesize that male and female activity patterns would differ, especially given the cyclical nature of sex steroids in females. The extensive animal literature (81) shows that female rodents, in general, are more active on a daily basis than male rodents, regardless of the measurement used. Interestingly, the majority of literature investigating this question in humans shows that human females, whether child, adolescent, or adult, are less active on a daily basis than males. Pate and colleagues, in an extensive review of physical activity in adolescents showed that female children and adolescents were generally less active than males (95). This trend continues into adulthood; Figure 5 represents findings from the 2001 (85, 86) and 2007 (1) United States Behavioral Risk Factor Surveillance Survey (BRFSS). In 2001, over 200,000 individuals were surveyed. A greater percentage of males expressed meeting the physical activity recommendations for moderate and/or vigorous exercise than did adult females. The 2007 dataset was expanded to include over 400,000 subjects, and resulted in an even larger activity gap between the sexes. These results also extended to overweight and obese adults. Recent accelerometer data (Figure 5) from Troiano et al. (134) from the 2003-04 National Health and Nutrition Examination Survey (NHANES) showed the same qualitative pattern—males were slightly more active than females—but compared to the BRFSS data, showed a significantly lower number of individuals

actually complete moderate activity. The same male-female differential activity pattern has also been observed in the non-technical Old Order Amish culture (8) as well as in several hunter/gatherer cultures (Figure 6 - the Nuñoa and Tamang being notable exceptions) indicating that in general, regardless of culture, males are more active than females (80, 93). Therefore, in contrast to the well-established rodent literature, the majority of the available literature suggests that human males are more active than human females (81). The causes of differential activity levels in humans are not entirely known; however, measurement of activity levels surrounding naturally or artificially induced changes in sex steroid concentrations may enhance our understanding of the sexually differentiated activity pattern.

Is there a difference between activity levels before and after menopause: The transition in female humans at menopause allows activity to be compared under two very different hormonal circumstances. Prior to menopause, the sex steroids circulate in higher concentrations compared to the concentrations experienced after menopause. Unfortunately, the scientific literature has yet to address this question directly or in sufficient detail. In one indirect study, Dorn et al. (21) investigated breast cancer risk in pre- and post-menopausal women with and without breast cancer. As a component of this analysis, the researchers asked the subjects to recall their strenuous physical activity patterns during the previous two years. Due to their illness, the patients with breast cancer likely had activity patterns that were deviant from normal and therefore were not considered for this review. Unfortunately, statistical comparisons were not made between the two control groups to directly evaluate pre- and post-menopause activity levels; however, these subjects estimated their yearly strenuous physical activity to be

77.3±127.8 $h \cdot yr^{-1}$ in women prior to menopause and 68.4±176.7 $h \cdot yr^{-1}$ in women after menopause (21). While it is tempting to infer a decrease in activity after reductions in steroid concentration following menopause, the considerable variation in the activity measurement and the varying age of the subjects significantly complicates this comparison. Thus, while the literature is limited, the use of both cross-sectional and longitudinal studies measuring activity levels pre- and post-menopause is certainly warranted.

Does physical activity change across the menstrual cycle: Examination of activity patterns in eumenorrheic women provides a hormonal concentration milieu that is unique. Tworoger and colleagues (137) reported the results of a carefully crafted correlational study that used data from 565 pre-menopausal women, ages 33-52 yrs, that were originally surveyed in the Nurses' Health Study II. The investigators measured a wide-variety of sex steroids in blood samples taken during the luteal and follicular phases of the menstrual cycle in woman that varied in amount of physical activity preformed during a typical week as gathered through self-report questionnaires. These authors noted that while in general there were no strong associations between physical activity and testosterone, progesterone, or follicular estradiol levels, there was an inverse association (p=0.05) between activity and luteal estrogen levels. Unfortunately, interpretation of any relationship between activity levels and estrogen levels in this study is difficult because body fat levels were lower in those women that were more active. Given that adipose tissue can increase estrogen levels (149), the lower body fat levels of the active women could have confounded the negative relationship between activity levels and estrogen levels in this study.

Do hormone replacement therapies or pharmacological interventions alter activity levels: Females— As noted earlier, there is extensive rodent literature suggesting that estrogen in particular, but also testosterone (81), increases activity levels via activation of the estrogen alpha-receptor pathway leading to downstream regulation of other physiological structures, potentially in the brain. While limited data exists to answer whether this is also true in humans, the studies that have been done have addressed the effect of hormone replacement therapy (HRT) on physical activity in women. Using a correlational design, Andersen et al. (3) observed that in the NHANES III data, women who had never used HRT reported higher levels of inactivity (40%) as compared to those women who had used HRT (28.5%) suggesting that women who had not used HRT (decreased estrogen) were more likely to be sedentary (decreased activity) when compared to those women that had used HRT. While supporting the hypothesis of sex steroid effects on activity levels, a major limitation of the NHANES III data was that it surveyed leisure time activity alone without regard for activities of daily living. Thus, any women that did not report any leisure time activity were classified as sedentary/inactive in spite of potential vigorous activity completed as part of their daily lives. Another research design issue with this data was the lack of control for the type of HRT or delivery method used (e.g. oral or transdermal; see below for further consideration).

Redberg et al. (102) stratified 248 post-menopausal women based on their HRT use and interviewed them regarding their physical activity, with physical activity levels estimated as Mets·h/wk (Figure 7A). The physical activity data were analyzed across four different groups (each subject was included in one or more of the experimental

groups): those women currently on HRT (n=108) versus those not currently on HRT (n=140) and those women who were currently or had been on HRT (n=158) versus those who had never used HRT (n=90). Ninety-four percent of the HRT subjects (n=101) used oral estrogen therapy while transdermal estrogen was used in six percent of the subjects (n=7). Of the group currently using HRT, 48% were on combined estrogen/progestin supplementation with the composition of the HRT for the remainder of the subjects not noted. Redberg and colleagues (102) reported large variability in physical activity in each group (Figure 7A) with no significant differences between any of the groups, thus concluding that activity was not affected by HRT status.

Anderson et al. (4) conducted a repeated measures trial on younger (45-55 yrs, n=18) post-menopausal and older (70-80 yrs, n=15) post-menopausal women investigating the effect of transdermally applied estrogen and estrogen plus vaginally-applied progesterone supplementation on a variety of energy balance measures with a two month clearance between treatments. This study was unique in that the estrogen and progesterone treatments were carefully titrated to result in near physiological circulating levels of both estrogen (76.89±4.43 - 80.50 ±5.43 $pg \cdot ml^{-1}$) and progesterone (7.09±0.91 $ng \cdot ml^{-1}$). Furthermore, the subjects were encouraged not to lose weight while in the experimental program. Figure 7B shows that Anderson and colleagues (4) observed no differences in physical activity level as measured by survey techniques across the treatments, thus supporting the lack of an effect of HRT on activity levels.

Kenny and colleagues (69) completed a three-year double blind, placebo-controlled trial investigating the effect of estrogen therapy on various muscle and physical functions in 167 older (average age at baseline ≈ 74 yrs) post-menopausal

women. The women were randomly assigned to either an estrogen treatment group (0.25 mg oral 17β-estradiol) or a placebo group (Figure 7C). Physical activity was estimated at the beginning of the study and then once per year for the three year trial using the Physical Activity Scale in the Elderly (PASE). Kenny et al. (69) reported that while the baseline activity scores were different between the groups, the rate of decrease in activity during the three years was the same in all of the groups.

Poehlman et al. (101) published a purported six-year controlled longitudinal study of HRT and energy balance that supposedly showed an increase in leisure time activity with HRT. Unfortunately, this article was subsequently retracted from the literature when the author admitted to fabricating the data in the study (100, 124, 125).

Males— In males, there are two possible avenues that could be used to artificially manipulate sex steroid levels in order to evaluate effects on activity. In one approach, the effects of antiandrogens (e.g. flutamide)—often prescribed for individuals at risk of or suffering from prostate cancer, as well as those suffering from a severe sexual disorder—on activity could be considered. However, there have been no published investigations on the effects of antiandrogens on physical activity in humans. A second avenue would be to consider the effects of supra-pharmacological doses of androgens on activity. While there are anecdotal statements of hyperactivity in individuals taking supra-pharmacological doses of androgens (115) there have been no studies that directly or indirectly measured physical activity levels in these subjects. There is a large body of literature concerning aggression with androgen supplementation in both men and women. Unfortunately, using the data on androgen supplementation and aggression to infer possible linkages between androgen supplementation and physical activity levels is

difficult due to the possible confounding effects of the various psychological states occurring with supra-pharmacological doses of androgens and the uncertainty regarding gonadal sufficiency of the subjects.

The human data available regarding the sex steroid effects on activity are sparse and the existing literature is limited to female subjects. While the female correlational data are split, with one study showing a positive relationship between lack of HRT and inactivity (3) and one showing no relationship (137), all of the available prospective studies (4, 69, 102) do not support the contention that estrogen administration alters physical activity in humans. These studies are strong from the standpoint that in each case, total daily physical activity levels were estimated as opposed to estimation of just leisure time activities as was the case with the NHANES III data (3). However, definitive conclusions are difficult to reach due to several possible confounding research design factors.

Are Research Design Issues the Reason for Species Difference in Sex Steroid Effects in Rodents and Humans?

Animals, especially rodents, have been used to model phenotypes or physiological phenomenon in humans with reasonable success. However, as has been discussed, the animal data show conclusively that sex steroids—specifically estrogen—exert powerful effects on daily activity, while the limited human data suggests that sex steroids play no role in the biological regulation of activity. Thus, the species difference in sex steroid effects on activity requires the consideration of several factors including 1) appropriateness of the animal model, 2) how dependency is assigned to variables, 3) whether rodent modes of activity directly translate to the measurement of activity in

humans, and 4) how the steroids are replaced or introduced to the organism. These factors help to outline the limitations of animal to human translation in physical activity level research and provide a foundation for future investigations in both humans and animal models.

Appropriateness of rodents as models of human physiology: Any consideration of species differences, especially differences between rodents and human models, must begin with questioning the appropriateness of using rodents to investigate human health questions. A variety of scientific organizations recognize the usefulness of rodents in modeling human physiology, especially with less than 1% of the mouse genome differing significantly from the human genome (146). Specifically, rodents are used frequently as models of human sex steroid physiology; in 2006 and 2007, the National Library of Medicine listed 881 articles that were published concerning mice and reproductive hormones. For example, rodent models have been used extensively to document the role of estrogen α and β-receptors (ERα and ERβ) in human cardiovascular physiology (5). Therefore, it is reasonable to suggest that data collected in rodents on the effect of sex steroids on physical activity can be applied to human physiology.

Independent versus dependent variables: Another factor that confounds the determination of whether sex steroids play a causative role in the regulation of daily activity in humans is experimental design issues, especially the assignment of independent/dependent variable relationships. While the animal literature has primarily studied the effect of sex steroids (i.e. independent variable) on physical activity (i.e. dependent variable), the majority of research in the human literature has approached physical activity as the independent variable, with sex steroid levels treated as dependent

variables. This approach is somewhat understandable given the belief that activity levels were controlled by voluntary directives and not the product of biological factors and thus, could not be affected by biological manipulation. With mounting evidence that 'voluntary' physical activity levels may actually be driven to a significant extent by biological/genetic factors (82, 83, 117, 130), it becomes important to understand the factors—such as sex steroids—that regulate daily activity. The understanding that there are discrete biological/genetic factors that drive 'voluntary' physical activity calls for further carefully controlled studies—in addition to the three studies currently available (4, 69, 102)—where activity level is the *dependent variable* with sex steroid level/supplementation as the *independent variable*. This approach would allow further understanding in the human model of the role of sex steroids—much as has been done in the animal literature.

Use of surveys to determine physical activity: The use of surveys or questionnaires to estimate physical activity levels in all of the current human studies limits conclusions. Numerous reviews (e.g. ref. 116) have observed that survey estimations of physical activity generally overestimate actual activity levels. This was reinforced recently by Troiano and colleagues (134) when they measured daily activity in a large population using accelerometers and found only a small percentage (3.5±0.3%) of adults completed moderate activity on a daily basis as compared to other survey estimates of adult activity levels (e.g. 45.4±0.2%, ref. 85). As noted in an extensive review of the reliability, validity, and sensitivity of physical activity questionnaires (116), correlation coefficients between measurement of physical activity (e.g. doubly-labeled water or accelerometers) and questionnaire results vary widely with few rising above 0.60 and

with the questionnaires generally overestimating activity levels. Specifically, two factors that often lead to the least valid estimates of activity are the length of time separating the recall from the activity and individuals whom have lower levels of activity (116). It is difficult to determine the possible effect of these two confounding factors on the conclusions of the currently available prospective HRT studies (4, 69) given that none listed the recall period that was used in their studies even though the instruments used by Kenny et al. (69) and Anderson et al. (4) were designed to estimate daily activity based on a seven day recall period. Therefore, to eliminate the possible confounds of using survey/questionnaire approaches which grossly overestimate daily activity, the use of direct measures of activity, such as was done in the NHANES 03-04 study (134) would considerably strengthen the investigation of sex steroid effects on daily activity levels.

Mode of hormone therapy used: The method of hormone delivery can significantly influence the circulating concentrations of sex steroid (135). For example, plasma concentrations of orally delivered steroids reach peak physiological levels approximately three hours after administration and then declines rapidly (135) exposing the individual to a non-physiological pattern of sex steroid concentration over the course of a 24 hour period. The use of transdermal administration provides a more stable plasma concentration of hormone, but to this point, only one of the prospective studies (4) used transdermal administration and verified constant, physiological concentrations of the sex steroids in their subjects.

Additionally, other than the Anderson et al. (4) study, no study has delineated the type of estrogen/progesterone used in prospectively assessing the effect of sex steroids on activity. Both the ERα and ERβ preferentially bind to certain estrogenic substrates. As

noted earlier, the estrogen-α receptor pathway has been shown to be the primary pathway involved in physical activity regulation. With different estrogens and progestins used in common HRT formulations (135) and the hypothesized activity inducing actions of the estrogen-α receptor pathway (81), it is possible that different HRT formulations produce different physical activity responses solely due to the lack of a compound with the necessary ability to bind to the estrogen-α receptor.

Thus, while the limited human data suggests that female activity patterns are not influenced by sex steroids, given the methodological limitations present, this conclusion is tentative at best and misleading at worst. Future studies controlling type and concentration of hormone replacement, the use of research designs that recognize that activity levels are influenced by biological factors, and the direct measurement of daily activity will provide further information to support or refute this hypothesis. However, while speculative, if we assume that the current human data are representative of the human activity response to sex steroids, the question of why there is a species difference in the activity patterns of female rodents versus humans remains enigmatic.

Summary and Conclusions

There is no doubt that increasing physical activity levels in adult populations, both nationally and internationally, should be a health-related priority and the fact that general levels of physical activity in both adults and children are less than optimal (e.g. refs. 95, 134) is troubling. Decades of activity promotion and research have not produced a noticeable reduction in the number of inactive individuals. Thus, these data present a clarion call for a greater understanding of the underlying mechanisms regulating voluntary daily activity and the roles that biological/genetic factors play in activity

regulation. The plethora of epidemiological evidence that suggests that human females, in most cases, are less active than males, along with increasing rates of cardiovascular disease in women (31), provides additional impetus for understanding all factors involved in activity regulation. In spite of the strong animal literature that suggests a probable physiological mechanism of activity regulation by sex steroids (81), human females are still generally less active than males. Thus, the question becomes and remains as to whether the environmental/cultural influences on physical activity override the increased physical activity drive inherent in women.

While there is some literature suggesting differential environmental, cultural, and psychological effects on activity levels between the sexes, there is no literature available investigating the interaction of these factors and biological effects on activity. Further study is warranted that specifically sets hormone concentration as an independent variable. Ideally, experimental modulation of hormone levels in a wider variety of individuals is necessary to completely understand the influence hormone levels have on activity. Such studies should capitalize on current therapeutic intervention used by medical professionals to manage endocrine related diseases and disorders including birth control administration, testosterone treatments, and hormone replacement therapy. The naturally occurring variation of hormone levels in both males and females throughout the life cycle, during distinct physiological conditions such as the luteal and follicular phases of the menstrual cycle, and between individuals may also add appropriate and measurable variability that allows independence to be assigned to hormone concentration. Additionally, the more reliable estimation of activity achieved through use of accelerometers and other quantifiable forms of locomotion measurement should be

utilized in place of traditional survey methods.

The clinical implications of expanding this research are potentially far reaching. Currently, a large body of literature exists regarding how extrinsic and environmental factors influence physical activity levels in humans; unfortunately, beneficial alterations to health indicators are minimal and physical inactivity and related conditions remain disproportionately high. Thus, physical activity management strategies and research agendas that focus solely on environmental or extrinsic factors are shortsighted. The overall goal of physical activity-sex steroid interaction research should be to further delineate biological pathways and identify enzymes/proteins/biomolecules involved in up-regulating activity levels in humans. In doing so, the medical community may in fact find ways in which to capitalize on these biomolecules and pathways leading to promotion of physical activity and increased beneficial health outcomes in the general population.

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Footnotes:

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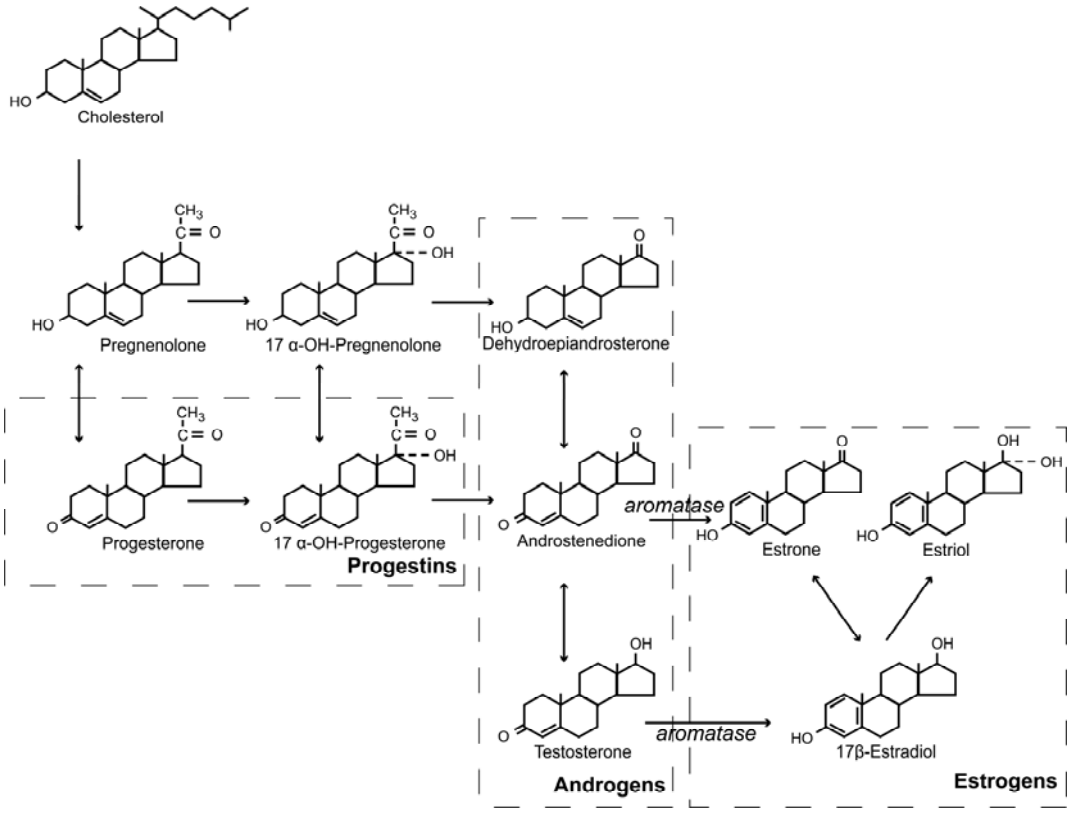

Figure 1: Basic pathways for sex steroid biochemistry in mammalian species. Double-headed arrows represent reversible reactions; single-headed arrows represent one-way reactions. The general sex steroid classes are grouped in dashed boxes.

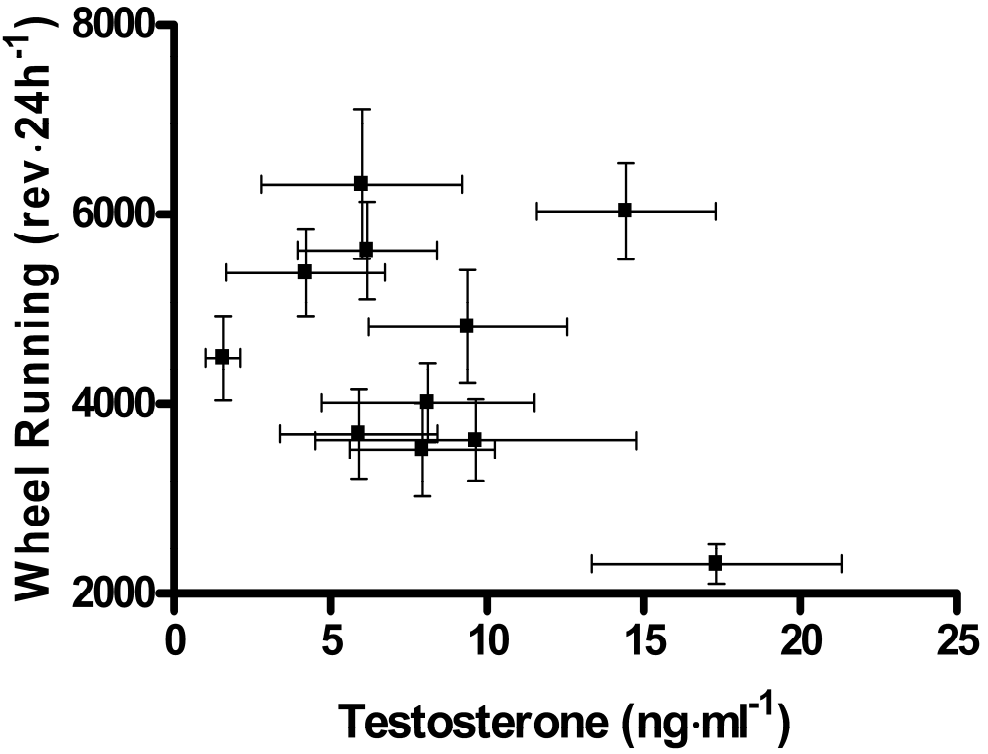

Figure 2: This figure shows the lack of a relationship between testosterone levels and wheel running activity in progenitor strains (BALB/cBy and C57BL/6By), F_1 hybrids ($B6CF_1$ and $CB6F_1$), and recombinant inbred strains (CXBD, CXBE, CXBG, CXBH, CXBI, CXBJ, CXBK). Figure redrawn from tabular data presented by Eleftheriou et al. (24).

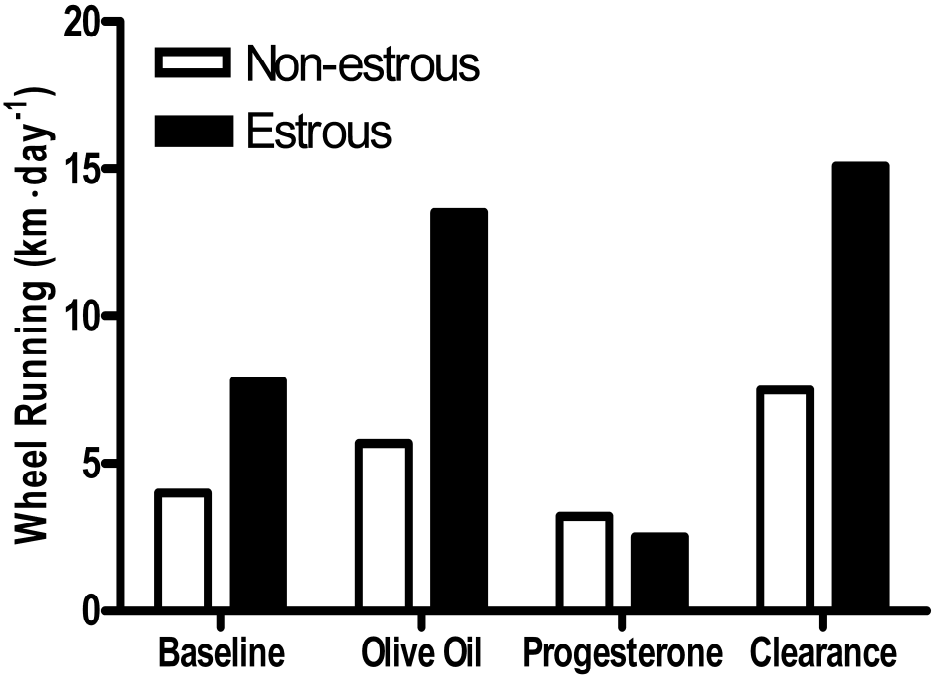

Figure 3: The effects of progesterone administration during estrous and non-estrous portions of the estrous cycle in female rats. Figure adopted from tabular data presented by Hervey (47). No statistical comparisons were made in the original dataset.

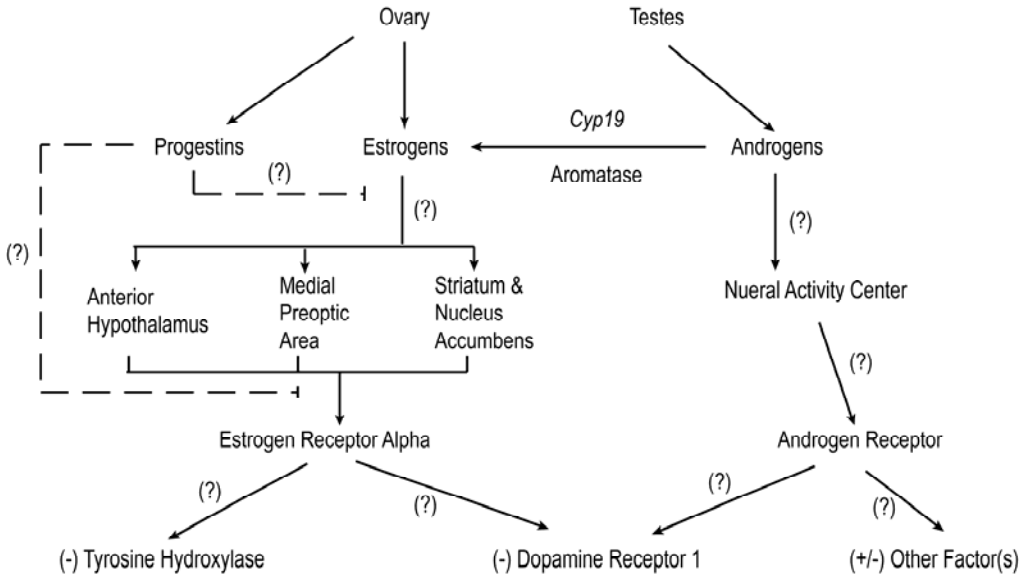

Figure 4: Schematic diagram of hypothesized estrogen, androgen, and progestin-derived pathways involved in the regulation of physical activity levels in male and female rodents.

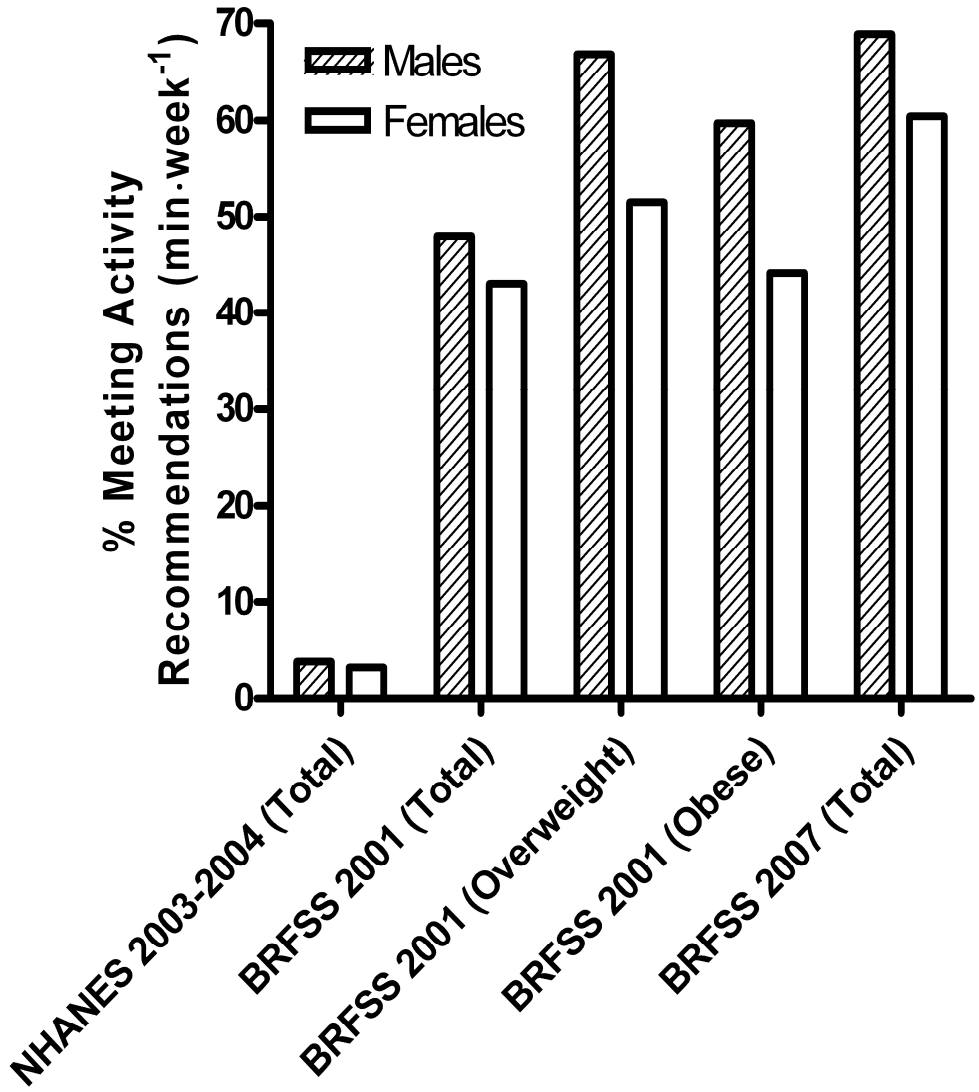

Figure 5: Percentage of U.S. adults meeting moderate/vigorous activity recommendations. Males are the hatched bars and females are the open bars. The total data included all subjects, overweight data included subjects with a BMI between 25.0 and 29.9 $kg \cdot m^{-2}$, and obese data included subjects with a BMI $\geq$ 30.0 $kg \cdot m^{-2}$. Data redrawn from United States 2001 (85, 86) and 2007 (1) Behavioral Risk Factor Surveillance Survey (BRFSS) and the 2003-2004 (134) National Health and Nutrition Examination Survey (NHANES). BRFSS data are presented as the whole dataset (1, 85) and subdivided by weight status (86).

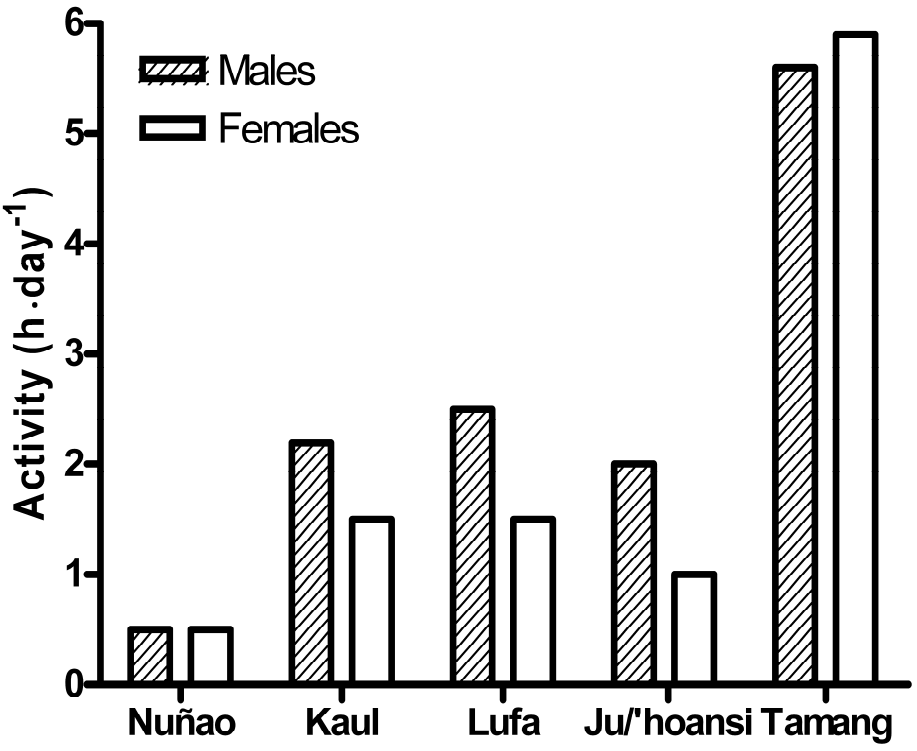

Figure 6: Hours per day of moderate-vigorous activity in Hunter/Gatherer cultures. Males are the hatched bars and females are the open bars. Data for the Nuñoa, Kaul, Lufa, and Ju/'hoansi populations are redrawn from Leslie et al. (80) and data for the Tamang population are redrawn from Panter-Brick (93). Note: The Ju/'hoansi people are referred to as !Kung in the manuscript (80) in which these data were taken. This terminology refers to the language spoken by the Ju/'hoansi peoples and is now considered offensive and derogatory (78).

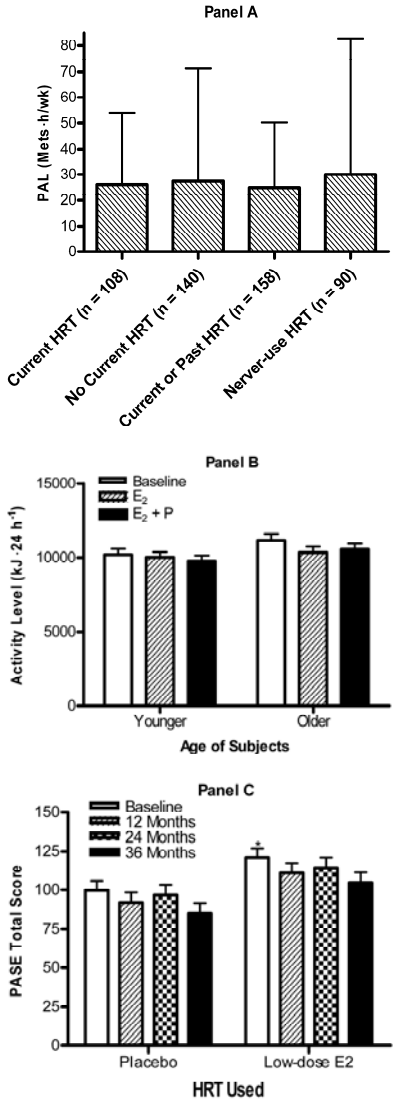

Figure 7: The effect of hormone replacement therapy (HRT) on physical activity. Panel A represents the lack of difference in physical activity estimates amongst any of the HRT and non-HRT groups and represents tabular data from Redberg et al. (102). Panel B shows the lack of an effect of HRT, both estrogen (E_2) and estrogen plus progesterone (E_2 + P) treatments, on estimated activity levels in younger menopausal and older menopausal subjects. Data taken from Anderson et al. (4). Panel C represents the lack of a difference in estimated physical activity between low-dose estrogen and placebo over a 36-month period. *Indicates a significant difference from placebo treatment at same time point. Data taken from Kenny et al. (69).

Table 1. The effects of ovariectomy, ovarian traumatization, and chemical gonadectomy on physical activity patterns in rodents

Study	*Species--Strain*	*Procedure*	*% difference from baseline or control animals*
Wang (141)	Rat	Ovariectomy	18.8
Durrant (22)	Rat--White	Ovariectomy	34.0
Bugbee & Simon (15)	Rat	Ovariectomy	69.5
Wang & Guttmacher (143)	Rat	Traumatization	9.1
Richter & Hartman (108)	Rat	Ovariectomy	16.7
Young & Fish (151)	Rat	Ovariectomy	15.0
Richter & Uhlenhuth (109)	Rat	Ovariectomy	5.4
Asdell et al. (6)*	Rat	Ovariectomy	200.0
Gorzek et al. (43)	Mouse--C57BL/6J	Ovariectomy	13.0
Hydock et al. (57)	Rat--Sprague Dawley	Chemical	42.9

*Asdell at al. (7) provided a correction to their 1962 paper, citing an inability to repeat all aspects of the previously published paper

Table 2. The effects of orchidectomy and castration on physical activity patterns in rodents

Study	*Species--Strain*	*Procedure*	*% difference from baseline or control animals*
Gans (34)	Rat	Fractional Castration	22.2
Gans (35)	Rat	Early Castration	110.0
Hoskins (54)	Rat	Castration	25.3
Hydock et al. (57)	Rat--Sprague Dawley	Chemical	47.1

CHAPTER 2:
EFFECTS OF SUPRA-PHYSIOLOGICAL DOSES OF SEX STEROIDS ON WHEEL RUNNING ACTIVITY IN MICE

Abstract

The regulatory mechanisms of physical activity are postulated to include environmental and biological/genetic factors. In particular, the sex steroids appear to have profound effects on wheel running in rodents. The purpose of this project was to investigate the effects of 17β-estradiol and testosterone on wheel running distance, duration, and speed in male and female C57BL/6J mice. The mice (N=46) were provided free access to running wheels interfaced with computers to track daily running distance, duration, and speed. Activity was assessed at baseline in intact mice, after surgical gonadectomy, and after replacement with either 17β-estradiol or testosterone. Upon removal of the gonads, physical activity levels were significantly reduced in both males and females. Distance (10-30% of baseline) and duration (20-47% of baseline) measures were most affected by the loss of endogenous steroids, while running speed (60-77% of baseline)—though significantly reduced—decreased by a much lower magnitude. Testosterone replacement fully recovered running distance, duration, and speed to pre-surgical levels in both sexes (100% of baseline). Distance (30-42% of baseline) and duration (43-47% of baseline) were partially recovered by 17β-estradiol, but not to baseline levels. Speed (100% of baseline) was fully recovered by 17β-estradiol replacement in males and females. This study suggests that physical activity in mice is

affected by endogenous steroids and can be altered by exogenous steroid replacement. The differences in the recovery abilities of 17β-estradiol and testosterone suggest that both estrogenic and androgenic pathways may be involved to variable degrees in activity regulation.

Introduction

Physical inactivity enhances the risk of many diseases including obesity, diabetes, many types of cancer, and heart disease (87). The US health care system is excessively burdened by hypokinetic related diseases, resulting in reduction in service and care. Furthermore, quality of life parameters are significantly degraded as inactivity-induced diseases progress. To understand the mechanisms inducing physical inactivity within the human population, efforts to elucidate the biological and genetic factors that alter either the motivation or ability to partake in increased activity are necessary.

The sex steroids have previously been shown to influence physical activity in rodents and may be important biological factors regulating activity levels. Gorzek et al. (43) altered 17β-estradiol levels in young female mice by surgically removing their ovaries followed by replacement in a capsulated form. The study showed a significant decrease in voluntary wheel running distance following ovariectomy and a recovery back to pre-surgery levels when capsulated 17β-estradiol was administered. A similar response has been shown in male rats after castration (144). Several other studies report comparable effects (15, 54, 55, 141, 151); however, with the exception of the study by Gorzek et al. (43), these studies were conducted in the 1920s prior to the discovery and purification of many of the chemicals involved in activity regulation. The results from these studies, though unique and novel for the early parts of the 20th century, are

outdated and require revision using newly available delivery techniques, measurement apparatuses, and purified steroid samples.

In a seminal study, Roy and Wade (114) administered dihydrotestosterone propionate, a non-aromatizable form of testosterone, to castrated male rats and found no significant change in activity levels suggesting that an estrogen-aromatase dependent mechanism was responsible for activity regulation by the sex steroids. Additionally, using estrogen receptor α (ERα) and β (ERβ) knock-out mice, Ogawa et al. (92) demonstrated alterations in wheel running via ERα, but not ERβ pathways. Finally, it has been hypothesized that changes to activity in murine systems due to changes in sex steroid levels may be due in part to undiscovered non-genomic effects and/or intricate interactions between estrogens, ERα, and dopaminergic neurons (81).

The purpose of this study was to systematically remove and replace both 17β-estradiol and testosterone in male and female mice allowing comparisons to be made between the steroids and sexes in the regulation of running wheel activity. Alteration in running distance or number of wheel revolutions has been the flagship measure defining physical activity levels in mouse models (15, 22, 43, 54, 55, 120, 142, 144, 151); newer techniques now allow running duration and speed to be quantified as well. Thus, a secondary purpose of this study was to evaluate the changes in running distance, duration, and speed in a murine model of physical activity under minimal and supra-physiological levels of circulating sex steroids.

Methods

Animals: This project conformed to standards of humane animal care and received approval from the UNC Charlotte Institutional Animal Care and Use Committee

prior to initiation. C57BL/6J inbred mice (Jackson Laboratory, Bar Harbor, ME) were used in this study due to their prevalent use in the scientific literature and because of their genetic homogeneity. Twenty-three male and 23 female mice were initially used in this study; however, five mice (male=1; female=4) showed signs of distress following the surgical gonadectomy procedures and were euthanized for humane purposes resulting in a total cohort of 41 animals for the remainder of the study. Prior to the start of this project, mice were group housed three to four per cage until they reached approximately 9 weeks of age. The mice were then individually housed and provided unlimited access to running wheels for the duration of the study. Whereas mice reach their activity zenith between 9 and 12 weeks of age (133), this study encompassed the most active parts of the lifespan. Through the entirety of the study, the mice were housed under a 12:12hr light:dark cycle initiating daily at 6am. Free access to water and standard mouse chow (Harlan Teklad, Madison, WI) was provided throughout the study. The chow provided to the mice during this study was not phytoestrogen-free. Several authors (36, 45, 46, 73, 74) have shown that phytoestrogens do not increase activity in gonadectomized mice.

Experimental Procedure Overview: The timeline for this project is displayed in Figure 8. Each mouse was randomly assigned to either an experimental group or a control group. This random assignment was stratified by sex and by the initial housing scheme in order to ensure that previous group housing effects would be minimized. After separation into individual cages, each mouse was supplied a running wheel. Wheel running distance, duration, and speed represented physical activity levels and were monitored under three experimental treatments including at baseline, after gonadectomy, and with supra-physiological steroid replacement (detailed below).

After an initial seven-day period to assess baseline wheel running, gonadectomy surgeries (detailed below) were performed to reduce circulating steroid levels. The control groups received sham surgeries. A 10-day recovery period, without wheels, allowed the surgical wounds to heal and remaining circulating steroids to clear from the system. After this recovery period, the wheels were replaced in the cages. Wheel running activity was then tracked for an additional seven days.

Next, the implant surgeries were completed and followed by two days for recovery and seven days to record activity. Silastic capsules (detailed below) containing 17β-estradiol were implanted in eight females and nine males. Silastic capsules containing testosterone were implanted in eight females and ten males. The animals in the control groups were given empty implants. The two-day recovery period allowed the animals to recover from surgical wounds and the silastic implants to deliver steroid into the bloodstream (17). After recovery, the mice were re-exposed to running wheels and their physical activity levels were monitored for seven days. At the end of each seven-day data collection period, body masses and percentage body fat measures were completed. A PIXImus 2.10 (Lunar, Madison, WI) was used to collect the percentage body fat measurements via dual energy x-ray absorptiometry.

Measurement of Wheel-Running Activity: Physical activity was measured by determining daily distance, duration, and speed of wheel running using standard protocols (82, 136). In brief, running wheels were mounted to the cage tops of standard rat cages and were equipped with a cycling computer (BC500, Sigma Sport, Batavia, IL) to record running distance and duration. Running wheels had a 450mm circumference and a 40mm wide, solid running surface. Running distance and duration data were

collected on a daily basis in the morning and average daily running speed was calculated from the corresponding distance and duration measures. The sensor and magnet alignment and freeness of the wheel were checked daily and adjusted as needed.

Surgical Procedures: Twenty males and twenty females received orchidectomies or ovariectomies. The remaining six mice (3 males; 3 females) acted as control animals and underwent sham surgical procedures. A preemptive dose (0.05 $mg \cdot kg^{-1}$) of buprenorphine was administered via intraperitoneal injection approximately 30 minutes prior to the gonadectomy procedures. All procedures were performed under light isoflurane anesthesia with a 300 $ml \cdot min^{-1}$ oxygen flow rate. Incisions were made under sterile conditions (10% betadine followed by 70% alcohol) with sterile surgical tools.

The gonadectomy surgery performed depended upon the sex of the mouse. Orchidectomy surgeries were preformed on the male mice and were initiated by making a small access incision in the skin directly proximal to the scrotal sac. Additional incisions were made in the fascia on either side of the scrotal sac. Slight pressure was applied just above the incision sites to expose the testes. Both the testes and epididymis were excised and discarded. The incision wound in the skin was closed with a surgical staple. Bilateral ovariectomies were preformed on the female mice. A small incision was made in the skin directly above the lumbar region. A small pocket was developed between the skin and muscle to allow unrestricted access to the animal's dorsolateral region. Small incisions were made in the fascia approximately 5 mm on either side of the spine just proximal to visible white fatty tissue. Each ovary was exposed and excised. The skin wound was closed with a surgical staple. The sham procedures preformed on the control animals were identical to the procedures described above minus the excision of the sex

organs.

Replacement Procedures: Two sets of implants were developed to release sex steroids into the bloodstream of the mice based on diffusion. The silastic implants were produced similar to the technique of Cohen and Milligan (17), except that dry powder without arachis oil was packed into silastic tubing. A 10 mm section of the silastic tubing (Dow Corning, Midland, MI) with an outer diameter of 2.16 mm and inner diameter of 1.02 mm was packed with either powder testosterone or powder 17β-estradiol (Sigma-Aldrich, St. Louis, MO). The ends of the tubing were covered with a small bead of weatherproof silicone glue. Each implant was washed in 70% alcohol for one minute and rinsed in deionized water. After washing, the implant was patted dry and stored in Eppendorf tubes at room temperature under dark, dry conditions. The implants for the control animals were prepared in the same way, but were left empty. Surgeries to implant silastic capsules to replace the sex steroids were preformed during the later stages of this project. A small incision was made on the lateral aspect of the neck. A cavity about 15 mm in depth and width was developed between the skin and muscle tissue. Forceps were used to insert a 10 mm long silastic implant into the cavity. The incision wound was closed with a surgical staple.

Sex Steroid Assays: The current project was performed in conjunction with two other related projects. Blood samples were taken on regular two week intervals during the studies and at the end of each project on live animals. The mice were immobilized in a decapicone bag with slots for the hind limbs. The medial aspects of the hind limbs were cleaned and blood was sampled from the saphenous vein via venipuncture. Plasma was retrieved after cold centrifugation and individual samples (n=3) from each

experimental condition were pooled. The blood sampled at the end of experiment was taken directly from the inferior vena cava. These samples were allowed to clot at room temperature and were then centrifuged. The serum was also pooled (n=3) based on common inclusion in a given experimental condition. The pooled samples were extracted in ethyl acetate (Sigma Aldrich, St. Louis, MO) that was then evaporated. The residue was re-suspended in steroid free serum (IBL America, Minneapolis, MN).

Testosterone ($ng \cdot ml^{-1}$) and 17β-estradiol ($pg \cdot ml^{-1}$) was measured via ELISA (IBL America, Minneapolis, MN) per the manufacture's instructions. The data were assessed for error and outliers and adjusted accordingly. Data points with high variation between duplicate measures or considerable (one data point exceeded the other data points in the group by greater than 500%) deviation from the condition mean were eliminated from analysis. Unfortunately, viable blood samples were not obtained from the female cohort, but vaginal smears and inspection of the uterine horns were completed to evaluate the effectiveness of each experimental intervention.

Statistical Analysis: Distance, duration, and speed were averaged for seven days per experimental treatment (i.e. baseline, gonadectomized, and replaced). Unpaired-sample t-tests were utilized to evaluate the overall mean differences attributable to sex. Separate two-way (group by treatment) analysis of variance (ANOVA) calculations were used to assess differences between the treatment levels for each physical activity variable. A three-way ANOVA was used to compare the body composition measures between groups, sexes, and experimental periods. Tukey's HSD *post-hoc* tests were used if the main effects or interactions from the initial ANOVA reached significance. The alpha value was set *a priori* to 0.05.

Results

Male Mice: While no difference was noted between the treatment groups during the baseline period (i.e. before gonadectomy), physical activity patterns of the male mice were markedly altered by removal and replacement of the sex steroids (Figure 9). After orchidectomy, both the testosterone and 17β-estradiol groups ran significantly less (10% of baseline, $p<0.05$) than the sham group and compared to baseline measurements. Replacement of testosterone recovered the activity pattern back to baseline and sham levels (90% of baseline). Replacement of 17β-estradiol partially recovered running distance (31% of baseline), but the distance remained significantly less ($p<0.05$) than baseline and sham values. Wheel running duration mirrored the distance results. At baseline, running durations were similar among the different experimental groups. Orchidectomy significantly reduced (19% of baseline) daily running duration compared to the sham group and baseline values ($p<0.05$). Testosterone replacement recovered duration to baseline levels (97% of baseline) while 17β-estradiol replacement failed to recover running duration to baseline levels (44% of baseline). Average wheel running speed was significantly influenced (59% of baseline) by sex gland removal ($p<0.001$) and recovered to near baseline after replacement. Running speed was significantly recovered during testosterone replacement (93% of baseline). 17β-estradiol replacement increased running speed to near baseline levels (74% of baseline), but did not recover running speed by the same magnitude as testosterone replacement.

Female Mice: Without regard to the steroidal or surgical conditions of the mice (all female data points compared to all male data points), the females ran farther ($t=3.87$; $p<0.001$), longer ($t=4.06$; $p<0.001$), and faster ($t=2.24$; $p<0.05$) than their male

counterparts. However, similar to the males, removal and replacement of the sex steroids altered the physical activity patterns of the female mice (Figure 10). The female mice did not show differences in the running pattern among the experimental groups at baseline. As in the male mice, both the surgical and replacement interventions influenced running distances. After ovariectomy, wheel running distance was partially reduced (31% of baseline, $p<0.05$); running activity was reduced less in females than males after surgery. Administration of testosterone fully recovered running distance (114% of baseline), while replacement of 17β-estradiol only slightly recovered running distance (43% of baseline). After removal of the ovaries, running duration was significantly reduced (37% of baseline, $p<0.05$). Testosterone increased running time to the highest levels recorded during the experiment (103% of baseline). After replacement of 17β-estradiol, wheel running duration remained significantly different compared to baseline values (46% of baseline, $p<0.05$). Running speed was significantly reduced after removing the ovaries (79% of baseline, $p<0.05$) in the 17β-estradiol treatment group, but not in the testosterone group (85% of baseline). Testosterone administration increased (107% of baseline) running speed slightly above the baseline and sham values. Replacement with 17β-estradiol slightly (86% of baseline) increased running speed.

Body Composition: The body mass (g) and body fat (%) measures are summarized in Table 3. The body masses varied between sexes and across the experimental periods. Males (25.1±1.9) weighed significantly more then females (20.9±2.0) throughout the study. The body mass increased significantly across all three periods (baseline: 21.8±2.9; after gonadectomy: 22.5±2.3; with replacement: 25.0±2.6). The percentage of body fat was also altered across experimental periods; percentage body

fat was significantly lower during replacement (11.14±0.90) compared to baseline (14.13±2.04) and gonadectomy (13.11±1.20).

Implant Efficacy: The functionality of silastic implants to deliver sex steroids in rodents has been previously shown and is a common technique in the endocrinology literature (11, 17, 20, 25, 36, 91, 99). Direct measurement of sex steroid blood plasma levels in the mice observed during study is difficult due to the necessary volume restrictions placed on survival blood draw techniques. Viable blood samples were obtained from male mice and ELISA data were summarized in Table 4. Plasma testosterone decreased after gonadectomy and 17β-estradiol remained about the same. With testosterone administration, both testosterone and 17β-estradiol levels were elevated to levels above the levels observed at baseline. Administration of 17β-estradiol did not affect testosterone levels, but dramatically increased plasma 17β-estradiol levels. The function of the implants and surgical success were confirmed via several direct observations in the female mice. First, the female mice receiving implants presented with visibly larger uterine horns when compared to control animals, suggesting circulating steroids were present in these mice (42). Secondly, the content of vaginal smears taken from individuals with steroid implants, were dominated by the presence of cornified epithelial cells, which also are indicative of estrogen replacement (42). Thirdly, the vaginal content of the mice after gonadectomy, but prior to implantation of silastic capsules was void of cellular debris. Thus, the successful use of silastic implants by past researchers to deliver steroid compounds to rodents (17) and the direct observations made during this study suggest effective delivery of 17β-estradiol and testosterone to the present cohort of mice.

In addition to the aforementioned observations, semi-quantitative measures of steroid release were made. Each implant contained approximately 3600 μg of powder steroid prior to placement in a mouse. Accurate post usage measurement of steroid containing devices is difficult due to the absorption of extraneous bodily fluids as the steroid moves into circulation. With the use of the current method of steroid delivery it was evident through direct observation that during the seven day period approximately half (1800 μg) of the powder had exited the implant upon retrieval of the capsule from the mice. Based on these observations it was estimated that between 200 and 300 μg was released from the capsules per day over the seven-day period. Comparing these data to the data of Cohen and Milligan (17), the capsules used in this study induced supra-physiological levels of the steroid in circulation as these authors demonstrated a five-fold increase in vaginal smear response and a nine-fold increase in uterine weight after an eight day exposure to silastic capsules containing 17β-estradiol (100 $\mu g \cdot ml^{-1}$). This method of steroid delivery was chosen to ensure adequate delivery of the steroids into the blood and the tissues involved in activity regulation. It has previously been shown that the blood plasma levels of the sex steroids are not equal to the levels of the steroids found in other tissue areas including the brain (37).

Discussion

In this study, running distance, duration, and speed were significantly reduced following surgical removal of the gonads in male and female C57BL/6J inbred mice. After the diminution of wheel running activity, attempts to recover activity levels via exogenous testosterone and 17β-estradiol produced variable magnitudes of recovery. Testosterone's propensity to boost the activity pattern in mice to normal levels after

gonadectomy (101% of baseline; average across all wheel running indices) was evident in both males and females and surprising given the limited and contradictory (114) literature available regarding testosterone replacement. As surprising, were the somewhat limited effects of administered 17β-estradiol which resulted in only partial recovery of the activity patterns of both sexes (54% of baseline; average across all wheel running indices). Interestingly, the present study indicates that both testosterone and 17β-estradiol influenced activity primarily by modulating running distance and duration as opposed to speed. Thus, while administration of estrogens has been shown previously to recover activity levels to some extent (43), these are the first data to suggest an equal or higher activity recovery level with testosterone administration.

Running Wheel Activity: This study evaluated physical activity patterns as a multifaceted character because running duration and speed have not been investigated in the previous sex steroid related literature. These physical activity indices have been suggested to contain a significant genetic component (77, 83) but the mechanisms are yet to be fully delineated. Given that the treatment we used was replacement of the sex steroids, the design of the present study allowed for further understanding of the interactions of the sex steroids and these physical activity indices. The novel aspect of the current study is that it examined both sexes under individual influences of both an estrogenic and androgenic compound via gonadectomy and replacement procedures.

The majority of activity studies present in the literature have used number of wheel revolutions or running distance (revolutions multiplied by wheel circumference) to evaluate changes to physical activity patterns and have mostly reintroduced estrogen analogs after sex gland removal. Gorzek et al. (43), who observed a decrease from

roughly 9.0 km·day^{-1} to less than 1.0 km·day^{-1} in a group of female C57BL/6J mice after ovariectomy and Hoskins, in 1925 (54) who observed a decrease from 15,142 rev·day^{-1} (≈ 14.6 km·day^{-1}) in normal male rats to 3,283 rev·day^{-1} (≈ 3.2 km·day^{-1}) in castrated male rats are just two examples of studies that have observed decreases in activity with removal of the sex steroids.

The preponderance of literature in this area has investigated the effect of estrogenic replacement on wheel running with some variability in post-surgical recovery of activity. Most recently, Gorzek et al. (43) observed recovery of wheel running activity to levels observed in control mice (≈ 85% compared to shams) of running distance in female mice with administered 17β-estradiol. Durrant (22) fed ovariectomized white rats a diet of glycerine prepared ovarian extracts and reported no effects on wheel revolution number suggesting limits to activity recovery with oral administration of steroids. Wang, Richter, and Guttmacher (144) demonstrated a robust increase from less then 1,000 rev·day^{-1} to between 6,000 and 8,000 rev·day^{-1} in castrated male rats treated with ovarian tissue grafts from female littermates. This response equated to a 50-100% recovery compared to intact control animals. Bugbee and Simon (15) found 100% of wheel revolutions were recoverable with repeated injections of ovarian follicular fluid, but noted that when the dosage was tripled, additional improvements in activity were not observed. The designs and age of past experiments have made it difficult to determine dose responses and thus, it is unclear how the estrogen replacement dosages used in previous literature compare to that used in the current study. However, the various estrogen replacement protocols in the literature have resulted in a 50-100% recovery rate for activity with the lower recovery rates reported by these studies (15, 22, 43, 144)

similar to those observed in the present study.

Potential Androgenic Effects: There are few studies regarding the androgenic influences on activity patterns in rodents available for comparison with the results of the current study. Before the discovery of testosterone, Hoskins (55) grafted testicular tissue into castrated rats, but did not observe changes in their activity patterns. In a similar study, Richter and Wislocki (110) used a more elaborate technique to introduce testicular grafts into male and female rats. The authors found a greater number of wheel revolutions in several of the animals and upon further histological investigations suggested that successful transplantation of the grafted tissue in most animals resulted in running at higher levels. Much later, Roy and Wade (114) investigated the effects of aromatizable testosterone propionate and found increased activity (from 2 $km \cdot day^{-1}$ to 4 $km \cdot day^{-1}$) in castrated male rats. To our knowledge, the data in the current study represent the first data available regarding the effect of testosterone replacement on activity patterns in both male and female mice and suggests that testosterone may play a larger role in regulating daily activity than previously suggested. Thus, our results suggest a broader picture of physical activity regulation by the sex steroids that also includes a yet to be outlined androgenic effect.

There are three lines of evidence commonly reported to support involvement of estrogen rather than testosterone compounds as the primary activity regulator in rodents. First, several experiments have shown a variable increase in activity in both male and female rodents when estrogenic compounds are delivered through a variety of administrative techniques (6, 15, 43, 107, 108, 120, 121, 141-144). Second, in studies of knockout mice of both sexes, Ogawa et al. (92) observed that activity levels were

dependent upon an interaction between an estrogenic compound and estrogen receptor α. In their study, no differences in activity were seen after implantation of β-estradiol 3-benzoate in animals lacking the α isoform of the estrogen receptor. Third and most notable, Roy and Wade (114) observed increased activity patterns only with the administration of testosterone propionate, an aromatizable androgen and not with administration of dihydrotestosterone propionate, a non-aromatizable androgen in castrated rats (114).

The mice in the present study that received 17β-estradiol capsules after gonadectomy would have elevated levels of the estrogens alone (Figure 11). Since the estrogens are not converted to testosterone, very little testosterone would be present in these mice. The mice receiving testosterone implants would fall into one of three categories regarding steroid levels in general circulation; 1) only estrogens are present (complete conversion of testosterone to estrogens via aromatization; Figure 11, panel a), 2) some of each steroid is present (some testosterone is aromatized to estrogens; Figure 11, panel b), and 3) only testosterone is present (no conversion to estrogens via aromatization; Figure 11, panel c).

Comparisons between the estrogen replacement group and the testosterone replacement groups mentioned above (no estrogen, some estrogen, all estrogen) suggest the presence of an androgenic regulatory effect on physical activity (Figure 11). The precise dimensions of a silastic implant allowed a constant volume (approximately 200 to 300 $\mu g \cdot day^{-1}$ as suggested earlier) of steroid to be present in each capsule, which led to the release of similar amounts of steroids to all mice. Therefore, the circulating levels of estrogens in mice with 17β-estradiol capsules would be equal only to the levels of mice

with testosterone capsules if the testosterone were converted completely to 17β-estradiol leaving no circulating testosterone (Figure 11, panels a, d, g). If the testosterone were completely converted to 17β-estradiol, it should be expected that the mice, regardless of steroid replaced, would not differ in activity performed. This was not the case in this study, as the mice receiving the testosterone capsules out performed the mice receiving the 17β-estradiol capsules. In reality, replaced testosterone is probably not entirely converted to 17β-estradiol (Figure 11, panel b). The residual levels of testosterone remain available to interact in an androgenic manner that can affect activity levels. These observations conflict with the results of Roy and Wade (114) who suggested that the androgens needed to first be converted to estrogens prior to influencing wheel running; we observed that testosterone's regulatory effects were not dependent upon estrogen.

Other Potential Effect Factors: The removal of the gonads, especially in female mice, not only removes the primary estrogen sources in these animals, but also a substantial progesterone source. While the influence of progesterone on wheel running activity was not the focus of this paper, past research has indicated a minimal effect of this steroid on activity regulation. Rodier (111) injected ovariectomized albino rats with 40 $mg \cdot kg^{-1} \cdot day^{-1}$ progesterone and found no changes to the activity pattern. In the same study, 8 $mg \cdot kg^{-1} \cdot day^{-1}$ of progesterone was given to intact female rats and was found to have a slight inhibitory effect (111). Based on these lines of evidence, the effects of progesterone loss due to ovariectomy in the present study was likely minimal.

The changes observed in the body composition variables appear to relate to natural differences between the sexes and appeared to follow temporal patterns rather than patterns attributable to changes in steroid status. Past research (45) has shown

changes in body composition characteristics with alterations in steroid levels; however, the brevity of the experimental periods in this study may have hindered full development of this effect. Furthermore, the effects of body mass on activity have been shown to be limited in non-obese rodents (32, 82, 136).

In conclusion, the current study investigated the influence of both testosterone and 17β-estradiol on physical activity patterns in male and female C57BL/6J mice. All three indices of activity were significantly decreased with removal of the sex steroids; running distance and duration were most responsive to alterations in circulating steroid levels. The differences in recovery of physical activity observed in mice implanted with 17β-estradiol (lower recovery) and testosterone (higher recovery) provided evidence that in mice, both steroids variably alter activity patterns. Based on the significantly larger magnitude of recovery with testosterone replacement, it is suggested that testosterone—believed from limited past studies to only regulate activity via an estrogen dependent mechanism—may also influence activity through a direct, androgenic mechanism.

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Footnotes:

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Journal Submission: At the time of publication of this dissertation, the following chapter was in review for publication in the journal: *Physiology & Behavior*

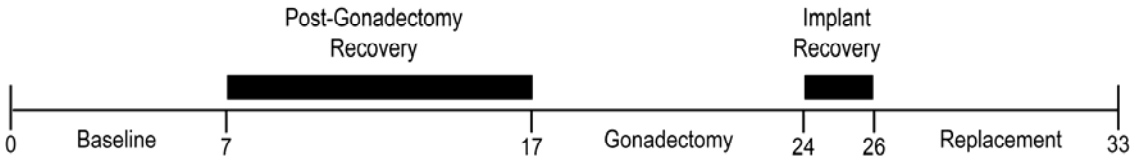

Figure 8. Experimental timeline (in days) for study assessing physical activity differences in C57BL/6J mice under various circulating sex steroid levels including normal physiological levels at baseline, low levels during gonadectomy phase, and elevated levels during replacement phase. Baseline, gonadectomy, and replacement were the experimental periods for this project.

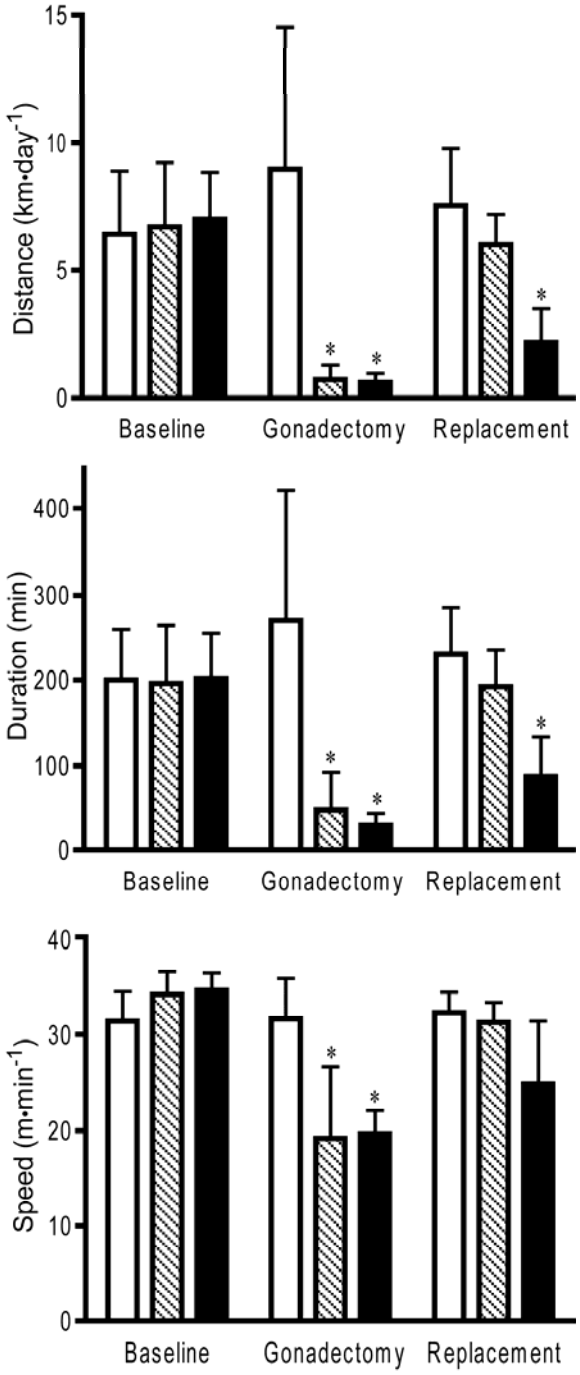

Figure 9. Male C57BL/6J mice wheel running distance, duration, and speed under normal physiological condition (Baseline), with low circulating steroid levels (Gonadectomy), and with artificially elevated steroid levels (Replacement). White bars=sham controls; Hatched bars=testosterone during replacement period; Black bars=17β-estradiol during replacement period; *=significantly different from sham controls and baseline treatments.

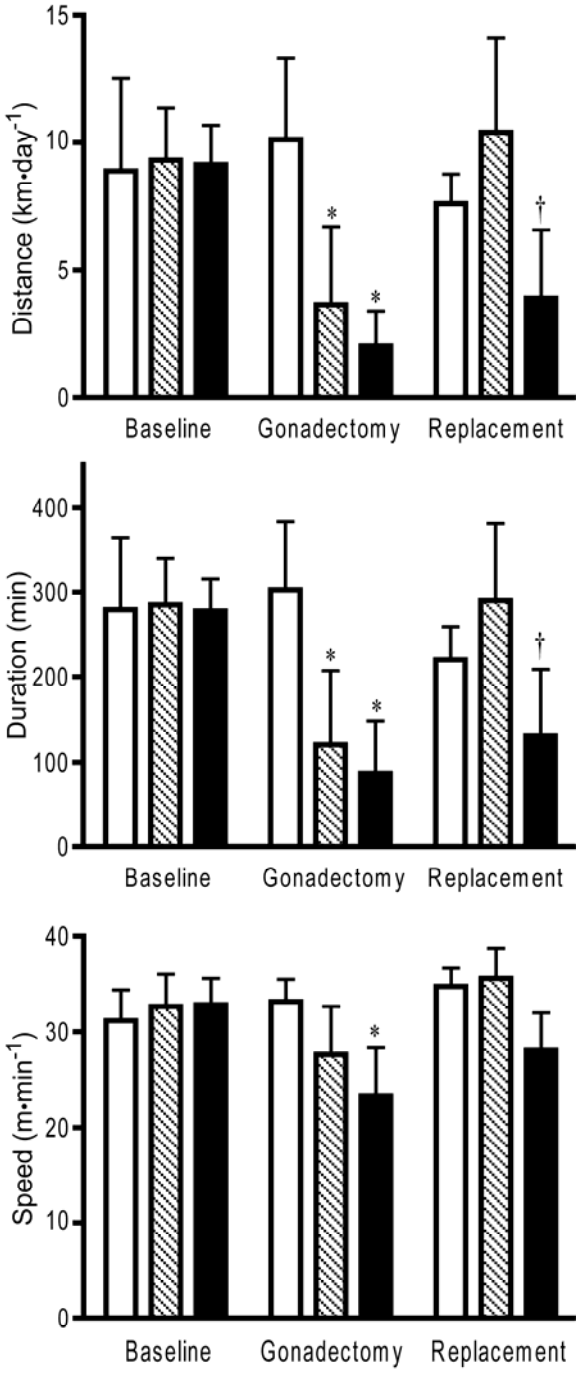

Figure 10. Female C57BL/6J mice wheel running distance, duration, and speed under normal physiological condition (Baseline), with low circulating steroid levels (Gonadectomy), and with artificially elevated steroid levels (Replacement). White bars=sham controls; Hatched bars=testosterone during replacement period; Black bars=17β-estradiol during replacement period; *=significantly different from sham controls and baseline treatments; †=significantly different from baseline.

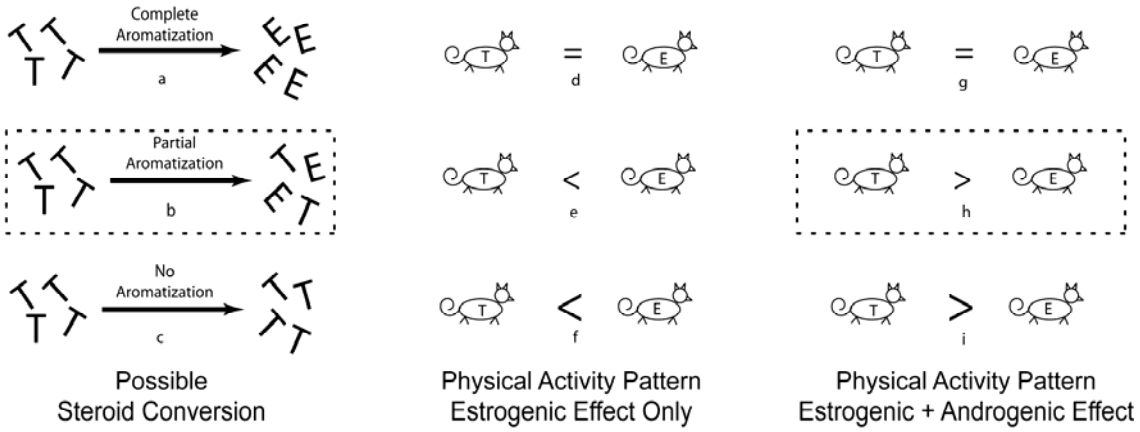

Figure 11. Schematic representation comparing mice receiving only 17β-estradiol (represented by mouse with 'E' on abdomen) to those receiving testosterone (represented by mouse with 'T' on abdomen) after surgical gonadectomy. Three scenarios (panels a-c) exist regarding testosterone's conversion and lead to three possible wheel running responses if the estrogens are the primary effecters of activity (panels d-f) and three possible responses if an androgenic effect is also present (panels g-i). T=Testosterone, E=17β-estradiol, dotted outline=observed outcome from present dataset.

Table 3. Body mass and % body fat in male and female C57BL/6J mice with altered sex steroid concentrations during wheel running

	Body Mass (g)			Body Fat (%)		
	Baseline	Gonadectomy	Replacement	Baseline	Gonadectomy	Replacement
			Males			
Sham (n=3)	24.0 ± 2.7	23.8 ± 2.9	26.7 ± 4.5	11.65 ± 1.05	12.19 ± 0.53	11.98 ± 2.48
Testosterone (n=10)	24.1 ± 1.3	24.0 ± 0.7	25.6 ± 1.3	12.63 ± 1.11	12.53 ± 1.56	12.33 ± 0.78
17β-Estradiol (n=9)	24.9 ± 1.1	24.7 ± 1.1	28.2 ± 1.1	13.79 ± 3.47	12.92 ± 1.27	12.36 ± 0.73
			Females			
Sham (n=3)	19.1 ± 0.6	18.5 ± 0.1	20.8 ± 0.5	13.43 ± 0.72	13.84 ± 0.94	12.51 ± 0.39
Testosterone (n=8)	19.7 ± 1.0	21.3 ± 1.2	23.6 ± 1.1	13.46 ± 1.15	12.79 ± 0.79	11.82 ± 0.76
17β-Estradiol (n=8)	18.9 ± 1.0	20.5 ± 1.2	23.4 ± 1.3	14.13 ± 1.86	13.11 ± 1.21	11.14 ± 0.51

Values are mean±SD. Baseline data represents normal characteristics, gonadectomy data represents no circulating sex steroids, and replacement represents reintroduced sex steroids. No significant difference found for the sex by group by treatment interaction. Individual main effects were reported in the results section.

Table 4. Sera/plasma testosterone and 17β-estradiol concentrations for mice* at baseline and under various placebo and experiment conditions

Condition (n=T/E_2)	Testosterone ($ng \cdot ml^{-1}$) mean±SD	17β-Estradiol ($pg \cdot ml^{-1}$) mean±SD
Baseline (n=3/3)	6.32±2.79	59.82±16.72
Placebo (n=15/15)	11.53±7.54	397.08±881.50
	Experimental	
ORCH (n=3/2)	min	109.79±29.71
ORCH+E_2 (n=2/3)	0.21±0.23	613.44±185.90
ORCH+T (n=3/2)	15.37±1.21	151.96±53.20

*Sera/Plasma from 3 mice were pooled together

Abbreviations: ORCH=Orchidectomy, E_2=17β-Estradiol Implant, T=Testosterone Implant, min=at the minimum of prediction curve

CHAPTER 3:
EFFECTS OF AROMATASE INHIBITION ON THE PHYSICAL ACTIVITY LEVELS OF MALE MICE

Abstract

Increasing activity levels in an inactive population can lead to associative increases in health and well-being. Both biologic and genetic factors have been identified that alter activity levels in humans and rodents. Currently, it is suggested that the androgens require conversion to estrogens prior to eliciting any effects on activity patterns. Recent data contradicts this assertion; thus, the purpose of this study was to evaluate the necessity of the aromatase complex in activity regulation. Wheel running was assessed in male C57BL/6J mice (n=80) under various sex steroid-disrupted and aromatase-inhibited conditions. Silastic capsules and surgical orchidectomies were used to alter circulating sex steroid levels. Inhibition of the aromatase complex was achieved through administration of an irreversible aromatase inhibitor. Wheel running was unaffected ($p>0.05$) by aromatase inhibition in reproductively intact and sex steroid supplemented mice. Orchidectomy significantly ($p<0.05$) reduced wheel running activity. Steroid replacement recovered wheel running to pre-surgical levels; however, aromatase inhibition did not affect wheel running levels ($p>0.05$). The persistence of wheel running in mice with compromised aromatase function suggests that the androgens—testosterone in particular—may directly affect wheel running patterns in male mice.

Introduction

Physical inactivity affects public health and unnecessarily burdens the American health care system. The risks of many diseases including obesity, diabetes, heart disease and several types of cancer are enhanced in individuals with habitually low physical activity levels (87). Although evidence exists suggesting that activity levels are determined by extrinsic and environmental factors, a growing number of literature sources suggest large genetic and biological influences also exist (63, 70, 77, 82, 83, 131). Androgens and estrogens have been the focus of extensive research relating to activity levels in rodents and the loss of testes or ovaries results in noted reductions in daily wheel running activity (43, 57, 81, 90, 92).

Currently, testosterone is suggested to require prior conversion to an estrogenic compound before any modulatory interactions to the wheel running response will occur. Roy and Wade (114) administered aromatizable and non-aromatizable forms of androgens to orchidectomized rats. The aromatizable androgen notably increased wheel running, but administration of the non-aromatizable molecule resulted in continued quiescence.

Supporting Roy and Wade's earlier study, Watai et al. (145) found that wheel running activity was hindered in an estrogen-deficient aromatase knockout mouse model. Conversely, Hill et al. (49), using a similar aromatase knockout model found that, the male knockout animals ran nearly twice as far as wild type animals, an observation that was reversed in three weeks with the administration of 17β-estradiol. While the use of knockout animals can lead to difficulties with interpretation due to issues arising during development due to the lack of the knocked out gene (16), it is interesting that the two

studies using aromatase knockout animals resulted in completely opposite results.

Thus, other experimental methods, such as the use of aromatase-inhibiting substances to circumvent the issues related to the use of knockout animals, is warranted for further elucidation of the roles of the sex steroids in physical activity regulation. With this approach, the functionality of particular physiological pathways can be altered without hindering normal development. The purpose of this study was to evaluate wheel running activity in the presence of irreversible and reversible aromatase inhibitors under normal physiological conditions and during artificial manipulation of endogenous sex steroid levels in male mice.

Methods

Animals: Eighty male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in three experimental procedures starting at approximately 63 days old. All mice were housed in an environmentally controlled animal husbandry facility under 12/12 h light/dark cycles with lights illuminating the housing room at 6:00am daily. After arrival at the research facility, mice were initially housed with six to eight littermates prior to initiating an experimental protocol. After acclimation and at approximately eight weeks of age (≈54 days old), the animals were individually housed in standard rat sized cages with metal running wheels during each experiment. The cages were equipped with a stainless metal food hopper and glass water bottle. The mice were allowed *ad libitum* access to both food and water. This project conformed to the ethical standards set forth by the scientific community and was approved by the UNC Charlotte Institutional Animal Care and Use Committee prior to initiation.

Wheel Running: Running wheels (450 mm circumference; Ware Manufacturing,

Phoenix, AZ) with a 40 mm wide running surface were attached to the metal tops of each cage and were equipped with cycling computers (BC500, Sigma Sport, Olney, IL) to track wheel running distance (km) and duration (min). Average speeds ($m \cdot min^{-1}$) for each day of analysis was calculated by dividing distance by duration. Each experimental epoch lasted seven days and wheel running data was collected every 24 hours. Average daily distance, duration, and speed were calculated for each seven day experimental epoch. Seven days of data were used in calculating each average for the epoch unless the recording computer was found non-functional. These data were removed from the calculation and the average was derived from five to six data points. Each computer was calibrated to the running wheel's circumference and was checked for proper connectivity on a daily basis by a research technician. Furthermore, the freeness of the wheel's rotation about the axle was checked daily and lubricated as needed. The wheels were sanitized every two weeks for the length of the experiments and were brushed when needed to keep the running surface free of debris (bedding, food, feces, etc.).

Injections: During this study, control and experimental injections were administered. Control injections consisted of 0.3% hydroxypropyl cellulose in phosphate buffered saline (HPC+PBS) and was administered in a 0.5 ml subcutaneous bolus. Experimental injections contained the aromatase inhibiting substance exemestane (Sigma-Aldrich, St. Louis, MO) suspended in HPC+PBS. The drug was administered subcutaneously at a dosage of 250 $mg \cdot kg^{-1}$ per 0.5 ml bolus. This dose schedule was developed to yield maximum inhibition of aromatase activity (44, 61). Prior to administration, steps were taken to maintain the sterility of the injection medium by using a standard liquid autoclave cycle prior to storage in a sterile lab container. The

exemestane was dissolved in methanol and passed through a 0.2 micron cellulose filter into a sterile mortar to remove impurities in the drug. The methanol was then evaporated and the residue exemestane was ground and added to an aliquot of sterile HPC+PBS to form a dispensable suspension for injection.

Alterations of Sex Steroid Levels: To vary the levels of circulating steroids, two procedures were employed. First, supplementation or replacement of steroids was achieved via silastic (Dow Corning, Midland, MI) implants. Testosterone and 17β-estradiol (Sigma-Aldrich, St. Louis, MO) were packed into 10 mm lengths of 1.02 mm internal diameter silastic tubing. The ends of the tubing were capped with clear silicone glue. The placebo implants were left empty. The implants were surgically inserted under isoflurane anesthesia in a small subcutaneous pocket on the lateral aspect of the neck between the skin and the muscle fascia. A two day recovery was allowed prior to reintroduction of running wheels.

The second technique for altering the levels of circulating steroids was the completion of bilateral orchidectomy surgeries to remove the testes, the major sex steroid producing tissue in male mammals. The surgeries were performed under isoflurane anesthesia after administration of the preemptive analgesic carprofen (5 $mg \cdot kg^{-1}$). A small incision was made in the midline of the scrotum just inferior to the penis. Each testis was exposed through the incision and was removed along with the epididymis. The incision was closed with a sterile wound clip and the animal was allowed to recover under a heating lamp. Placebo animals received a sham procedure; the testis were exposed but were not excised. The surgical procedures were followed by ten days of wheel free recovery.

Experimental Procedures: The experimental timeline for each experiment is shown in Figure 12. Twenty mice were used in the first experiment. Mice, stratified by original group housing, were randomly assigned to a placebo (n=10) or experimental (n=10) group. In both groups, wheel running was monitored under three conditions. First, both groups underwent baseline screening for seven days to assess normal physiologically wheel running activity. Next, each mouse received either placebo or exemestane injections and wheel running was monitored for an additional seven days. Lastly, injections ceased and mice were allowed three days of unmonitored wheel running followed by a final seven days to assess wheel running during drug clearance.

Thirty mice were used in experiment two to evaluate the effects of supplemented sex steroids on wheel running activity during exemestane injections in mice with fully functional reproductive organs. One mouse was euthanized at the onset of the experiment due to an injury sustained during the preliminary group housing phase. The mice were divided into control (n=9), experimental one (n=10), and experimental two (n=10) groups. Wheel running was again assessed at baseline under normal physiological conditions for seven days. During the next seven days of wheel running activity, the mice received exemestane (experimental one and two) or placebo (control) injections. In the final seven days of this experiment, the mice received silastic implants containing testosterone (experimental one) or 17β-estradiol (experimental two). The control animals received empty implants. After a brief two day recovery, exemestane and control injections were resumed and wheel running was evaluated for seven additional days.

The third experimental procedure utilized thirty mice and evaluated the effects of

orchidectomy and aromatase inhibition on wheel running activity. Replacement strategies (via silastic implants) were employed to reintroduce the sex steroids after removal of the gonads. The experimental groupings were as stated above in experiment two. Baseline data was collected at the onset of the experiment. Double and sham orchidectomies were performed and were followed by a ten day recovery period. Wheel running was evaluated at the end of the orchidectomy recovery period for seven days and then continued for seven more days while placebo or exemestane injections were administered every 24 hours. Silastic implant surgeries were performed followed by two days of recovery and a final seven day wheel running assessment period. Testosterone, 17β-estradiol, and blank implants were again utilized during this final period.

Blood Collection and Sex Steroid ELISAs: The present study was conducted in close succession with two additional projects with similar placebo and experimental conditions; thus, blood was sampled throughout these experiments, stored, and assayed conjunctively. Blood was sampled on regular 14-day intervals via venipuncture and directly from the inferior vena cava at the time of sacrifice. Each mouse was restrained in a decapicone bag with openings to expose the hind limbs. The medial surface of the exposed limbs were shaved and cleaned. The saphenous vein was exposed through application of light pressure to the proximal segment of the hind limb and was punctured using a 25 gauge sterile needle. The blood was collected into EDTA or heparinized microvette capillary tubes and stored on ice for up to three hours. Plasma was separated from the red cell mass via cold centrifugation and was stored until assay. Samples collected at the end of the experiments were allowed to clot and were then centrifuged at room temperature. The serum was stored until assay.

Sera/plasma samples (n=3) were pooled together based on the experimental condition at the time of collection. An extraction procedure was performed on the pooled samples prior to assay. Pooled samples (200 µl) were combined with 1000 µl of ethyl acetate (Sigma-Aldrich, St. Louis, MO) and were frozen over night. The ethyl acetate was then decanted and evaporated. The dry, extracted residue was reconstituted in steroid free serum (IBL-America, Inc., Minneapolis, MN) and was used in all assay procedures.

Testosterone and 17β-estradiol concentrations were measured via ELISA (IBL-America, Inc., Minneapolis, MN). Calibrator samples were used to estimate the concentration of the unknown samples. All samples were measured in duplicate and the average value was used as each samples' concentration. A data point was eliminated from analysis if duplicate measurements were highly variable or if an individual concentration was different (500% difference between a single data point compared to other data points in the group 500%) from the group mean.

Letrozole Experiment: Exemestane has both aromatase inhibiting and androgenic properties. It was speculated that the effects from the first three experiments utilizing exemestane might be due to the androgenic rather than the aromatase inhibiting effects; therefore, the reversible aromatase inhibitor letrozole (Fisher Scientific, Pittsburgh, PA) was used to validate the results achieved in experiment one of the current project. Twenty untreated C57BL/6J mice were used in the confirmatory study and the methodological techniques were identical to experiment one. In brief, placebo (n=10) or letrozole (n=10) injections were given to reproductively intact mice. Wheel running indices were monitored prior to injections, during injections, and after cessation of

injections—each phase lasting seven days. Letrozole was administered via sub-cutaneous injections at a concentration of 0.5 µg per 100 µl of 0.3% HPC+PBS for seven days. Placebo injections consisted of 0.3% HPC+PBS.

Statistical Analysis: The physical activity data collected during each of the four experiments were analyzed using individual two-way (group by condition) analysis of variance (ANOVA) tests for each wheel running indices (distance, duration, or speed). A Tukey's *post-hoc* test was used to assess significant main effects or interactions. The ELISA data for all experiments were assessed by one-way (condition) ANOVA. The alpha level was set *a priori* at 0.05.

Results

Physical Activity: Wheel running indices (experiment one) for male C57BL/6J mice at baseline, receiving exemestane or placebo injections, and during a 10-day clearance period are shown in Figure 13. The aromatase blocking substance was not an effective inhibitor of activity and did not significantly alter any of the wheel running indices at the administered dosage (250 $mg \cdot kg^{-1}$). Distance (p=0.61), duration (p=0.38), and speed (p=0.69) did not change with exemestane injections. The power values for these analyses were low (distance: power=0.10, duration: power=0.07, duration: power=0.15).

Running distance, duration, and speed for experiment two are shown in Figure 14. Wheel running was assessed at baseline, with exemestane injections, and after implantation of testosterone or 17β-estradiol containing capsules. The difference across experimental conditions and groups were non-significant (distance: p=0.66, duration: p=0.61, speed: p=0.56) and neither testosterone nor 17β-estradiol altered the running

response. Power values for these analyses was low (distance: power=0.19, duration: power=0.21, speed: power=0.23).

Wheel running indices (experiment three) at baseline, after surgical or sham orchidectomies, with placebo, testosterone, or 17β-estradiol implants, and with injections of placebo vehicle or exemestane are shown in Figure 15. Wheel running was significantly altered by these experimental interventions (distance: F=4.65, p=0.0001, duration: F=4.82, p=0.0001, speed: F=6.63, p=0.0001) and Tukey's HSD *post-hoc* tests revealed that several interventions altered the three wheel running activity indices measured (see Figure 15). Orchidectomies significantly reduced all three indices of wheel running and testosterone replacement recovered wheel running back to baseline levels; however, 17β-estradiol failed to engender the same level of recovery. Administration of exemestane had limited effect on activity in the orchidectomized mouse receiving either steroid.

Testosterone and 17β-Estradiol ELISAs: Testosterone and 17β-estradiol levels assessed in pooled sera/plasma samples across all three experiments in the present project are displayed in Table 5. The testosterone and 17β-estradiol concentrations were highly variable, but not statistically different among the various conditions. The widely different mean concentrations however, indicate very different endocrinological functionality among the conditions.

Letrozole Experiment: Wheel running distance for control and letrozole-treated mice are displayed in Figure 16. The wheel running response under this reversible aromatase inhibitor was similar to the response observed with exemestane inhibition. All wheel running parameters were unaffected by letrozole administration and did not deviate

from the levels measured in the control animals ($p>0.05$).

Discussion

The present results demonstrated that activity remains primarily unaffected by the administration of aromatase inhibitors in a dosage previously shown to maximally inhibit the aromatase complex (44, 61). This project evaluated two manipulative strategies: 1) supplementation of both testosterone and 17β-estradiol in intact mice receiving the aromatase inhibitors; and 2) replacement of testosterone and 17β-estradiol in orchidectomized mice receiving the aromatase inhibitor. Surprisingly, the results of this study demonstrate that the hypothesis of a primary estrogen-derived activity regulating mechanism is probably incorrect. However, we are cautious in our conclusions due to the high variability in sex steroid concentrations observed in this study, which may mask the complete delineation of the mechanisms of sex steroid derived activity regulation in mice.

Aromatase Inhibition and Physical Activity Levels: A physiologically normal C57BL/6J mouse runs vigorously when exposed to an in-cage running wheel. Alterations to the sex steroids interrupt this normal running pattern (54, 56, 106, 144), an effect expected to occur through modulation of estrogen levels via the aromatase complex based on previous research (114). However, in the current project, inhibition of the aromatase complex did not significantly alter wheel running activity indicating that a functional aromatase complex may not be required for activity levels to be modulated. While contradictory to previous speculations, little direct evidence exists that indicates activity regulation—via the sex steroids—requires the presence of estrogenic compounds.

Roy and Wade (114) assessed the ability of testosterone propionate (an

aromatizable androgen) and dihydrotestosterone propionate (a non-aromatizable androgen) to affect activity levels in castrated male rats. Administrations of the aromatizable androgen increased activity to the levels observed in estrogen treated animals, but the non-aromatizable androgen had little effect (114). In addition, changes to wheel running levels were also different. Roy and Wade (114) noted a partial recovery of wheel running with testosterone administration compared to estradiol benzoate administration. In the current study, wheel running not only returned to the levels observed at baseline, but exceeded the levels induced by 17β-estradiol administration. This difference maybe accounted for by the modes of steroid delivery used in each project. The steroidal compounds were delivered via silastic implants in the current project, but Roy and Wade (114) delivered the steroids via daily injections in sesame oil. The injection methods employed by Roy and Wade (114) required daily contact with the animals which has been shown to induce higher levels of stress and changes to wheel running activity (104). The effects of human interaction on wheel running behavior observed by Richter (104) were not as dramatic as the differences noted between Roy and Wade (114) and the current project; therefore, other mechanisms may also be influencing the activity patterns in the current study. Unfortunately, steroid concentrations were not quantified by Roy and Wade (114); therefore, further direct comparison is difficult.

Watai et al. (145) evaluated activity in an aromatase (*Cyp19*) knockout mouse model and observed very low activity levels in these mice noting a requirement for estrogens to be present in order for activity levels to match those observed in normal animals. The use of gene knockout models are not without adversity as developmental differences in mice can result in an obscured representation of reality (16). The

difference between the current study and Watai et al. (145) may be due to such effects and thus, may be due to a abnormal physiological phenomenon rather than relevant deviations in the mechanisms affecting activity levels.

Hill et al. (49) evaluated wheel running activity in estrogen deficient male mice, similar to the mice utilized by Watai et al. (145) and observed compulsory wheel running activity that exceeded the levels observed in wild type controls. Interestingly, this effect was not observed in female *Cyp19* knockout animals and compulsory wheel running was ameliorated after the administration of 17β-estradiol (49). The non-essential need for a functional aromatase complex to maintain normal activity levels observed in the present study and by Hill et al. (49) is contrary to the observations of Roy and Wade (114) and Watai et al. (145). The technical difference noted in these studies may account for some of the observational disparities; however, further study detailing the underlying estrogenic and androgenic mechanisms is required.

Potential Androgenic Mechanisms of Activity Regulation: The presence of an activity modulating mechanism regulated by androgenic steroids remains unclear with minimal supporting data in the literature. The current study suggests that, at a minimum, an androgenic mechanism of activity regulation exists in mice. Two primary lines of evidence from the current project support the hypothetical presence of an androgenic activity regulator. First, blockade of the aromatase complex via either reversible or irreversible aromatase inhibiters did not significantly reduce wheel running in reproductively intact or sex steroid treated orchidectomized mice. Second, testosterone replacement to orchidectomized mice increased wheel running activity back to baseline levels, while 17β-estradiol increased wheel running to only 50% of baseline levels

despite higher concentrations of estrogens levels in the 17β-estradiol treated animals.

The current research represents the second attempt to evaluate a direct androgenic mechanism in non-genetically modified mice. Flynn et al. (29) exposed mice to the antiandrogenic fungicide vinclozolin at gestational age seven in dam's milk and continued exposure via food after weaning. With exposure to vinclozolin, both males and females exhibited decreased wheel running levels; however, only the females reached a statistically significant decrease in wheel running activity at the highest dose of the chemical. The authors (29) concluded that the depressive effects of vinclozolin on activity levels were caused by an inhibitory interaction between the fungicide and the androgen receptor. Thus, the data of Flynn et al (29) partially supports our hypothesis regarding the existence of an androgenic physical-activity regulating mechanism.

While several authors (18, 37, 53, 66, 140) have identified high malleability of the activity response in animals treated with estrogens directly to the brain, the permeability of the rodent brain to androgens is also high (94) suggesting a potential pathway for testosterone to effect a central activity regulator. To date, only one paper (50) has evaluated brain morphology in aromatase compromised mice. Hill et al. (50) noted lower levels of dopaminergic neurons in the medial preoptic area and arcuate nucleus of male knockout mice. This response would tentatively explain their noted increased activity levels of the aromatase knockout mice based on recent work from Knab et al. (70) that suggested an increased activity level was due to down-regulated dopamine 1 receptor levels; i.e. the animals were more active to drive the reward signaling from the dopamine receptors higher. Thus, we can speculate that testosterone influences activity levels in male mice through interactions with the androgen receptor leading to alteration of central

dopamine function.

Study Limitations: The periodic measurement of sex steroids from the same mouse over a matter of weeks, as was completed in this project, is very difficult. The low plasma/sera volumes obtained from an individual mouse are often too small for assessment of sex steroid concentrations in each mouse using any known assay procedure. In this project, this issue was circumvented with pooling techniques that allowed the samples for a given experimental condition to be increased to an appropriate volume for assay detection. However, the errors extending from the pooling technique are limited by the use of genetically identical mice. Thus, the variations observed in hormone concentrations were due to environmental sources and the experimental manipulation rather than inherent difference in steroid levels between mice.

The blood sampling techniques employed in this project required close contact with the mice on the day of blood collection for a prolonged period. Interactions between animal handlers and small rodents have been shown to induce a stress response in small rodents (104) which could artificially alter sex steroid levels in the bloodstream. Steps were taken to minimize the significant stress on the animals in this study for humane purposes and to limit the potential to induce aberrant sex steroid concentrations. During blood sampling, animals were handled using controlled and secure techniques, monitored for signs of elevated stress (vocalizations, biting, increased mobility, etc.), and directly exposed to technicians for a minimal amount of time. Most blood draws from initial immobilization to release back into the home cage were less than 10 minutes.

Exemestane is an irreversible aromatase inhibiting drug and it is expected that administration of this pharmacological substance should lead to a decrease in estrogen

content, but effect testosterone levels very little. Surprisingly, we showed that the concentrations of both steroids are highly variable in the presence of the drug. Sex steroid levels in exemestane treated animals have not been quantified by others (44, 61) and therefore, the source of the variability in hormone concentrations are difficult to identify. In addition to its inhibitory effects, exemestane has been shown to possess androgenic characteristics in clinical situations including an affinity to bind the androgen receptor (62). The dosage used in this project was previously shown to have physiological effects on bone characters and tumor morphology, effects that could have been due to the androgenic rather than the aromatase inhibitory nature of exemestane. In order to parse the androgenicity and aromatase inhibitory effects of the drug, the results from the first experiment were repeated using letrozole, a reversible aromatase inhibitor that does not have androgenic effects. In this repeated experiment, very similar results were observed indicating that the aromatase inhibiting effects of the drugs did not alter wheel running behavior and that physical activity regulation via estrogenic sources was not an absolute requirement in our model.

Conclusions: The present study evaluated the effects of two aromatase inhibitors on wheel running activity in male C57BL/6J mice. Neither aromatase inhibitor altered wheel running activity in intact or orchidectomized animals. These data, in conjunction with wheel running measures in aromatase knockout mice (49) and vinclozolin treated mice (29), suggests that aromatization of testosterone is not needed for activity regulation as has been earlier postulated (114) and that a direct androgenic mechanism regulates activity levels in mice. The complexity of such regulatory mechanisms and the low number of available research studies provide ample basis for reinvigoration of this

research area. In particular, studies that partition the androgenic and aromatase inhibitory effects of the irreversible aromatase inhibitors will provide valuable insight into this mechanism. Future research should also utilize pharmacological and chemical methods to manipulate the function of the aromatase complex and androgen receptors of intact and gonadectomized mice while monitoring wheel running. Physical inactivity has reached epidemic proportions in the developed world leading to increasing rates of obesity and hypokinetic diseases, thus identifying and understanding the biological mechanisms that regulate activity levels can profoundly influence human health worldwide.

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Footnotes:

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Journal Submission: At the time of publication of this dissertation, the preceding chapter was in preparation for review in the journal: *Endocrinology*

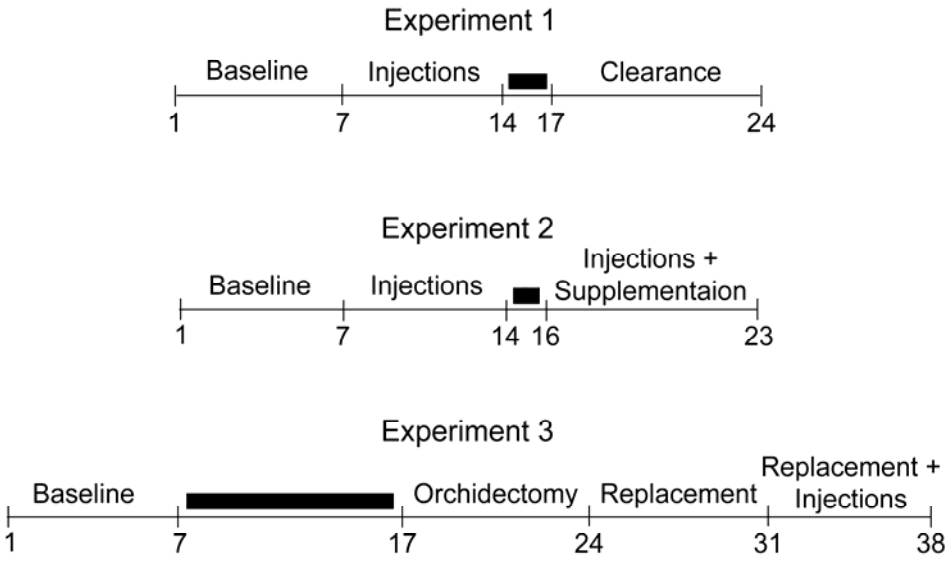

Figure 12. The experimental timelines (days) for assessing physical activity levels in aromatase inhibited and sex steroid modified mice. Black bars represent recovery periods—after surgeries and after cessation of injections—in which running wheels were not in the cages.

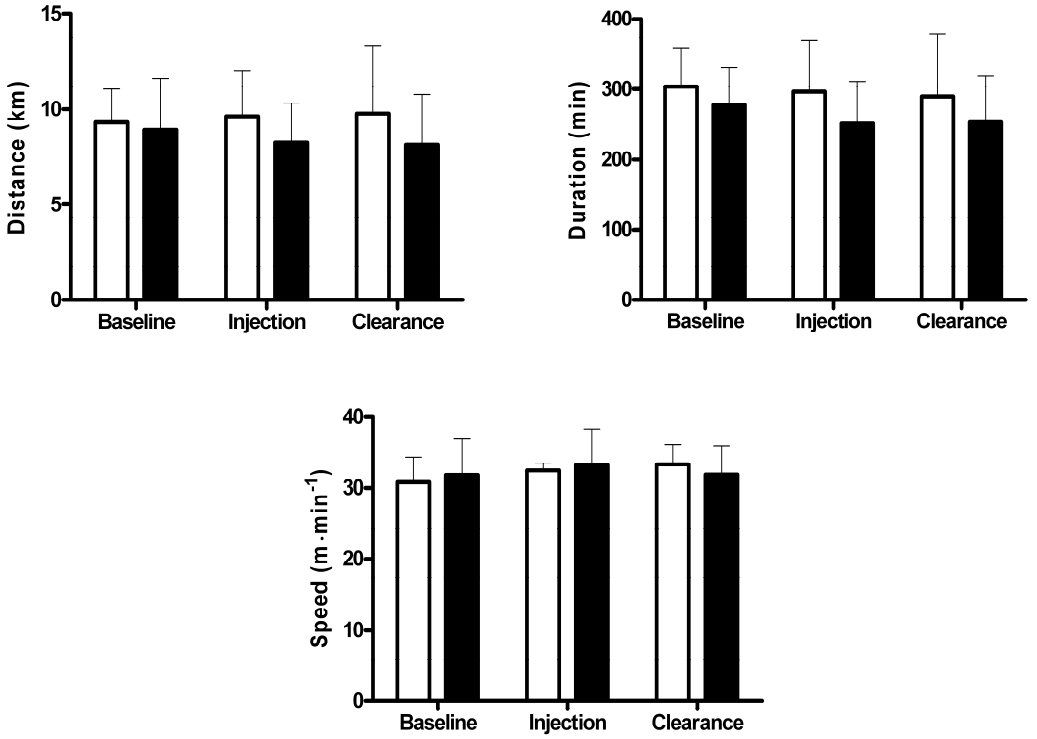

Figure 13. Wheel running indices (distance, duration, and speed) for male C57BL/6J mice at baseline, during injections, and during post-injection clearance. White bars denote control mice (n=10) that received vehicle injections and black bars denote experimentally treated mice (n=10) that received exemestane injections.

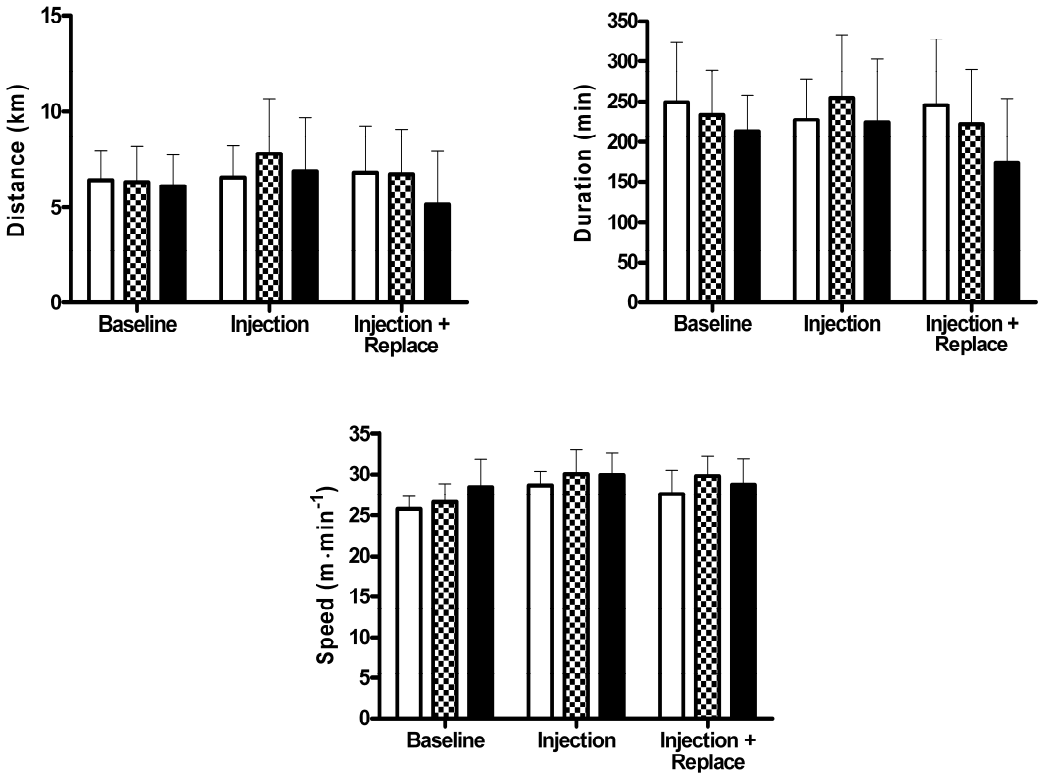

Figure 14. Wheel running indices (distance, duration, and speed) for male C57BL/6J mice at baseline, during injections, and during injections supplemented with either testosterone or 17β-estradiol. White bars denote control mice (n=10) that received vehicle injections and blank silastic implants. Checkered bars denote experimentally treated mice (n=10) that received exemestane injections and silastic implants containing testosterone. Black bars denote experimentally treated mice (n=10) that received exemestane injections and silastic implants containing 17β-estradiol.

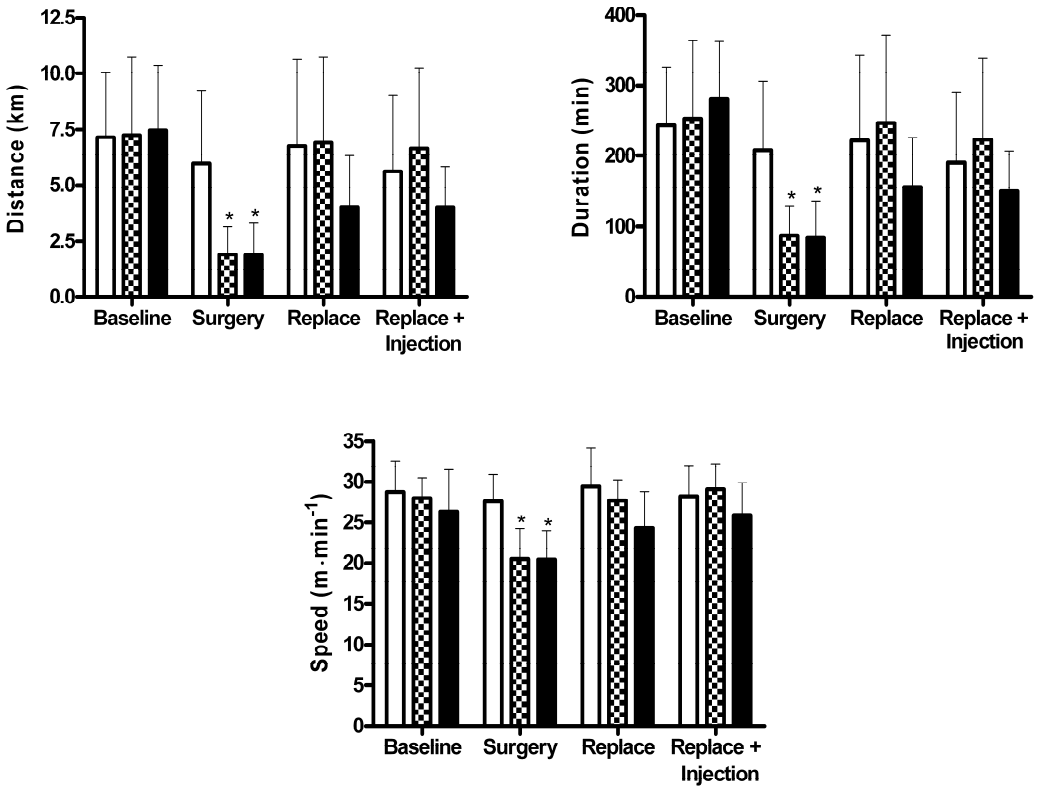

Figure 15. Wheel running indices (distance, duration, and speed) for male C57BL/6J mice at baseline, during injections, during steroid replacement, and during steroid replacement with injections. White bars denote control mice (n=10) that received vehicle injections and blank silastic implants. Checkered bars denote experimentally treated mice (n=10) that received silastic implants containing testosterone and exemestane injections. Black bars denote experimentally treated mice (n=10) that received silastic implants containing 17β-estradiol and exemestane injections. *=significantly different from controls and baseline values.

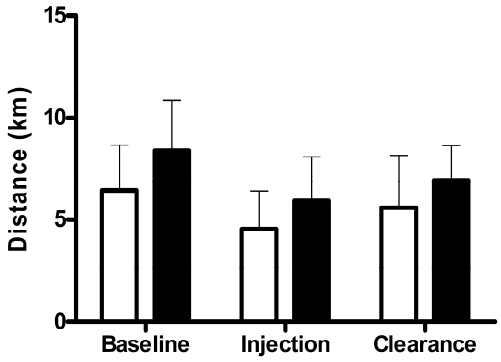

Figure 16. Wheel running distance in C57BL/6J mice at baseline, after injection of letrozole (reversible aromatase inhibitor), and during a seven day drug clearance period. The white bars represent the control mice (n=10) and black bars represent the experimental mice (n=10).

Table 5. Sera/Plasma testosterone and 17β-estradiol concentrations from mice* at baseline and under various placebo and experimental conditions

Condition (n=T/E_2)	Testosterone ($ng \cdot ml^{-1}$) mean±SD	17β-Estradiol ($pg \cdot ml^{-1}$) mean±SD
Baseline (n=3/3)	6.32±2.79	59.82±16.72
Placebo (n=15/15)	11.53±7.54	397.08±881.50
	Experimental	
EXEM (n=2/1)	9.97±1.82	472.82
EXEM Clearance (n=2/1)	13.92±9.89	57.31
EXEM+T (n=2/1)	15.69±7.54	660.26
EXEM+E_2 (n=2/2)	min	121.07±21.90
ORCH (n=3/2)	min	109.79±29.71
ORCH+T (n=3/2)	15.37±1.21	151.96±53.20
ORCH+E_2 (n=2/3)	0.21±0.23	613.44±185.90
ORCH+T+EXEM (n=2/2)	14.08±1.34	346.55±116.04
ORCH+E_2+EXEM (n=2/2)	11.70±9.55	680.91±262.98

*Sera/Plasma from 3 mice were pooled

Abbreviations: EXEM=Exemestane Injections, T=Testosterone, E_2=17β-Estradiol, ORCH=Orchidectomy, min=at the minimum of prediction curve

CHAPTER 4:
THE SEX STEROIDS DO NOT INFLUENCE PHYSICAL ACTIVITY LEVELS THROUGH THE DOPAMINE 1 RECEPTOR

Abstract

Physical activity levels are highly associated with lower occurrences of chronic disease and disability; therefore, increased physical activity may provide substantial benefit to the individual and society. Previous literature has suggested that testosterone and dopaminergic functioning may interact to affect physical activity. Thus, the purpose of this project was to investigate the effects of testosterone and D1-like antagonism on the physical activity levels of orchidectomized mice. Thirty C57BL/6J mice were used in this project. At the onset, 20 mice were orchidectomized and ten received sham surgeries. Wheel running was monitored for seven days after a post-surgery recovery period. Next, testosterone and a D1-like antagonist were administered to the orchidectomized mice (n=10 per group) and wheel running was monitored for seven more days. At the end of experiment, the nucleus acumbens and striatum were assessed for dopamine 1 receptor (*Drd1*) mRNA expression. The experimental treatment affected all three wheel running indices (p=0.0001). The orchidectomized mice ran less than controls and testosterone replacement increased wheel running to control levels. The D1-like antagonist did not recover wheel running back to control levels. End of experiment *Drd1* mRNA expression did not differ between the experimental conditions (p=0.83). In conclusion, though it appears that testosterone regulates activity levels in

mice, the effect is not related to the availability of *Drd1* genetic transcripts.

Introduction

Increasing physical activity levels is an important health related goal for the developed world and has been shown to reduce the risks associated with cardiovascular disease (33, 148), various types of cancer (132), diabetes (148), and obesity (150). Mokdad et al. (87) found physical inactivity and poor diet were major actual causes of death (approximately 15.2%) in the United States and was the second highest actual cause of death behind tobacco use (approximately 18.1%). In addition, the trend toward inactivity is increasing and is expected to become the leading cause of actual death in a few years (87). This trend toward inactivity and chronic disease persists despite a 25+ year foundation of research focused on environmental regulators of activity in adults (147) and adolescents (27). This adverse trend may occur for at least two reasons; the translational application of scientific research is inefficient or there are more powerful activity influences overriding the inputs of the environmental factors. Certainly, the former is a barrier experienced by all types of scientific discoveries and may be resolved through fervent application of public heath policy, alteration of urban development trends, or modification of socioeconomic perception; however, the latter has recently received more attention from the scientific community resulting in several remarkable findings.

Several studies have indicated important genetic and biologic pressures influencing activity and exercise traits in both humans (64, 76, 130, 131, 137) and rodents (28, 43, 70, 77, 79, 82, 83, 136, 139, 145). In particular, testosterone and 17β-estradiol have been shown to significantly modify wheel running in rodents (see ref. 81 for a brief

review). The surgical removal of the gonads in either sex results in a consistent reduction of activity levels that is recovered to various extents upon reintroduction of testosterone or 17β-estradiol (13, 14, 19, 43, 90, 96, 99). Currently, the mechanistic effectors transmitting the sex steroids' effects have not been substantially identified, but the effect appears to be centrally regulated (18, 53, 66, 68, 70, 139).

Several areas in the brain and factors acting within these areas have been suggested to influence activity levels in rodents. Kennedy (66) induced inactivity via ovariectomy in rats and observed increased activity after administration of estrogens. The recovery abilities of the estrogens were inhibited in rats with lesions to the ventrolateral hypothalamus (66). Colvin and Sawyer (18) observed increased wheel running in rats receiving estrogens directly to the medial forebrain bundle, but found no evidence that the ventrolateral hypothalamus was involved in regulation of activity. Hitt and Gerall (53) implanted electrodes directly into 14 distinct regions of the brain and observed reduced wheel running during stimulation of central hypothalamus structures; stimulation of the posterior and anterior regions of the hypothalamus did not effectuate similar results. The authors speculated that the electrical stimulation blocked estrogen's ability to interact with neurons in the central hypothalamus resulting in lower activity levels (53). Fahrbach et al. (26) confirmed the work of Hitt and Gerall (53) with direct implantation of estradiol to various structures in the anterior hypothalamus and pre-optic areas, but also noted variable responsiveness to the implants. More recently, Knab et al. (70) and Verhagen et al. (139) noted variations in activity patterns that associated with low dopamine 1 receptor (*Drd1*) mRNA expression levels and dopamine/serotonin release in the nucleus acumbens respectively. The dopamine system of the nucleus

acumbens and striatum in rodents also shows a significant sexual dimorphism, an effect that may occur through G-protein-coupled receptors and may be stimulated by interactions with the sex steroids—directly through estrogens or indirectly through the aromatic conversion of testosterone (9). Moreover, Hill et al. (50) observed an apoptotic reduction in dopaminergic neurons in estrogen-deficient aromatase knockout mice.

While dopaminergic neurons have been shown to be responsive to changes in sex steroid levels, no studies have investigated the interaction between testosterone and dopamine receptors to determine if there is an interactive effect on activity levels. Therefore, the purpose of this paper was to evaluate the changes to *Drd1* mRNA expression levels in orchidectomized mice after administration of testosterone or a D1-like antagonist. In addition, the activity levels were evaluated in orchidectomized mice receiving testosterone or a D1-like antagonist. We hypothesized that *Drd1* levels would not differ from the levels observed in control mice resulting in wheel running levels that were comparable to the levels observed in the non-orchidectomized control animals.

Methods

Animals: This project utilized 30 male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME), which were divided into three experimental groups (Control, ORCH 1, ORCH 2). Upon arrival at the animal housing facility the mice were group housed seven or eight per cage. The mice were randomly assigned to individual cages at nine weeks of age and were allowed *ad libitum* access to water and food (Harlan Teklad, Madison, WI). All animal use conformed to ethical standards set by the scientific community and was approved by the UNC Charlotte Institutional Animal Care and Use Committee.

Protocol Overview: Double orchidectomies were performed two days after

individually housing the mice. The control animals (n=10) received sham procedures and the two experimental groups (n=10 per group) received real orchidectomies. These surgeries were followed by a 10-day recovery period. In brief, all animals received a pre-emptive dose of carprofen ($5mg \cdot kg^{-1}$). All surgeries were performed under 2% isoflurane anesthesia mixed with oxygen at a flow rate of 600 $ml \cdot min^{-1}$. A small incision was made on the midline of the scrotal sac just inferior to the penis after the area was sterilized with betadine and 70% alcohol scrubs. Each testicle was exposed and removed along with the epididymis. The surgical wound was closed with a sterile surgical wound clip. The sham procedures were identical to the real procedures, but the testicles and epididymis were left intact. All procedures were performed by an experienced technician.

At the end of the post-operative 10-day recovery period, metal running wheels (Ware Manufacturing, Phoenix, AZ) were installed in each cage. The wheels had a 40 mm running surface, 450 mm circumference, and were equipped with bicycle computers (BC500, Sigma Sport, Olney, IL) specifically calibrated to measure running distance (km) and duration (min). Speed was calculated from these variables ($m \cdot min^{-1}$). The running wheel data were collected every 24 hours and averaged over seven days. Wheel resistance was checked daily and the axles were lubricated with oil as needed. The data from maladjusted wheels or improperly functioning computers were eliminated from that dataset on the day the error was noted.

The mice were allowed two days to acclimate following introduction of the wheels. For the following seven days, post-surgery wheel running indices were assessed. After this first seven day period, silastic (Dow Corning, Midland, MI) capsules (17) were implanted into the control and ORCH 1 groups. The control animals received empty

capsules and experimental animals received testosterone (Sigma-Aldrich, St. Louis, MO) filled capsules. The capsules were 10 mm long and had an internal diameter of 1.02 mm. The ends of the implants were sealed with silicone glue. A small incision was made in the skin on the lateral aspect of the neck and a pocket was created between the skin and the fascia to contain the implant. All procedures were performed under the same conditions as the orchidectomy surgeries, except that no pre-emptive analgesic was necessary for this procedure.

The mice were allowed two days to recover from the surgical wounds prior to reevaluating wheel running. At the onset of this period, the second experimental group (ORCH 2) received daily injections of a D1-like antagonist (SCH23390; Tocris Bioscience, Ellisville, MO), in sterile saline (0.3 ml) at a concentration of 0.10 $mg \cdot kg^{-1}$ as an intraperitoneal injection on the animal's right side. Each preparation was filtered through a 0.2 micron cellulose filters prior to injection. Control mice received 0.3 ml sterile saline injections.

At the end of the study, all mice were euthanized and the brains were prepared for further analysis. Specifically, the nucleus acumbens and striatum regions (NA/S) of the brain were analyzed for dopamine 1 receptor sub-form A (*Drd1*) mRNA expression levels. Quantitative real time polymerase chain reaction (qRT-PCR) experiments were completed to estimate the levels of *Drd1* expression as previously described (41). Total RNA was extracted from the right portion of the NA/S using the Trizol method (TRI Reagent, Sigma-Aldrich, St. Louis, MO) per the manufacturer's specifications. Total RNA was reverse transcribed to cDNA via a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD). The quantity of cDNA was assessed via

spectrophotometery (NanoDrop, Wilmington, DE).

Drd1 specific primers (forward: ttctcctttcgcatcctcac, reverse: tgtcgaaacctgatgacagc) were designed in Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA) and manufactured (Integrated DNA Technologies, San Diego, CA) to flank an intron region. Real Time PCR experiments were completed using an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Carlsbad, CA). The *Drd1* mRNA expression levels were assessed relative to β-actin. The β-actin primers (forward: agacttcgagcaggagatgg, reverse: aaggaaggctggaaaagagc) were designed and manufactured similar to the *Drd1* primers. Samples were analyzed in triplicate and the two nearest data points per sample were operationally defined as the true crosspoint value for that sample and were used in a modified version of the Pfaffl (98) method to calculate relative expression levels of *Drd1* mRNA. The efficiency values (*E*) were determined, as previously described (98), from the slope of a log-linear dilution curve (input concentrations=1:1, 1:10, 1:100, 1:1000) and the expression ratios calculated using the following equation:

$$\text{Expression Ratio} = \frac{(E_{\text{ref}})^{\text{CP}_{\text{ref}}}}{(E_{\text{target}})^{\text{CP}_{\text{target}}}}$$

where E=efficiency of the PCR reaction for the target and reference genes, and CP=the crosspoint thresholds of the target and reference genes.

Blood Sampling and ELISA Analyses: The current project was completed in conjunction with two other related projects. Blood was sampled from all mice in these projects and pooled based on the experimental condition at the time of blood sampling. For this project, blood samples were acquired prior to the gonadectomy surgery, 14 days after the surgery, and at the end of the study. Blood samples were collected in microvette

capillary tubes from the saphenous veins in conscious mice or were collected directly from the inferior vena cava at the end of the experiment. The sera/plasma was separated from the red cell mass via centrifugation. All samples were stored at −80°C until steroid quantification via enzyme-linked immunosorbent assays (ELISA) could be performed. Three samples from each experimental and control group were assayed for testosterone and 17β-estradiol (IBL-America, Inc., Minneapolis, MN) across the three time points. A steroid extraction procedure (ethyl acetate extraction) was used prior to running each assay. Optical absorbance was measured in duplicate for each sample and calibrator solution. The concentrations of unknown values were predicted based on the calibrators' optical absorbance and known concentration. The duplicate measures were averaged to represent the actual concentration value from each sample.

Statistical Analysis: Three (control, ORCH1, ORCH2) by two (post-surgery and post-replacement) analysis of variance (ANOVA) tests were used to compare differences in wheel running activity and *Drd1* mRNA expression. Tukey's HSD *post-hoc* tests were used to assess individual differences when a main effect or an interaction was present. An *a priori* alpha level of 0.05 was considered significant.

Results

The results for wheel running are shown in Figure 17. All three wheel running indices changed in response to the experimental treatments applied to this cohort of mice (p=0.0001). Wheel running distance, duration, and speed were lower in the orchidectomized mice than in the placebo animals. Testosterone administration recovered the orchidectomy-induced wheel running deficit in all indices. Distance and duration remained significantly lower with the D1-like antagonist than the levels

observed in both the placebo and testosterone treated animals; however, speed was increased in this group to the levels observed in the placebo animals immediately following sham surgical orchidectomies. The observed speed increase in the D1-like antagonized mice, however, remained lower than the speed levels observed in the testosterone treated animals and the control animals during the interventional stage of the experiment.

The relative mRNA expression of the dopamine 1 receptor in the nucleus acumbens and striatum was not statistically different (p=0.83) between groups at the end of the study (Figure 18). The average relative *Drd1* mRNA expression levels (% of β-actin) for placebo (mean=0.80, SD=0.29), testosterone (mean=0.72, SD=0.38), and D1-antagonist (mean=0.72, SD=0.34) were homogenously distributed across the entire sample population despite the significant differences in wheel running activity. Furthermore, the wheel running indices did not correlate (distance: r=0.042, duration: r=0.077, speed: r=-0.102) with relative *Drd1* mRNA expression levels.

Testosterone and 17β-estradiol concentrations are listed in Table 6. Statistically the groups did not exhibit significantly different concentrations for either steroid; however, the steroid concentration amongst the control and experimental groups were physiologically distinct.

Discussion

Castration or orchidectomy has been shown to be a robust inhibitor of wheel running vigor in the male rodents (20, 34, 35, 54-56, 99, 106). A similar effect was observed in the current study in which male C57BL/6J mice ran very little after orchidectomy surgery compared to reproductively intact control animals. Sex steroid

administration in various forms has been shown to reverse the gonadectomy-induced wheel running deficit in both male and female rodents (20, 43, 54, 55, 60, 99, 141, 144). In the current study, testosterone administration via silastic implants resulted is recovery rates—equal to the levels of control mice—which is similar to the levels reported by others for estrogens (43) and testosterone (99). The D1-like antagonist failed to increase wheel running to the level observed in controls. *Drd1* mRNA levels were similar between the experimental and control groups despite the noted differences in activity levels, thus the hypothesized interaction between testosterone and dopaminergic activity resulting in higher levels of activity was unfounded.

Sex Steroids, Dopamine and Activity: The modulation of steroid levels via surgical procedures and replacement strategies is speculated to affect morphology and function in specific parts of the brain (81). Several authors have investigated these potential alterations through direct and indirect manipulation of neurological circuits in various areas of the brain. Consequently, most of the direct manipulation studies have been completed in female rats and have utilized various estrogens (26, 53, 66, 68). The current project is novel in that it evaluated the effects of a primarily androgenic sex steroid noting large increases in wheel running after testosterone administration without changes to *Drd1* mRNA in the nucleus acumbens and striatum. This observation suggests that no interactions between *Drd1* and the sex steroids were necessary to manipulate the activity response in male mice; however, several past studies suggest that *Drd1* function and/or quantity are vitally important in the regulation of activity levels in rodents. Our findings suggest that the sex steroids and dopamine system modulate physical activity through separate physiological pathways. This interpretation is

complicated by the fact that in our mice the aromatase complex was fully functional; therefore, we can assume that some estrogen was present after conversion by the aromatase complex. However, we have shown that presence of a functioning aromatase complex does not affect activity; therefore, the presence of estrogens should not confound our interpretation.

Rhodes et al. (103) evaluated several neurological modulating compounds in mice artificially selected for high wheel running. In particular, blockade of *Drd1* via SCH23390—the D1-like antagonist used in the present study—resulted in reduced wheel running in control lines and high activity selected lines; however, the magnitude of the decrease was much less in the high activity mice. The authors concluded that D1-like receptors were expressed much less in the high activity line, and thus, showed a lower sensitivity and response to the antagonist (103). Supporting Rhodes et al.'s (103) suggestion of dopaminergic involvement, Knab et al. (70) evaluated mRNA expression levels of several dopamine genes in low (C3H/HeJ) and high (C57L/J) activity inbred mice and found that *Drd1*and tyrosine hydroxylase mRNA were expressed at lower levels in the high active mice (70).

Recently, dopamine and serotonin levels were evaluated in a mouse model of activity-based anorexia during food-anticipatory activity and feeding (139). The levels of both neurotransmitters were elevated during feeding but remained low during the food anticipatory behavior portion of the study—when the mice were most active. It is noteworthy that the associative relationship regarding general dopaminergic functionality noted by Knab et al. (70) was similarly repeated in the data of Verhagen et al. (139) in the nucleus acumbens. The current project, on the contrary, did not demonstrate this

relationship after reintroducing testosterone.

Sex Steroids and Central Control of Activity: The brain is highly permeable and responsive to changes in sex steroid levels (94). Furthermore, changes to the anterior hypothalamus, medial pre-optic area, nucleus acumbens, and striatum have been shown to associate with activity levels in various kinds/strains of mice (53, 66, 68, 70, 103, 139). Taken in concert, the sex steroids are realistic intermediaries between physiological function and phenotypic expression of activity levels in rodents. The current project demonstrated high levels of activity in steroid treated mice that did not associate with *Drd1* mRNA expression levels in the nucleus acumbens and striatum. At a minimum, this suggests that *Drd1* transcription in the nucleus acumbens and striatum is unaffected by changes in sex steroid levels and were not involved in regulating activity levels in the current cohort of mice.

It has been suggested that biologically regulated activity levels are most likely centrally controlled (113); therefore, though the dopamine 1 receptors in the nucleus acumbens and striatum appeared uninvolved in regulating activity levels in the current project, other receptors and brain regions may be of greater consequence in considering the role of sex steroids in physical activity. The hypothalamus and medial pre-optic area have been the targets of several studies in which portions of the brain have been directly exposed to sex steroids—estrogens in particular. Lesions to the ventrolateral aspect of the hypothalamus resulted in low activity levels that were unresponsive to estrogen administration (66, 68). Hitt and Gerall (53) noted a higher propensity for rats to be active during the estrogen peak if the hypothalamus and pre-optic areas were unencumbered. The function of these brain areas were not investigated in the current

cohort of C57BL/6J mice and are potential future targets of investigations into activity regulation.

Study Limitations: The current project evaluated activity levels in D1-like antagonized mice with orchidectomy-induced physical inactivity and though the D1-like antagonists have been shown to decrease activity levels in some mice (103), the current cohort of mice appeared unaffected by the drug. The numerous differences in the aforementioned literature—the current project included—indicate that the effect of the dopaminergic system on activity is far from consistent. Given the dissimilarities, interpretation and mechanism identification is likely strain specific and requires extreme caution.

The injection protocol used in the current project was developed based on past literature (103); however, several other studies have utilized cannula systems or electrodes to apply compounds or stimuli directly to specific brain regions. These types of systems have primarily been used to directly administer sex steroids to the hypothalamus (53, 66, 68). Application of these direct manipulation systems to the nucleus acumbens or striatum may induce different results than we observed by avoiding issues related to inadequate drug delivery to the brain or premature drug clearance via the liver or kidneys.

The results of this study are limited to male mice, and thus, extension of the current data to female populations are uncertain. Though untested in the current project, the genetic and biological mechanism relating to central regulation of physical activity could have sex specificity. In fact, physical activity displays a significant sexual dimorphism in all three wheel running indices: distance, duration, and speed (52, 82).

However, we have observed that in spite of differing baseline activity levels, both sexes responded in a similar manner to supplements of testosterone and estrogen suggesting that the sexual dimorphism may effect baseline activity values and not the amount of alteration in activity induced by supplements.

Conclusions: The present study evaluated the effects testosterone and a D1-like antagonist (SCH23390) on physical activity patterns in male orchidectomized C57BL/6J mice. Wheel running indices were low in the orchidectomized animals, but were increased with administration of testosterone. Blocking the *Drd1* receptors did not affect physical activity levels in orchidectomized mice and the *Drd1* mRNA levels in control, testosterone-treated, and D1-like antagonized animals were not different. Therefore, our data do not support an interaction between the sex steroids and *Drd1* levels in activity regulation in C57BL/6J male mice. Future research will focus on evaluating the sex steroids' effects on other brain regions and neurotransmitter systems in the context of physical activity regulation.

Acknowledgments:

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Footnotes:

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Journal Submission: At the time of publication of this dissertation, the preceding chapter was in preparation for review in the journal: *Endocrinology*

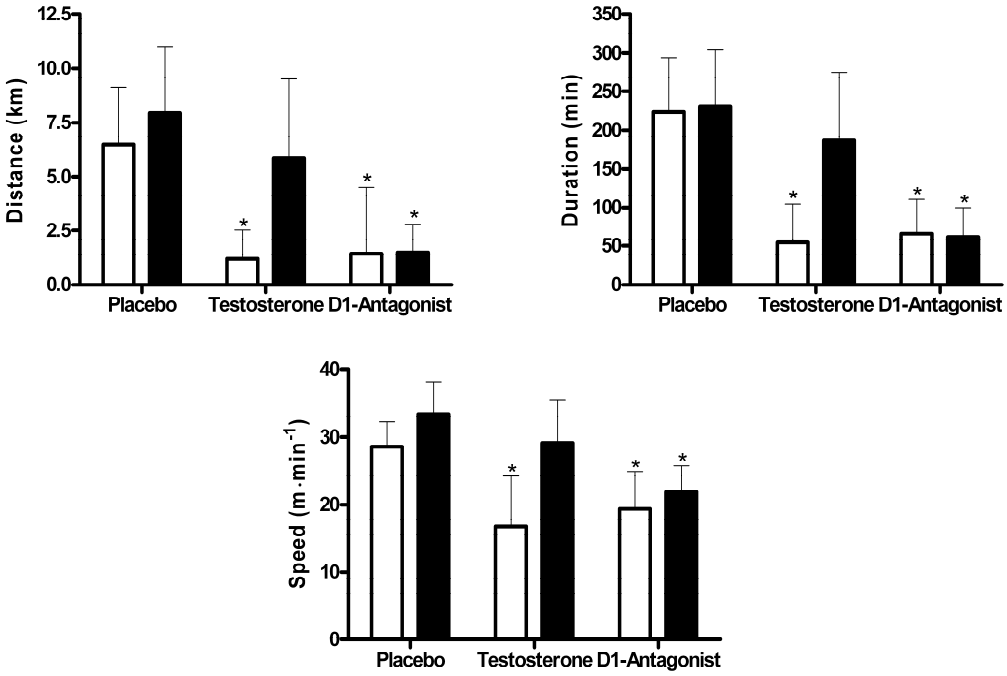

Figure 17. Wheel running indices for C57BL/6J mice after sham or surgical orchidectomy and administration of control implants, testosterone implants, or D1-like antagonist (daily injections). White bars=daily average wheel running after surgery; black bars=daily average wheel running with interventional strategies. *=significantly different from placebo mice and testosterone treated orchidectomized mice.

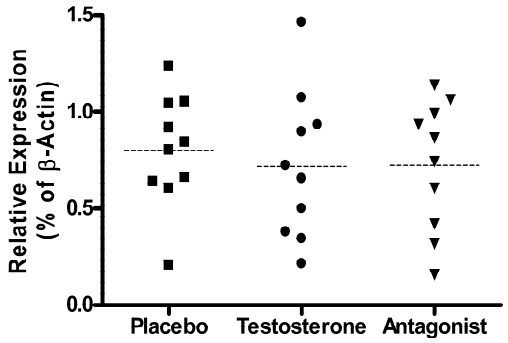

Figure 18. End of experiment relative mRNA expression levels for dopamine 1 receptors in the nucleus acumbens and striatum of placebo, testosterone, and D1-like antagonist treated male C57BL/6J mice.

Table 6. Sera/Plasma testosterone and 17β-estradiol concentrations for mice* at baseline and under various placebo and experiment conditions

Condition (n=T/E_2)	Testosterone ($ng \cdot ml^{-1}$) mean±SD	17β-Estradiol ($pg \cdot ml^{-1}$) mean±SD
Baseline (n=3/3)	6.32±2.78	59.82±16.72
Placebo (n=15/15)	11.53±7.54	397.08±881.50
	Experimental	
ORCH (n=3/2)	min	109.79±29.71
ORCH+SCH23390 (n=2/1)	4.85±7.20	291.09
ORCH+T (n=3/2)	15.37±1.21	151.96±53.20

*Sera/Plasma from 3 mice were pooled

Abbreviations: ORCH=Orchidectomy, T=Testosterone Implant, min=at the minimum of prediction curve

SUMMARY

Physical activity levels inversely correlate with risks for obesity, certain cancers, diabetes, hypertension, and other hypokinetic diseases. The understanding of how activity levels are regulated, thus, remains an important health related goal. The sex steroids—given their ability to alter activity levels in rodents—are potent biological regulators of physical activity levels.

The projects described in this dissertation evaluated the effects the sex steroids have on physical activity regulation. Physical activity levels in rodents were measured via running wheels under various sex steroid modulated physiological conditions. I observed that the removal of endogenous sex steroid producing tissues resulted in greatly depressed wheel running in both male and female mice. Replacement of testosterone or 17β-estradiol reversed the gonadectomy induced wheel running deficit in both sexes, with testosterone replacement recovering pre-surgery activity levels in all mice.

It has been suggested that sex steroid alterations to wheel running occur through estrogenic pathways. However, I found that inhibition of the aromatase complex—the enzyme involved in primary production of estrogens via testosterone in male mice—did not result is significant changes to wheel running. These results indicate that an "estrogen-only" dogma of sex steroid activity regulation is likely inaccurate. Our results strongly suggest that androgenic pathways regulating activity exist and strongly influence physical activity levels in rodents.

Current literature suggests that an organism's general level of physical activity may be regulated by the nucleus acumbens in the brain, and in particular, by dopamine 1 receptors. Other literature has implicated a complex interaction between sex steroids and

the dopamine system, thus suggesting that this interaction may be altered with changes in sex steroid levels. When I reintroduced testosterone to orchidectomized mice, they recovered wheel running to normal levels, but they did not show altered dopamine 1 receptor mRNA in the nucleus acumbens or striatum. Furthermore, administration of a D1-like antagonist to orchidectomized mice did not reinvigorate activity suggesting that testosterone's abilities to alter activity are not propagated through this receptor.

In this set of dissertation studies, I have provided evidence that the sex steroids regulate activity levels in mice through independent androgenic and estrogenic pathways. The androgenic affects do not appear to occur through the dopamine 1 receptor in the nucleus acumbens and striatum. In conclusion, the sex steroids are potential targets for therapeutic strategies aimed to enhance population wide activity patterns leading to overall health benefits, reduced utilization of the health care system, and improved quality of life.

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