Effects of Aromatase Inhibition on the Physical Activity Levels of Male Mice

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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 physical activity levels in humans and rodents with an extensive early literature regarding sex steroid effects on physical activity. 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 under various sex steroid-disrupted and aromatase-inhibited conditions. Inhibition of the aromatase complex was achieved through administration of two different aromatase inhibiting substances—letrozole and exemestane. Wheel running was unaffected by aromatase inhibition in reproductively intact and sex steroid supplemented mice. Orchidectomy significantly reduced wheel running activity. Steroid replacement recovered wheel running to pre-surgical levels; however, aromatase inhibition did not further affect wheel running levels. The recovery of wheel running in mice with androgen supplementation and the further 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.

Keywords: Sex Steroid; Sex Hormone; Locomotion; Exemestane; Letrozole

Introduction

Physical inactivity affects public health and unnecessarily burdens the 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 [1]. Although evidence exists suggesting that activity levels are determined by extrinsic and environmental factors, a growing number of scientific studies suggest large genetic and biological influences also exist [2-8]. Androgens and estrogens have been the focus of extensive research relating to activity levels in rodents. Notably, the surgical removal of testes or ovaries results in noted reductions in daily wheel running activity [9-13].

Currently, it is suggested that testosterone requires conversion to an estrogenic compound before any modulatory interactions to the wheel running response will occur. Roy and Wade [14] 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. [15] found that wheel running activity was hindered in an estrogen-deficient aromatase knockout mouse model. Conversely, Hill et al. [16], 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 [17], 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, are warranted for further elucidation of the roles of the sex steroids in physical activity regulation. With a pharmacological approach, the functionality of particular physiological pathways can be altered without hindering normal development. Therefore, 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.

Materials and Methods

Eighty male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in three experimental procedures. All mice were housed in an environmentally controlled animal husbandry facility under a 12/12 h light/dark cycle 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 experimental protocols. 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 allowing *ad libitum* access to both food and water. Activity data collection began when the mice were 63 days of age. Physical activity levels are near a maximum and demonstrate low levels of variation

across the ages utilized in this experiment [18] minimizing the effects of age on wheel running indices. 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.

Running wheels (450 mm circumference; Ware Manufacturing, Phoenix, AZ) with a 40 mm wide solid 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·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. 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.). The physical activity indices measured in this project have previously been shown to exhibit a high level of repeatability in our hands [19].

During this study, control and experimental injections of aromatase inhibitors were administered. Control injections consisted of 0.3% hydroxypropyl cellulose in phosphate buffered saline (HPC+PBS) and were administered in a 500 µl subcutaneous bolus over a two minute period to ensure full delivery and absorption of the solution. Experimental injections contained the irreversible aromatase inhibitor exemestane (Sigma-Aldrich, St. Louis, MO) suspended in HPC+PBS. Exemestane aggressively inhibits aromatase activity in a wide array of tissues including the brain and adipose tissue [20-21]. The drug was administered subcutaneously at a dosage of 250 mg·kg^{-1} per 500 µl bolus. This dosing schedule and administration technique has previously been shown to yield maximum inhibition of aromatase activity [22-24]. 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 pulverized and added to an aliquot of sterile HPC+PBS to form a dispensable suspension for injection.

Exemestane has both aromatase-inhibiting and androgenic properties. It was speculated that any observed exemestane effect 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 the exemestane phase of the project. 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 using; the dosing schedule and administration techniques used for letrozole injections followed well established methods [25]. Placebo injections consisted of 0.3% HPC+PBS.

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. Our silastic implant technique has a long established and validated record of supplementation/replacement and has previously been shown to modulate steroid levels in rodents [26-33]. Thus, testosterone and 17β-estradiol (Sigma-Aldrich, St. Louis, MO) were packed into 10 mm lengths of silastic tubing (Dow Corning, Midland, MI) with an internal diameter of 1.02 mm, external diameter of 2.16 mm, and wall thickness of 0.56 mm. The ends of the tubing were capped with clear silicone glue. 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/back between the skin and the muscle fascia. A two day recovery after the silastic implant surgery 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 preemptive administration of the analgesic carprofen (5 mg·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 recovery without access to running wheels.

Experiment One (Figure 1): Twenty 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 wheel running activity. Next, each mouse received either placebo or exemestane injections; wheel running was monitored for an additional seven days. Lastly, mice were allowed three days of unmonitored wheel running followed by a final seven days to assess wheel running during drug clearance.

To verify the results of experiment one, twenty untreated C57BL/6J mice were used in a confirmatory study with methodological techniques 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. Upon confirmation, further experiments were conducted utilizing exemestane because the androgenic effects of the drug were considered negligible.

Experiment Two (Figure 1): 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 randomly divided into control (n=9), experimental A (n=10), and experimental B (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 A and B groups) or placebo (control) injections. In the final seven days of this experiment, the mice received silastic implants containing testosterone (experimental A group) or 17β-estradiol (experimental B group). 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.

Experiment Three (Figure 1): 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
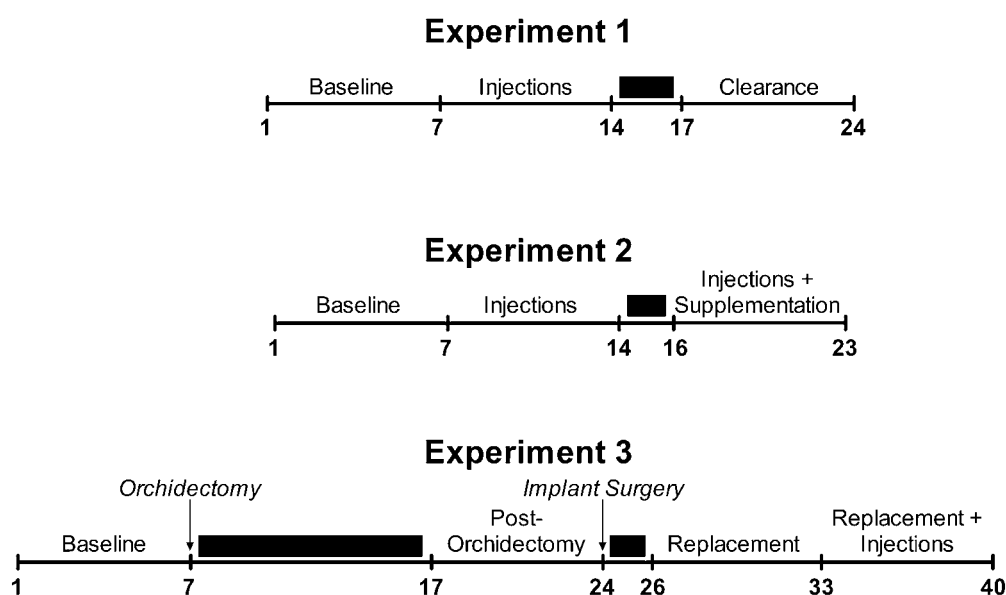
Figure 1: The experimental timelines (days) for assessing physical activity levels in aromatase inhibited and sex steroid modified mice. Black bars represent recovery periods—after surgeries and after cessation of injections—in which running wheels were not in the cages.

after removal of the gonads. The experimental groupings were the same as experiment two. Baseline data was collected at the onset of the experiment over a seven day period. 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. Silastic implant surgeries were performed followed by two days of recovery. Seven more days of wheel running was assessed following the recovery period. Testosterone, 17β-estradiol, and blank implants were again utilized during this period. A final seven days of wheel running was assessed while placebo or exemestane injections were administered every 24 hours. The silastic capsules remained in place.

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 alpha level was set *a priori* at 0.05.

Results

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 2a-c. Blocking aromatase did not inhibit activity and did not significantly alter any of the wheel running indices (distance: $p=0.61$, duration: $p=0.38$, or speed: $p=0.69$) at the administered dosage (250 mg·kg^{-1}).

The wheel running response under a 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 (distance: $p=0.24$, duration: $p=0.11$, or speed: $p=0.34$). The data for wheel running distance are depicted in Figure 2d; trends for the duration and speed indices were similarly non-significant.

Running distance, duration, and speed for experiment two are shown in Figure 3. 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: $p=0.56$) with neither testosterone nor 17β-estradiol altering the running response.

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 4. 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 (Figure 4). 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. There was no significant alteration to activity with administration of exemestane in orchidectomized mice receiving either steroid.

Discussion

Our results demonstrate that activity remains unaffected by the administration of aromatase inhibitors at a dosage of 250 mg·kg^{-1}. The results of this study call into question the limited data that has generated the hypothesis of a primary estrogen-derived activity regulating mechanism. These data add additional impetus for further investigation into the effect of sex-steroids on physical activity in both rodent and human populations, with a needed subsequent focus on pathway identification and molecular mechanisms. Additional studies that focus on the effects the aromatase complex has on activity level regulation is warranted. The current project represents a seminal *modus operandi* in this new avenue of hormonal and steroidal research.

A physiologically normal C57BL/6J mouse runs vigorously when exposed to a running wheel (see Figures 2-4; baseline running data). Alterations to circulating sex steroid concentrations interrupt this normal running pattern [34-37], an effect postulated to occur through the modulation of estrogen levels via the aromatase complex; an assertion primarily based on limited previous research [14]. However, 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 or maintained at normal levels in the reproductively intact rodent. While contradictory to previous speculations, other than Roy and Wade's [14] data, little direct evidence exists that indicates activity regulation—via the sex steroids—requires the presence of estrogenic compounds.

Roy and Wade [14] assessed the ability of testosterone propionate (an aromatizable androgen) and dihydrotestosterone propionate (a non-aromatizable androgen) to affect activity levels in castrated male rats. Administration of the aromatizable androgen increased activity to the levels observed in estrogen treated animals, but the non-aromatizable androgen had little effect on activity level [14]. Roy and Wade [14] noted only a partial (~ 45%) recovery of wheel running with testosterone administration (~4000 revolutions) compared to estradiol benzoate administration (~9000 revolutions). With testosterone administration, we observed that wheel running not only returned to the levels observed at baseline, but also was notably elevated above the levels induced by 17β-estradiol administration (Figure 4). The difference in testosterone-driven activity between our and Roy and Wade's study may be accounted for by the modes of steroid delivery used. The steroidal compounds were delivered via silastic implants in the current project, but Roy and Wade [14] delivered the steroids via daily injections in sesame oil. The injection methods employed by Roy and Wade [14] required daily contact with the animals, which has been

shown to induce higher levels of stress with resultant decreases in wheel running activity [38]. However, the effects of human interaction on wheel running behavior observed by Richter [38] were not as dramatic as the differences noted between our results and Roy and Wade's [14]. Therefore, though this certainly may explain some of the differences between our study and Roy and Wade's [14], it is likely that other mechanisms also exist that account for this discrepancy.

Watai et al. [15] evaluated activity in an aromatase (*Cyp19*) knockout mouse model and observed very low activity levels in these mice. Replacement of 17β-estradiol elevated activity in male knockout mice suggesting a requirement for estrogens to be present in order for activity levels to match those observed in normal animals [15]. The use of gene knockout models are not without adversity as developmental differences in mice can result in an obscured representation of reality [17] as well as potential elimination of neighboring regulatory genomic regions in the knockouts [39]. Thus, the results of Watai et al. [15] may be due to such unintended effects and therefore the result of abnormal physiological phenomenon rather than relevant deviations in the mechanisms affecting activity levels.

Conversely, Hill et al. [16] evaluated wheel running activity in estrogen deficient male mice, similar to the mice utilized by Watai et al. [15] and observed compulsory wheel running activity that exceeded the levels observed in wild type controls suggesting that estrogen was inhibiting activity in this model. Interestingly, this effect was not observed in female *Cyp19* (aromatase) knockout animals. In addition, compulsory wheel running was ameliorated after the administration of 17β-estradiol in the male, but not female knockout mice [16]. The non-essential need for a functional aromatase complex to maintain normal activity levels observed in the present study and by Hill et al. [16] is contrary to the observations of Roy and Wade [14] and Watai et al. [15]. The technical difference noted in these studies may account
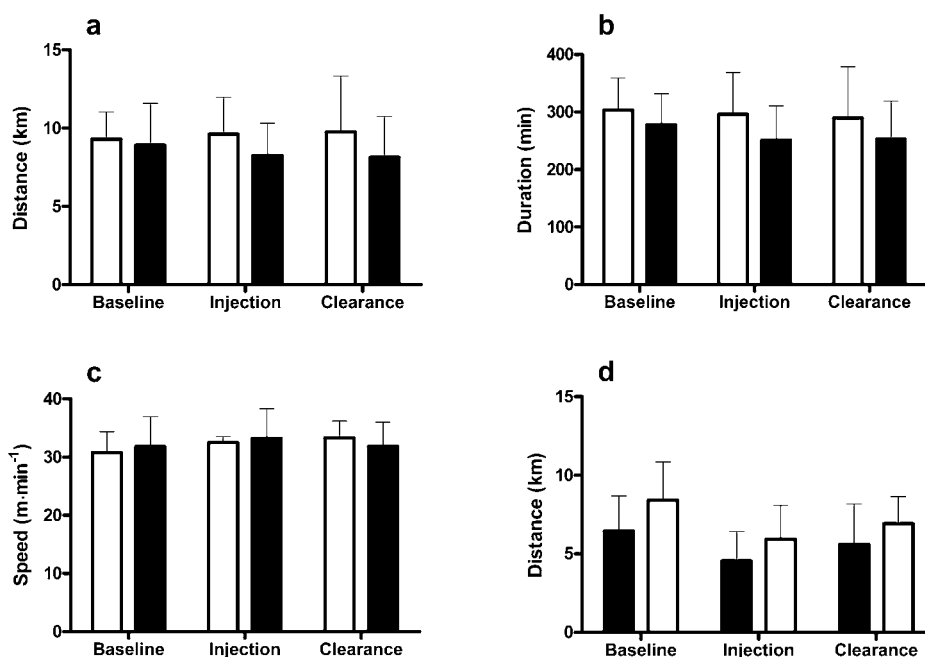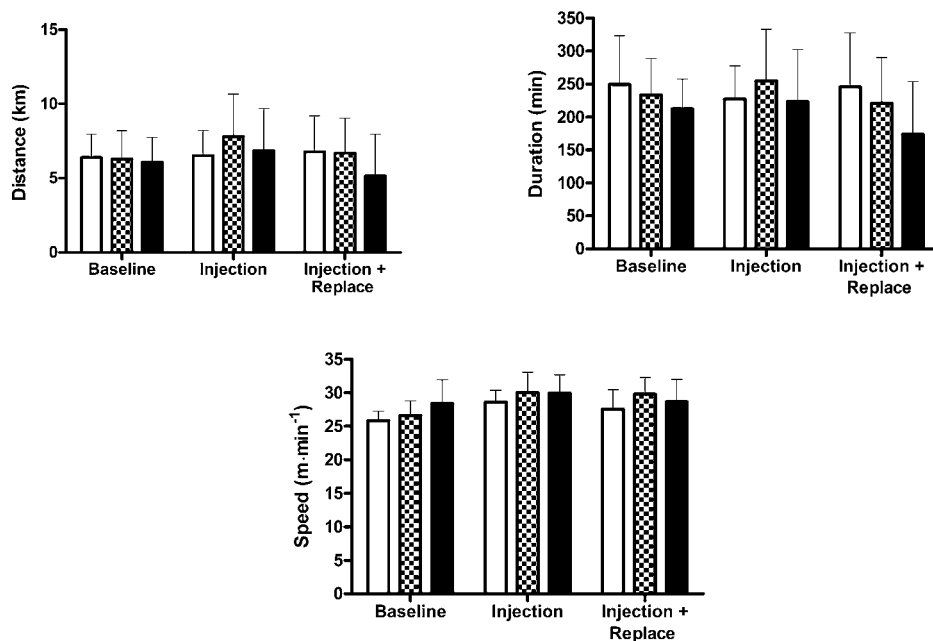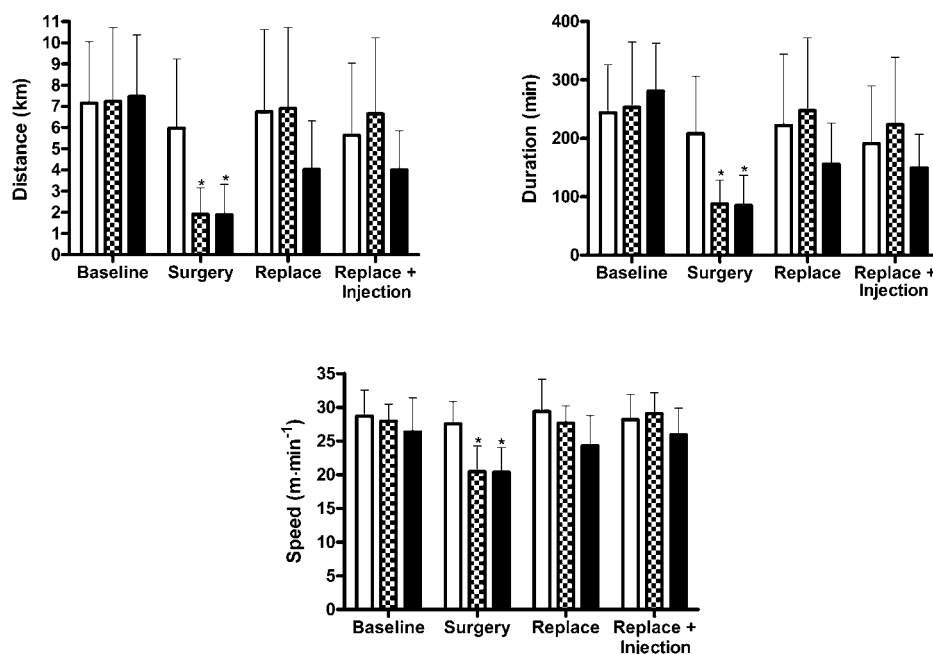

Figure 2: Wheel running indices for male C57BL/6J mice at baseline, during injections, and during post-injection clearance. White bars denote control mice (n=10) that received vehicle injections and black bars denote experimentally treated mice (n=10) that received exemestane (panels a-c; distance, duration, speed) or letrozole (panel d; distance) injections.

Figure 3: 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=9) that received vehicle injections and empty 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 4: 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 empty 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.

for some of the observational disparities; however, it is obvious that testosterone may have a stronger effect on physical activity than previously understood thus justifying further study detailing the underlying estrogenic and androgenic mechanisms.

The potential identity of the androgenic steroid mechanisms modulating physical activity remains unclear. Two primary lines of evidence from the current project support the 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 male mice. Second, testosterone replacement in orchidectomized male mice increased wheel running activity back to baseline levels, while 17β-estradiol increased wheel running to only 50% of baseline levels. Our data represent the second study to suggest the existence of a direct androgenic mechanism in non-genetically modified mice. Flynn et al. [40] exposed mice to the anti-androgenic fungicide vinclozolin at gestational age seven in dam's milk and continued exposure via food after weaning. With exposure to vinclozolin and the resultant androgenic-inhibition, 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 [40] 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. [40] partially supports our hypothesis regarding the existence of an androgenic physical-activity regulating mechanism.

While several authors [41-45] have suggested that the high malleability of the activity response in animals treated with estrogens is a centrally-located (i.e. brain) function, the permeability of the rodent brain to androgens is also high [46] suggesting a potential pathway through which testosterone affects physical activity. To date, only one paper [47] has evaluated brain morphology in aromatase compromised mice and noted lower levels of dopaminergic neurons in the medial preoptic area and arcuate nucleus of male knockout mice. This response would tentatively explain the noted increased activity levels of the aromatase knockout mice based on recent work from Knab et al. [7] that suggested an increased activity level was due to down-regulated dopamine 1 receptor levels. It is hypothesized by Knab et al. [7] that dopamine receptor reward signaling is decreased due to the lower number of receptor containing neurons and to compensate, mice run more, thus initiating a reward signal on a more frequent basis through the reduced number of receptor containing neurons. Therefore, we speculate that testosterone influences activity levels in male mice through interactions with the androgen receptor leading to alteration of central dopamine functioning.

The injection and sampling techniques employed in this project required close contact with the mice several times throughout the study. Interactions between animal handlers and small rodents have been shown to induce a stress response in small rodents [38] which could artificially alter sex steroid levels and wheel running patterns. Steps were taken to minimize stress in this study for humane purposes and to limit the potential to induce aberrant sex steroid concentrations and unnatural running patterns. During all procedures, 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 contact from initial immobilization to release back into the home cage was less than three minutes. The effectiveness of drug administration to reduce aromatase activity in the study's mouse population was assured through use of routine and previously described techniques. As stated before, the dosage and technique used in this project are a common methodology to reduce aromatase activity in rodents [22-24]. The only major concern was a potential bias induced by multiple handlers during injection events. To circumvent these issues, an individual technician prepared and performed delivery of both pharmaceutical agents using the described simple, effective methods. This well-trained technician is a highly proficient animal handler.

Exemestane is an irreversible aromatase inhibiting drug. 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 [48]. The dosage used in this project was previously shown to have physiological effects on bone characteristics, tumor morphology, and estrogen production. These effects 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. Using letrozole, similar results were observed as with exemestane 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.

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, supplemented, or orchidectomized animals. These data, in conjunction with wheel running measures in aromatase knockout mice [16] and vinclozolin treated mice [40], suggests that aromatization of testosterone is not an absolute requirement for activity regulation as has been earlier postulated [14] and that a direct androgenic mechanism regulates activity levels in mice. This proposed androgenic mechanism is likely an additional effect supplementing and/or offsetting the effects of the estrogenic compounds. 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 could profoundly influence human health worldwide.

Acknowledgments

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