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Agonistic behavior in males and females: Effects of an estrogen receptor beta agonist in gonadectomized and gonadally intact mice

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Summary Affiliative and agonistic social interactions are mediated by gonadal hormones. Research with estrogen receptor alpha (ERα) or beta (ERβ) knockout (KO) mice show that long-term inactivation of ERα decreases, while inactivation of ERβ increases, male aggression. Opposite effects were found in female αERKO and βERKO mice. The role of acute activation of ERα or ERβ in the agonistic responses of adult non-KO mice is unknown. We report here the effects of the ERβ selective agonist WAY-200070 on agonistic and social behavior in gonadally intact and gonadectomized (gonadex) male and female CD-1 mice towards a gonadex, same-sex intruder. All 15 min resident—intruder tests were videotaped for comprehensive behavioral analysis. Separate analyses assessed: (1) effects of WAY-200070 on each sex and gonadal condition; (2) differences between sexes, and between gonadally intact and gonadex mice, in untreated animals. Results show that in gonadally intact male and female mice, WAY-200070 increased agonistic behaviors such as pushing down the intruder and aggressive grooming, while leaving attacks unaffected. In untreated mice, males attacked more than females, and gonadex animals showed less agonistic behavior than same-sex, gonadally intact mice. Overall, our detailed behavioral analysis suggested that in gonadally intact male and female mice, ERβ mediates patterns of agonistic behavior that are not directly involved in attacks. This suggests that specific aspects of aggressive behavior are acutely mediated by ERβ in adult mice. Our results also showed that, in resident—intruder tests, female mice spend as much time in intrasexual agonistic interactions as males, but use agonistic behaviors that involve extremely low levels of direct attacks. This non-attack aggression in females is increased by acute activation of ERβ. Thus, acute activation of ERβ similarly mediates agonistic behavior in adult male and female CD-1 mice.

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1. Introduction

Aggression, and its underlying neurobiological mechanisms, have long been a topic of interest and a target of research (e.g., Howard, 1920; Craig, 1921). Aggression research with laboratory rodents has typically focused on males' behavior, usually employing the resident–intruder test (e.g., Thor and Flannelly, 1976; Brain et al., 1981; Simon and Whalen, 1986), where an intruder is introduced into the home cage of another, usually isolated, animal (the resident). Measures of fighting or overt aggression such as latency to, or frequency of, attack, are taken (e.g., Thor and Flannelly, 1976; Simon and Whalen, 1986; Parmigiani et al., 1999; Morè, 2008), sometimes together with other measures of "agonistic" behavior, which include both the aggression and the reaction to it (Olivier et al., 1989, 1991; Alleva, 1993; Pietropaolo et al., 2004; Branchi et al., 2006). In mice, this paradigm takes advantage of the fact that males will often establish and maintain an exclusive territory or dominate a shared territory under natural conditions (Miczek et al., 2001, 2007; see Latham and Mason, 2004 for a review). Male laboratory mice of various inbred (e.g., C57BL/6J, C-3H agoutis, BALB/C albinos) and outbred (e.g., Swiss CD-1, Swiss-Webster, CF-1, Tuck TO, unspecified albino) strains, as well as wild-descended mice, consistently attack male intruders, especially following isolation or housing with a female, although wild-descended and outbred Swiss CD-1 males show greater aggression than some inbred strains (e.g., Uhrich, 1938; Ginsburg and Allee, 1942; Scott and Fredericson, 1951; Edwards, 1969; Peters et al., 1972; Brain and Bowden, 1979; Parmigiani et al., 1999).

There has been much less research conducted on aggression or agonistic behavior in female than male mice. Most of this research has focused on circumstances related to reproduction, such as following housing with a male or when protecting their litter, in so-called maternal or postpartum aggression (e.g., Haney et al., 1989; Haney and Miczek, 1989; Palanza et al., 1995, 2005). Studies with outbred Swiss albino mice show that this type of female aggression is qualitatively different from the typical intrasexual aggression studied in males, as it is less ritualized and more violent (Svare and Gandelman, 1973; Al-Maliki et al., 1980). Non-reproduction-related female aggression, while less frequently studied than males, also exists. Virgin or non-reproductively active outbred albino females will fight during group formation, following isolation, or if the intruder is a lactating female (Uhrich, 1938; Brain and Haug, 1992; Morè, 2008), although virgin females of some inbred strains (e.g., C57BL/6J, 129) only rarely attack same-sex intruders (Ogawa et al., 1998a, 2004).

Higher levels of intrasexual aggression are observed in wild or wild-descended females than in both inbred and outbred laboratory mice, suggesting that interfemale aggression has been largely eliminated from these domesticated mice (Scott, 1966; Parmigiani et al., 1999; Palanza et al., 2005). Alternatively, these domesticated females may use other agonistic behaviors than attacks, such as chasing, pinning, or aggressively grooming the intruder (Grant and Mackintosh, 1963; Alleva, 1993; Clipperton et al., 2008) that are often not assessed in tests for aggression. These behaviors would not lead to the violent exclusion of the intruder from the territory, but rather would be aimed at establishing dominance over the intruder. The study of female agonistic interactions in tests of aggression therefore requires the use of a comprehensive analysis, often referred to as an "ethological" analysis, based on the mouse's full behavioral repertoire (the ethogram; Grant and Mackintosh, 1963) and that involves more than just measures of attacks (Mos and Olivier, 1989; Olivier et al., 1989; Miczek et al., 2001; Pietropaolo et al., 2004; Branchi et al., 2006).

Steroid hormones are known to mediate social and aggressive interactions in mice. It has long been known that castration eliminates intermale aggression in wild as well as inbred and outbred laboratory mice (e.g., Uhrich, 1938; Beeman, 1947; Luttge, 1972; Brain and Bowden, 1979; Simon and Whalen, 1986). The administration of the prototypical male gonadal hormone, testosterone (T), will restore aggression in these castrated males (Beeman, 1947; Tollman and King, 1956; Luttge, 1972; Brain and Bowden, 1979; Simon and Whalen, 1986).

Testosterone can affect behavior either directly, or indirectly through one of its two main metabolites: the androgen dihydrotestosterone (DHT) and the estrogen estradiol. In castrated males of some strains (e.g., Swiss-Webster, CD-1, Tuck TO; Luttge, 1972; Luttge and Hall, 1973; Brain and Poole, 1976; Brain and Bowden, 1979; Simon and Whalen, 1986), DHT restored intermale aggression near pre-castration levels, while in other strains these effects of DHT were not found (e.g., CFW, CF-1; Simon and Whalen, 1986; Cologer-Clifford et al., 1999).

Estrogens were also shown to mediate aggression. Estradiol administration reinstated aggression in castrated male mice of a number of strains (CF-1, CFW, Tuck TO, Swiss-Webster; Edwards and Burge, 1971; Brain and Poole, 1976; Bowden and Brain, 1978; Brain and Bowden, 1979; Simon and Whalen, 1986). This effect was weaker in castrated male CD-1 mice (Finney and Erpino, 1976; Simon and Whalen, 1986). Similarly, anti-estrogen treatment reduced aggression in castrated CD-1 or Tuck TO males given T replacement (Luttge, 1979; Clark and Nowell, 1979). Furthermore, male mice in which the gene for aromatase has been inactivated, who therefore cannot convert T to estradiol, show an estradiol-reversible reduction in aggression (Toda et al., 2001). Thus both androgens and estrogens have been shown to be involved in the mediation of aggressive behavior in a number of strains of mice.

Work with C57BL/6J and 129 inbred mice and Swiss black mice in which either of the two intranuclear estrogen receptors (ER), ERα or ERβ, had been made non-functional (so-called "knockout," KO, mice) suggests that the two ERs are differently involved in the mediation of aggression (Ogawa et al., 1998a,b, 1999; Clipperton et al., 2008; Choleris et al., 2008). Male αERKO mice showed reduced aggression towards an olfactory bulbectomized (OBX) or intact male intruder. In addition, αERKO males did not show T-enhanced aggressive responses (Ogawa et al., 1998b) and displayed female-directed aggression (Scordalakes and Rissman, 2003). Unlike αERKO males, male βERKO mice showed increased aggression in puberty and early adulthood towards OBX male intruders, and had normal estrogen-induced aggression (Nomura et al., 2002, 2006). In male mice, therefore, activation of ERα appears to increase aggression, while ERβ seems to decrease it in a manner that is dependent upon the experience and age of the animal. Both effects on aggression may be related to

the impairment of the αERKO and βERKO mice in social recognition (Choleris et al., 2003, 2006, 2009).

There have been few studies on female aggression using ER-specific KO mice. In one study, virgin αERKO females showed increased aggression towards ovariectomized (ovx), hormone-primed same-sex intruders, but not toward OBX males, and this aggression was reduced by estrogen replacement following ovariectomy (Ogawa et al., 1998a). Gonadally intact female βERKO mice showed no aggression towards ovx female intruders, and exhibited reduced aggression following T administration (Ogawa et al., 1999, 2004). Chronic KO of the genes for ERα and ERβ thus have the opposite effects in males and females, suggesting that intrasexual aggression may be differentially mediated by estrogens in the two sexes. However, these studies with KO mice do not allow dissection of developmental from activational effects, nor do they allow the contributions of flanking genes or compensatory mechanisms to be ruled out (Waddington et al., 2005; Choleris et al., 2008).

Single acute administration of the ERβ agonist 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY-200070; Malamas et al., 2004) to adult ovx female CD-1 mice reduced dominance scores and increased submissive behavior during their interactions with a familiar, ovx same-sex cagemate (Clipperton et al., 2008). This suggests that the acute activation of ERβ in adult mice affects aggressive behavior. In the present study, we used the resident–intruder test to investigate the effects of acute administration of WAY-200070 on the responses of gonadally intact and gonadectomized (gonadex) male and female mice to unfamiliar, gonadex same-sex intruders. We combined the use of a standardized test for aggression with a comprehensive behavioral analysis, to assess the effects of WAY-200070 on different types of agonistic behavior.

2. Methods

2.1. Animal housing

Adult CD-1 mice (*Mus musculus*) were obtained from Charles River, QC, Canada. Mice used as intruders were housed in same-sex group cages of four to six (46 cm × 26 cm × 21 cm). Mice used as residents were individually housed for 2 weeks in order to develop a home cage territory in clear polyethylene cages (26 cm × 16 cm × 12 cm). All cages were provided with corn cob bedding, environmental enrichment (plastic containers and paper nesting material), and food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, Madison, WI) and tap water *ad libitum*. The mice were held in the Central Animal Facility at the University of Guelph, under a 12:12-h reversed light/dark cycle (lights on at 2000 h) at a temperature of 21 ± 1 °C.

2.2. Experimental subjects

Subjects were 132 female and 152 male CD-1 mice. Seventy-seven of the female mice were ovariectomized (ovx), and 95 of the male mice were castrated, both at 2–3 months of age by the supplier, 1 or 2 days before being shipped to the University of Guelph, and at least 2 weeks before the experiment. The vaginal cytology of the ovx mice confirmed that they were not cycling.

Since both T and estrogens have been implicated in aggression, 28 ovx and 40 castrated males were randomly selected to be intruders. The use of group-housed, same-sex, gonadex mice results in more "standardized" intruders with uniformly low levels of circulating sex hormones. In males, this avoids possible confounding effects due to the fact that T both produces and elicits attacks (Mugford and Nowell, 1970; Luttge, 1972; Mugford, 1974; Simon and Whalen, 1986). The use of ovx female intruders eliminates the estrous cycle dependent variability that has been shown to affect aggression in outbred, wild-descended mice (Hyde and Sawyer, 1977). Furthermore, as both outbred and inbred gonadex mice typically initiate little aggression themselves (e.g., Beeman, 1947; Gray et al., 1978), using them as intruders enabled us to assess the level of aggression initiated by the resident mouse, while preventing the encounter from escalating to the point at which testing would have to be interrupted. This allowed us to perform a full behavioral analysis over the entire duration of the test in all sexes and gonadal conditions. Intruders were reused no more than four times, at a minimum interval of 3 days between tests, and were replaced when they reached 4 months of age.

The remaining 104 female and 112 male mice were used as residents. No cage changes took place for at least 3 days prior to testing to allow the residents to establish a territory in their home cages. No resident experimental mouse was reused, and each resident was assigned to an independent treatment group, as outlined in Table 1. Intact female residents were at mixed stages of the estrous cycle. This research was conducted in accordance with the regulations of the Canadian Council on Animal Care and was approved by the Institutional Animal Care and Use Committee of the University of Guelph.

2.3. Apparatus

Social interactions between residents and intruders took place in the resident's home cage during the dark phase of

Table 1 Distribution of treatment groups. Numbers indicate mice remaining in each group after the removal of outliers (number in parentheses).

Treatment	Gonadally intact males	Gonadally intact females	Castrated males	Ovariectomized females
Uninjected	*n* = 9 (0)	*n* = 10 (3)	*n* = 12 (0)	*n* = 10 (1)
Sesame oil vehicle	*n* = 13 (0)	*n* = 11 (1)	*n* = 10 (0)	*n* = 8 (2)
30 mg/kg WAY-200070	*n* = 11 (1)	*n* = 12 (0)	*n* = 13 (0)	*n* = 10 (0)
90 mg/kg WAY-200070	*n* = 10 (1)	*n* = 10 (0)	*n* = 12 (0)	*n* = 10 (1)
180 mg/kg WAY-200070	*n* = 12 (0)	*n* = 12 (0)	*n* = 8 (2)	*n* = 11 (1)

the light/dark cycle. Clear Plexiglas lids were used to allow videotaping from above with an 8 mm Sony Handycam (in Nightshot) for subsequent behavioral analysis of the interactions.

2.4. Drug

The selective estrogen receptor β agonist 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY-200070), which is 78 times more selective for ERβ than ERα in mice (compound 92 in Malamas et al., 2004; Harris, 2007), was obtained courtesy of Wyeth Laboratories, Inc., Madison, NJ, and was suspended in sesame oil.

For each of the four conditions (gonadally intact males, gonadally intact females, castrated males, and ovx females), five independent groups of resident mice were formed: a sesame oil vehicle control (OIL) and three drug-treated groups, each receiving one of three doses of WAY-200070, 30 mg/kg (LOW), 90 mg/kg (MID), or 180 mg/kg (HIGH). In addition, in order to assess potential effects of sesame oil on behavior (Anton et al., 1974; Curtis and Wang, 2005; Colafranceschi et al., 2007), an uninjected group was included for each gonadal condition. This also allowed us to compare gonadex and gonadally intact mice in untreated animals. The distribution of doses in each sex and gonadal condition is outlined in Table 1. Treatment was administered via a single intraperitoneal (IP) injection, at a volume of 10 ml/kg, approximately 72 h prior to the experiment. Each treatment group consisted of a unique set of resident mice, and each resident mouse was tested only once. This pattern of administration of the drug allows for the assessment of the acute, rather than chronic, behavioral effects of steroid hormones (Priest et al., 1997; Mickley and Dluzen, 2004; Walf and Frye, 2005; Jasnow et al., 2007; Choleris et al., 2009) and parallels the administration procedure that showed effects of this drug on social learning and agonistic behaviors between familiar ovx females (Clipperton et al., 2008). IP administration of compounds dissolved in sesame oil has been used in a number of other studies (e.g., Valentovic et al., 1994; Eroschenko et al., 1997; Rankin et al., 1997; Mahieu et al., 2004; Curtis and Wang, 2005; Hoyk et al., 2006; Chu et al., 2006; Löfgren et al., 2008; Clipperton et al., 2008; Cragg, Fissore, Pfaff, and Choleris, unpublished observations) and was chosen to ensure the mice were receiving the proper dosage, since in preliminary investigations we observed excessive leakage of the oily substance following subcutaneous injections.

The specific doses of WAY-200070 and the time of administration were chosen on the basis of information obtained directly from the provider. Lower doses of WAY-200070 do not cause nuclear translocation of ERβ receptors and do not seem to be biologically active (Hughes et al., 2008). Consistently, in previous research both single and repeated administration of lower doses had no effects on either social (Choleris et al., 2009; Cragg, Fissore, Pfaff, and Choleris, unpublished observations) or non-social behavior (Hughes et al., 2008). The acute IP administration of the doses used in this study, however, did affect social learning, social interactions (Clipperton et al., 2008; Choleris et al., 2009) and social recognition (Choleris et al., 2009; Cragg, Fissore, Pfaff, and Choleris, unpublished observations). These effects were different from those of a highly specific ERα agonist (Clipperton et al., 2008; Choleris et al., 2009; Cragg, Fissore, Pfaff, and Choleris, unpublished observations). These behavioral results, together with the finding that high doses of WAY-200070 do not affect ERα-rich tissue (Malamas et al., 2004; Mewshaw et al., 2005), indicate ERβ-specific effects of WAY-200070 at these doses.

2.5. Procedure

Three days before testing, the resident mice of each of the four conditions (gonadally intact males, gonadally intact females, castrated males, and ovx females) were randomly assigned to one of the five groups and administered treatment (uninjected control, sesame oil vehicle, 30 mg/kg WAY-200070, 90 mg/kg WAY-200070, or 180 mg/kg WAY-200070). To assist in identification, the intruder mice were colored with black magic marker at least 12 h prior to testing. At this time, all residents and intruders were moved into the testing room and left undisturbed for at least 12 h.

During their active phase, each resident mouse had a same-sex, gonadex intruder placed into his or her home cage. The mice were left undisturbed to freely interact for 15 min while being videotaped.

The videotaped social interactions were scored by two trained observers, who each watched half of each treatment group, were unaware of the animals' treatment, and had an interobserver reliability correlation of 0.92. The interactions were analyzed for 21 behaviors based on the ethogram by Grant and Mackintosh (1963; see Table 2, modified from Clipperton et al., 2008, for behavior descriptions) using The Observer Video Analysis software (Noldus Information Technology, Wageningen, the Netherlands). The behavioral analysis focused on the treated mouse, the resident. The behavior of the intruder was collected only in relation to the behavior of the resident (i.e., attacks received, reciprocal attacks, aggressive postures, social inactivity), and in the reciprocal pairs of behaviors (dominant/submissive behavior and chasing/avoidance of the intruder). Dominant behavior on the part of one mouse was typically met by submissive behavior on the part of the other.

Ten categories of individual behaviors were formed (see Table 3, modified from Clipperton et al., 2008) in order to assess potential non-specific effects of the drug on the total activity and total social and non-social explorations of the mice, as well as on the overall levels of agonistic behavior displayed during the interaction. These categories are meant to provide an overall view of the animals' behavior, and thus allow comparison to non-ethological types of analysis (e.g., Cushing et al., 2008; Walf et al., 2008; Sanchis-Segura et al., 2000). However, with this paper we wish to emphasize the importance of looking at the component behaviors of these categories, as this will determine whether the effects observed are due to specific individual behaviors or a general effect across a category. This is particularly true in the case of aggression, as an increase in total levels of agonistic behavior may be due to effects on types of aggression-related behavior that have potentially different functional implications, such as overt attacks for the establishment of territories and/or behaviors aimed at establishing a dominance hierarchy within a shared territory. The direction of the agonistic behavior was defined whenever possible, and a "dominance score" was also calculated for each pair of mice by subtracting the amount of agonistic behavior received

Table 2 Description of scored behaviors.

Social behaviors	
Chasing the intruder	The resident mouse actively follows, or pursues and chases the intruder; reciprocal to avoid.
Dominant behaviors	The resident mouse is in control; includes pinning of the intruder, aggressive grooming, crawling over or on top, and mounting attempts.
Attacks delivered	Physical attacks, including dorsal/ventral bites. Only the frequency of attacks was measured.
Aggressive postures	Physical attacks which include box/wrestle, offensive and defensive postures, lateral sideways threats and tail rattle.
Reciprocal attacks	Physical attacks with a locked fight including tumbling, kick-away and counterattack where the attacker cannot be identified.
Avoidance of the intruder	The resident withdraws and runs away from the intruder while the intruder is chasing.
Submissive behaviors	The intruder mouse is in control; includes crawl under, supine posture (ventral side exposed), prolonged crouch, and any other behavior in which the intruder is dominant (e.g., the intruder pins, aggressively grooms, etc., the resident).
Attacks received	Physical attacks including bites to dorsal/ventral regions. Only the frequency of attacks was measured.
Defensive upright posturing	Species-typical defensive behavior; upright with the head tucked and the arms ready to push away.
Social inactivity	Includes sit/lie/sleep together.
Oronasal investigation	Active sniffing of the intruder's oronasal area.
Body investigation	Active sniffing of the intruder's body.
Anogenital investigation	Active sniffing of the intruder's anogenital region.
Stretched approaches	Risk assessment behavior; back feet do not move and front feet approach the intruder. Only the frequency of stretched approaches was measured.
Approaching and/or attending to the intruder	Often from across the cage; the resident's attention is focused on the intruder, head tilted toward the intruder and movements toward the intruder; this becomes "chasing the intruder" once along the tail or sniff if resident is within 1.5 cm of the intruder.
Non-social behaviors	
Horizontal exploration	Movement around the cage; includes active sniffing of air and ground.
Vertical exploration	Movement to investigate upwards, both front feet off the ground; includes sniffing, wall leans and lid chews (less than 3).
Digging	Rapid stereotypical movement of forepaws in the bedding.
Abnormal stereotypies	"Strange" behaviors, including spinturns, repeated jumps/lid chews/head shakes (more than 3).
Solitary inactivity	No movement; includes sit, lie down and sleep.
Self-grooming	Rapid movement of forepaws over facial area and along body.

from the amount of agonistic behavior delivered (as described in Table 3 and modified from Clipperton et al., 2008). The dominance score thus took into account the reciprocity of "dominant" and submissive behavior involved in agonistic interactions.

To determine the phase of the estrous cycle in intact females, and to confirm that the ovx mice were not cycling, vaginal smears were taken from all females immediately following testing. These slides were stained with Giemsa stain (Sigma–Aldrich, Oakville, ON) and examined under a microscope using 100× magnification. Proestrus was defined as consisting of predominantly nucleated cells, estrus of primarily non-nucleated, cornified cells, and diestrus of chiefly leukocytes.

2.6. Statistical analysis

The 15 min social interaction period was divided into three 5 min intervals in order to assess the time course of the behaviors. The frequency (FREQ), duration (DUR), and latency (LAT) of each behavior were measured. If a behavior was not performed, a latency of 900 s was assigned. For numerous behaviors, Levene's test for equality of variance revealed that there were significant differences between group variances (Levene statistics between 10.39 and 2.63, p values from less than 0.001–0.045), therefore violating the assumption of equality of variance necessary for parametric statistics. Results were thus analyzed using non-parametric statistics, which do not require assumptions to be made about the underlying population. Specifically, we used the non-parametric equivalent of analysis of variance, the Kruskal–Wallis test, followed by the non-parametric Mann–Whitney U test for binary comparisons. When the results of the analyses on DUR and FREQ were in agreement, only those of DUR are reported; thus, if not specified, results are DUR. Only the main results are reported in the text. All other results, including the time course data, are in the online Supplemental Material. The data from 14 mice that

Table 3 Descriptions of grouped and composite behaviors.

Total activity	All behaviors involving activity, both social and non-social. Excluded from this group are inactive alone, inactive together, and self-groom.
Total social behaviors	Follow intruder, dominant behaviors, attacks delivered, aggressive postures, reciprocal attacks, avoid intruder, submissive behaviors, attacks received, defensive upright posturing, inactive together, oronasal investigation, body investigation, anogenital investigation, stretched approaches, and attend to/approach intruder.
Total agonistic behaviors	Follow intruder, dominant behaviors, attacks delivered, avoid intruder, submissive behaviors, attacks received, defensive upright posturing, aggressive postures, and reciprocal attacks. This composite behavior represents the overall levels of agonism present in the resident–intruder interactions and does not indicate the direction of the agonistic behavior (i.e., whether agonistic behavior is directed toward the resident or toward the intruder).
Agonistic behaviors delivered	Follow intruder, dominant behaviors, and attacks delivered.
Agonistic behaviors received	Avoid intruder, submissive behaviors, attacks received, and defensive upright posturing.
Dominance score	Total agonistic behavior delivered minus total agonistic behavior received. A negative score indicates that the resident was the submissive animal in the pair, while a positive score signifies that the resident was the dominant animal.
Social investigation	Oronasal investigation, body investigation, anogenital investigation, stretched approaches, and attend to/approach intruder.
Non-social behaviors	Horizontal exploration, vertical exploration, dig, stereotypies, inactive alone, and self-groom.
Non-social locomotor behaviors	Horizontal exploration, vertical exploration, and dig.
Non-social non-locomotor behaviors	Inactive and self-groom.

were two standard deviations from the mean on four or more behaviors were removed; these mice were distributed across conditions and treatment groups (see Table 1).

Two sets of analyses were performed to assess (1) the effects of drug treatment on each mouse condition separately, and (2) differences between sexes and between intact and gonadex same-sex mice in untreated animals. First, for each condition (gonadex or intact male or female mice), the effects of the drug are reported in comparison to the sesame oil control group. Effects of sesame oil *per se* are reported in comparison to the uninjected group. The variable of interest in this analysis was the treatment (sesame oil, 30 mg/kg WAY-200070, 90 mg/kg WAY-200070 and 180 mg/kg WAY-200070). For the gonadally intact females, a second variable, the phase of the estrous cycle (proestrus, estrus and diestrus), was included.

The second set of analyses was performed to assess sex differences and to compare gonadex and gonadally intact mice in the uninjected control groups. Variables were sex (gonadally intact male and gonadally intact female) and gonadal condition (gonadally intact, gonadex; analyzed separately for each sex). In the gonadally intact females, effects of the estrous cycle (proestrus, estrus and diestrus) were also analyzed.

In all of the analyses in which it was assessed, the phase of the estrous cycle did not have significant effects, neither as a main factor nor in interaction with drug treatments, probably because of the low number of mice in each group. Consequently, the phase of the estrous cycle was removed from the models, and results of the analyses of the data obtained from gonadally intact females without this variable are reported below. All analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

For each experimental group (gonadally intact or gonadex males and females) we report the main results observed below. Additional statistically significant minor results are reported in detail in the online Supplemental Material.

3.1. Effects of WAY-200070 in gonadally intact males

Overall, in gonadally intact males WAY-200070 specifically increased agonistic behavior other than overt attacks and reduced anxiety-like behavior without affecting non-agonistic social behavior or non-social activity.

Dominant behaviors were increased by HIGH ($U = 42.00$, $z = -1.96$, $p = 0.050$; see Fig. 1a). Stretched approaches were decreased by HIGH ($U = 39.00$, $z = -2.15$, $p = 0.039$; see Fig. 1b). OIL also had some effects, e.g., increasing aggressive postures ($U = 25.00$, $z = -2.27$, $p = 0.023$) and decreasing the FREQ of agonistic behaviors received ($U = 28.00$, $z = -2.04$, $p = 0.041$). Please see Supplemental Material, Table 1 for additional results.

3.2. Effects of WAY-200070 in gonadally intact females

Overall, in gonadally intact females, WAY-200070 increased both agonistic and non-agonistic social behaviors as well as non-social activity.

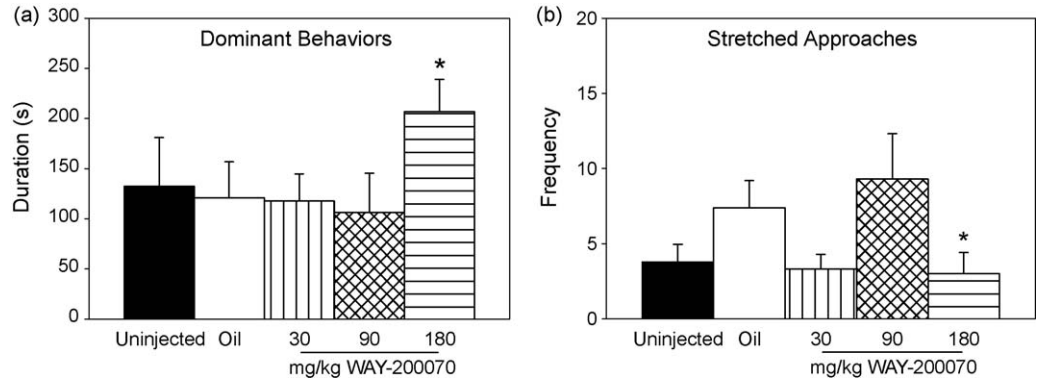

Figure 1 WAY-200070 effects on gonadally intact males. (a) Duration of dominant behaviors. (b) Frequency of stretched approaches. (*) Indicates a significant difference from the sesame oil control group, $p < 0.05$.

There was an effect of treatment on total activity ($\chi^2(4) = 11.72$, $p = 0.020$), both the DUR ($U = 32.00$, $z = -2.09$, $p = 0.036$; see Fig. 2c) and FREQ ($U = 10.00$, $z = -3.45$, $p = 0.001$) of which were increased by HIGH. This was predominantly due to effects of treatment on the frequencies of total social behaviors ($\chi^2(4) = 24.72$, $p < 0.001$; $U = 8.00$, $z = -3.57$, $p < 0.001$; see Fig. 2d) and social investigation ($\chi^2(4) = 20.54$, $p < 0.001$; $U = 6.00$, $z = -3.69$, $p < 0.001$; see Fig. 2e), which

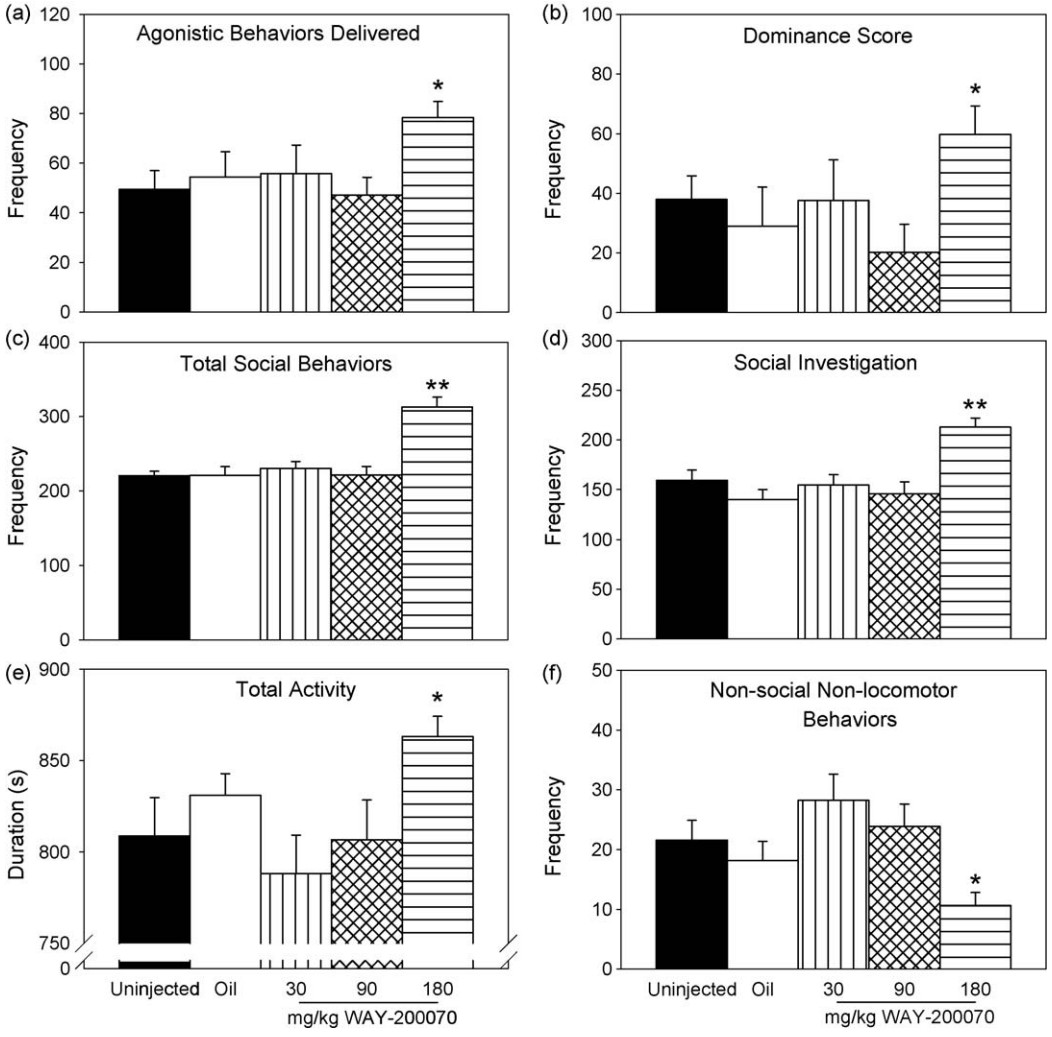

Figure 2 Effects of WAY-200070 on gonadally intact females. (a) Frequency of agonistic behaviors delivered. (b) Frequency of dominance score. (c) Frequency of total social behaviors. (d) Frequency of social investigation. (e) Duration of total activity. (f) Frequency of non-social non-locomotor behaviors. (*) Indicates a significant difference from the sesame oil control group, $p < 0.05$. (**) Indicates a significant difference from the sesame oil control group, $p < 0.01$.

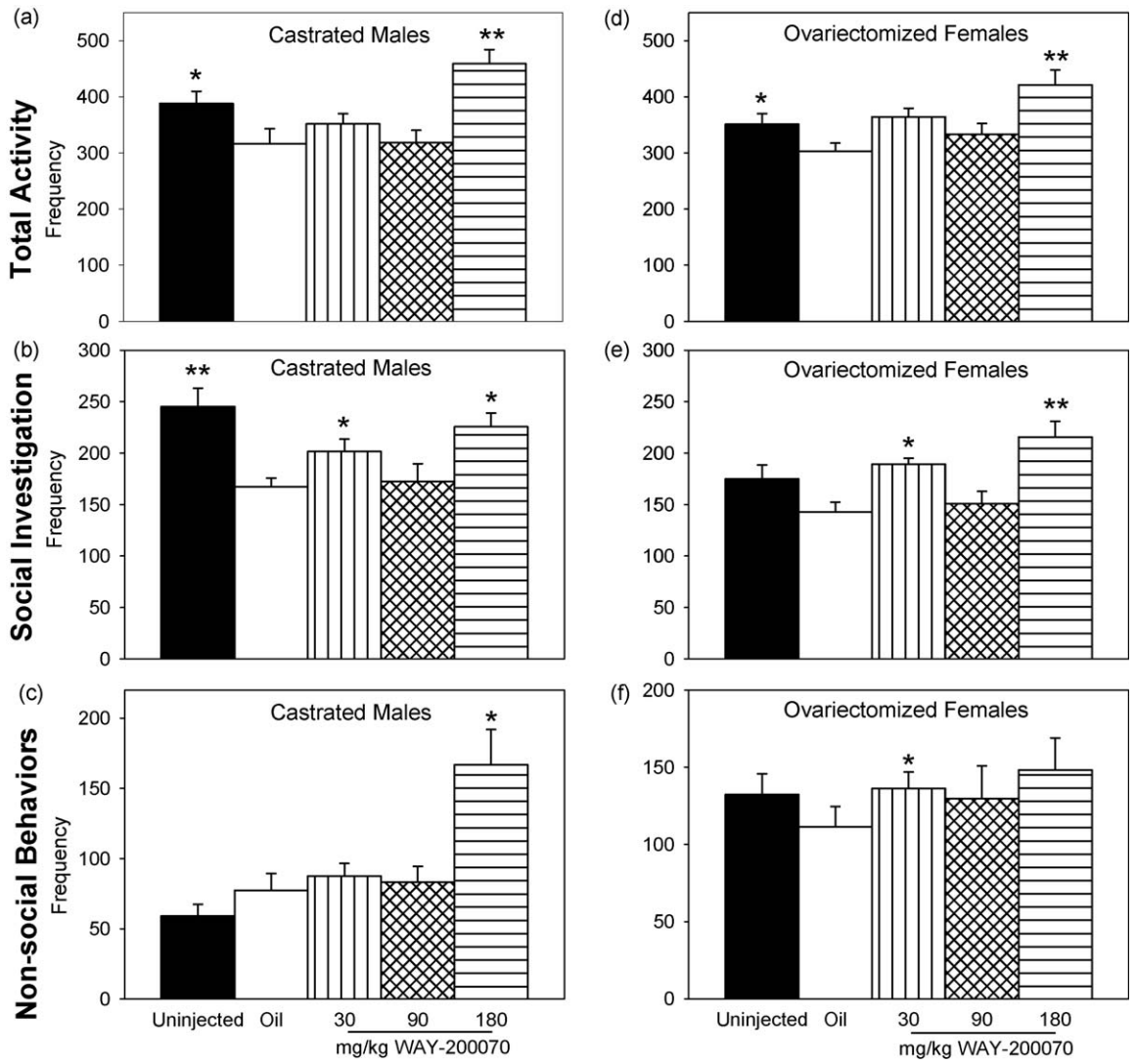

Figure 3 WAY-200070 effects on gonadectomized male and female mice. (a) Frequency of total activity in castrated males. (b) Frequency of social investigation in castrated males. (c) Frequency of non-social behaviors in castrated males. (d) Frequency of total activity in ovx females. (e) Frequency of social investigation in ovx females. (f) Frequency of non-social behaviors in ovx females. (*) Indicates a significant difference from the sesame oil control group, $p < 0.05$. (**) Indicates a significant difference from the sesame oil control group, $p < 0.01$.

were increased by HIGH. There was a treatment effect on the FREQ of agonistic behaviors delivered ($\chi^2(4) = 11.24$, $p = 0.024$) and the FREQ of both this category ($U = 28.50$, $z = -2.31$, $p = 0.021$; see Fig. 2a) and the dominance score were increased by HIGH ($U = 33.50$, $z = -2.00$, $p = 0.045$; see Fig. 2b). There was a treatment effect on the FREQ of non-social non-locomotor behaviors ($\chi^2(4) = 15.88$, $p = 0.003$), which were decreased by HIGH ($U = 30.00$, $z = -2.22$, $p = 0.026$; see Fig. 2f), and this dose also increased the FREQ of the non-social locomotor behavior digging ($U = 34.00$, $z = -2.08$, $p = 0.038$). OIL also affected a number of behaviors, including increasing total social behaviors ($U = 16.00$, $z = -2.75$, $p = 0.006$) and decreasing digging ($U = 23.00$, $z = -2.35$, $p = 0.019$). Please see Supplemental Material, Table 2 for additional results.

3.3. Effects of WAY-200070 in castrated males

Overall, WAY-200070 effects on castrated males are similar to those in females and show that non-social as well as social non-agonistic behaviors were increased by treatment, although unlike gonadally intact females, there were few effects on agonistic social behaviors.

The FREQ of total activity ($\chi^2(4) = 14.80$, $p = 0.005$; $U = 8.00$, $z = -2.84$, $p = 0.004$; see Fig. 3a) was affected by treatment and increased by HIGH, as was the FREQ of non-social behaviors ($\chi^2(4) = 12.14$, $p = 0.016$; $U = 13.00$, $z = -2.40$, $p = 0.016$; see Fig. 3c) and several other elements of total activity. The FREQ of social investigation was affected by treatment ($\chi^2(4) = 19.37$, $p = 0.001$; see Fig. 3b), decreased by OIL ($U = 9.00$, $z = -3.36$, $p = 0.001$) and increased by LOW ($U = 33.00$, $z = -1.99$, $p = 0.047$) and HIGH ($U = 33.00$, $z = -1.99$, $p = 0.047$). The DUR of Social Investigation was also affected by treatment ($\chi^2(4) = 12.80$, $p = 0.012$), but was decreased by HIGH ($U = 16.00$, $z = -2.13$, $p = 0.033$). OIL had a number of effects, including decreasing total activity ($U = 21.50$, $z = -2.54$, $p = 0.011$) and the FREQ of social investigation ($U = 9.00$, $z = -3.36$, $p = 0.001$), and increasing total non-social behaviors ($U = 29.00$, $z = -2.04$,

$p = 0.041$). Please see Supplemental Material, Table 3 for additional results.

3.4. Effects of WAY-200070 in ovariectomized females

Overall, WAY-200070 had similar effects on ovx females to those observed in castrated males, with both non-social and social non-agonistic behaviors being increased.

The FREQ of total activity was affected by treatment ($\chi^2(4) = 11.14$, $p = 0.025$; see Fig. 3d), with OIL decreasing ($U = 16.00$, $z = -2.13$, $p = 0.033$), and the LOW ($U = 13.50$, $z = -2.36$, $p = 0.018$) and HIGH ($U = 12.00$, $z = -2.64$, $p = 0.008$) doses of the drug increasing this behavior, likely due to increases in social and non-social behaviors. The FREQ of total social behaviors was affected by treatment ($\chi^2(4) = 11.24$, $p = 0.024$) and increased by HIGH ($U = 10.00$, $z = -2.81$, $p = 0.005$), in part because of similar results in the FREQ of social investigation, which was also affected by treatment ($\chi^2(4) = 13.06$, $p = 0.011$; see Fig. 3e) and increased by LOW ($U = 17.00$, $z = -2.05$, $p = 0.041$) and HIGH ($U = 11.50$, $z = -2.69$, $p = 0.007$). The FREQ of total non-social behaviors was increased by LOW ($U = 16.50$, $z = -2.09$, $p = 0.037$; see Fig. 3f). Additionally, total activity was decreased by OIL ($U = 16.00$, $z = -2.13$, $p = 0.033$). Please see Supplemental Material, Table 4 for additional results.

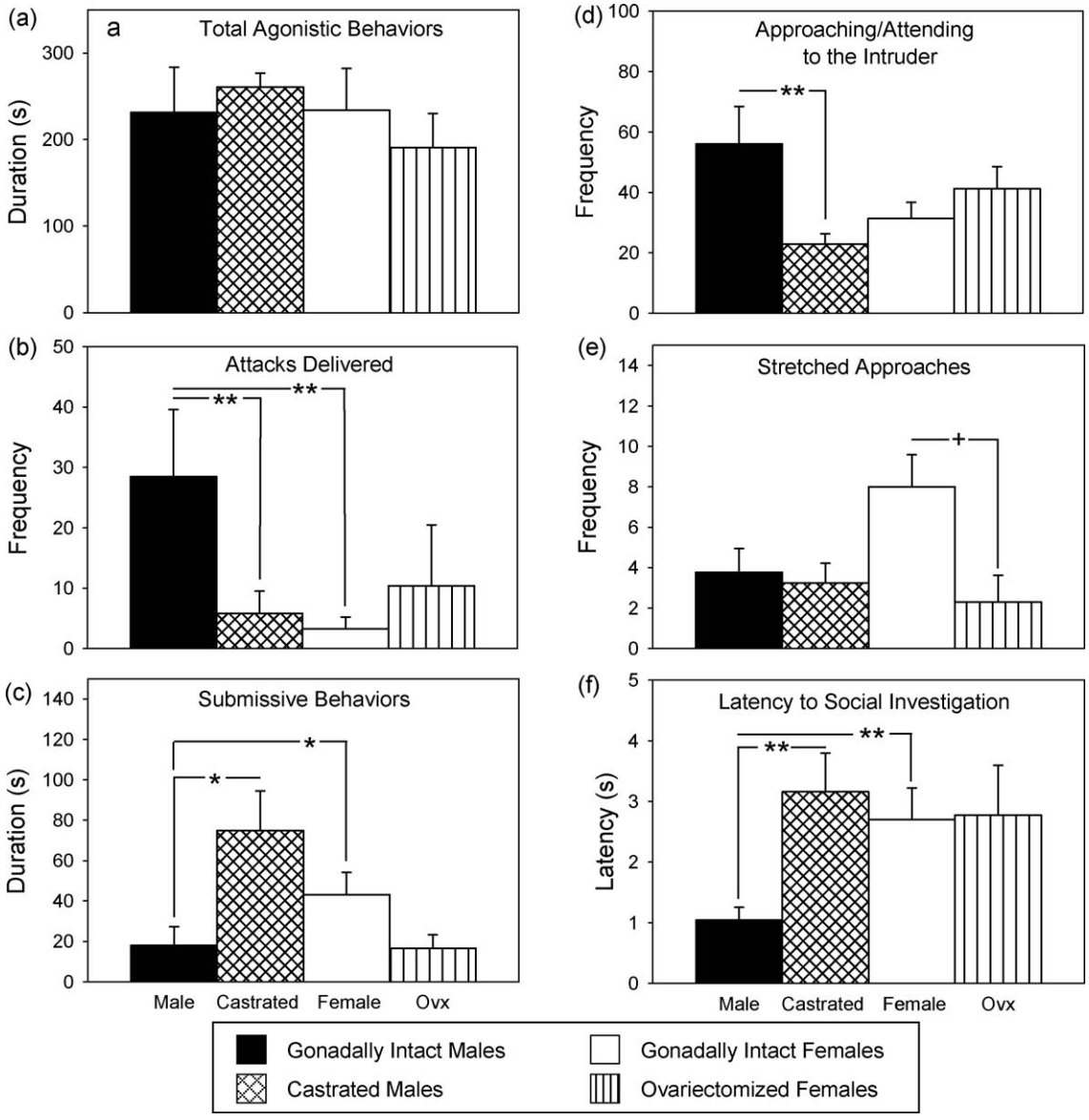

Figure 4 Sex differences and gonadectomy effects on agonistic behaviors. (a) Duration of total agonistic behaviors. (b) Frequency of attacks delivered. (c) Duration of submissive behaviors. (d) Frequency of approaching/attending to the intruder. (e) Frequency of stretched approaches. (f) Latency to social investigation. (*) Indicates a significant difference from gonadally intact males, $p < 0.05$. (**) Indicates a significant difference from gonadally intact males, $p < 0.01$. (+) Indicates a significant difference from gonadally intact females, $p < 0.05$.

3.5. Sex differences in uninjected, gonadally intact mice

Overall, while males and females showed qualitatively different types of agonistic behavior, the two sexes devoted a similar amount of time to agonistic responses to the intruder. In uninjected, gonadally intact CD-1 mice, males showed more reciprocal attacks ($U = 10.00$, $z = -3.30$, $p = 0.001$), and a shorter LAT to reciprocal attacks ($U = 12.00$, $z = -3.56$, $p < 0.001$) and to aggressive postures ($U = 32.50$, $z = -2.05$, $p = 0.041$) than females. Males also performed attacks delivered, which females did not ($U = 10.00$, $z = -2.91$, $p = 0.004$; see Fig. 4b), while females showed more submissive behaviors ($U = 21.00$, $z = -1.96$, $p = 0.0499$; see Fig. 4c). However, despite these differences in the components of the categories, gonadally intact male and female CD-1 mice did not significantly differ in the DUR of total agonistic behaviors (see Fig. 4a) or agonistic behaviors delivered. Additionally, males switched between behaviors more frequently ($U = 16.00$, $z = -2.37$, $p = 0.018$), and females showed a longer LAT to Social Investigation ($U = 13.50$, $z = -2.89$, $p = 0.004$; see Fig. 4f). Please see Supplemental Material, Table 5 for additional results.

3.6. Differences between gonadally intact and castrated uninjected males

Castrated males were more like gonadally intact females than gonadally intact males in some respects. Castrated males performed fewer attacks delivered ($U = 14.00$, $z = -2.89$, $p = 0.004$; see Fig. 4b), less approaching and/or attending to the intruder ($U = 9.00$, $z = -3.20$, $p = 0.001$; see Fig. 4d), and more submissive behaviors ($U = 21.00$, $z = -2.35$, $p = 0.019$; see Fig. 4c) than the gonadally intact males. Castrated males also showed a longer LAT to social investigation ($U = 17.00$, $z = -2.63$, $p = 0.008$; see Fig. 4f) than the gonadally intact males. Please see Supplemental Material, Table 6 for additional results.

3.7. Differences between gonadally intact and ovariectomized uninjected females

Ovx females appeared to be less anxious than gonadally intact females, showing fewer stretched approaches ($U = 21.50$, $z = -2.19$, $p = 0.029$; see Fig. 4e) and more frequent oronasal investigation ($U = 21.00$, $z = -2.20$, $p = 0.028$) than the gonadally intact females. Please see Supplemental Material, Table 7 for additional results.

4. Discussion

By performing an ethological analysis where we looked at a range of behaviors, we were able to obtain a comprehensive assessment of the effects of the ERβ agonist WAY-200070 on agonistic and non-agonistic social and non-social behavior in mouse responses to a gonadectomized (gonadex), same-sex intruder, and the specific behaviors that make up these categories (e.g., the specific type of agonistic behavior performed). It also allowed us to compare the responses of male and female mice in the same testing conditions and to determine that, over a 15 min test, CD-1 females do not spend less time in agonistic interactions with a gonadex, same-sex intruder than males, but rather respond to an opponent using agonistic behaviors other than attacks.

4.1. WAY-200070 effects

The ERβ agonist WAY-200070 affected behavior in the sexes and gonadal conditions differently. WAY-200070 increased intrasexual agonistic behavior in both gonadally intact CD-1 males and females while having little effect on agonistic behavior in gonadex CD-1 mice. In both gonadally intact and ovariectomized (ovx) CD-1 females, WAY-200070 increased overall activity, but this effect was on both some social and some non-social behaviors in gonadally intact females, and specific to non-agonistic behaviors in ovx females. Similarly, WAY-200070 increased several social behaviors in castrated males while specifically increasing agonistic behavior, and no other social behaviors, in gonadally intact males.

Our results showing that acute activation of ERβ increased non-attack agonistic behavior appear to be in contrast to the increased aggression seen in βERKO males of the same age (but a different strain) as the CD-1 mice in the current study (Nomura et al., 2002). This difference suggests that the effects seen in the βERKO males are at least in part developmental, not activational, or that the timing of the ERβ manipulation may affect its mediation of aggression. Alternatively, ERβ may be differently involved in different types of agonistic behaviors, as suggested by the present finding that WAY-200070 had clear enhancing effects on non-attack agonist behaviors while having no effects on direct attacks in gonadally intact CD-1 mice of both sexes. A similar pattern of effects was found in ethopharmacological studies of maternal aggression in rats, in which diazepam affected agonistic behaviors but not biting attacks (Mos and Olivier, 1989), suggesting that attacks and non-attack agonistic behaviors may be differently regulated. It is possible that similar effects on non-attack agonistic behaviors occurred with the βERKO mice, but were simply not recorded. Clearly, more studies conducting comprehensive behavioral analyses are needed before a full understanding of the involvement of ERβ in different aspects of agonistic behaviors can be achieved.

In gonadally intact males, WAY-200070 reduced the number of stretched approaches performed towards the intruder, suggesting an anxiolytic effect (Blanchard et al., 1993; Choleris et al., 2001). This is in agreement with findings that activation of ERβ reduced anxiety-like behaviors in mice on numerous different tasks (see Choleris et al., 2008 for a review). Thus, our WAY-200070-treated gonadally intact males may have been more likely to perform agonistic behaviors because they were less afraid of the intruder. As WAY-200070 did not affect the amount of non-social active behaviors or non-agonistic social behaviors in gonadally intact males, neither the increase in agonistic behavior nor the decrease in anxiety-like behavior can be ascribed to generalized effects on overall activity.

Like the gonadally intact males, intact females treated with WAY-200070 showed increased agonistic behavior and had a higher dominance score, indicating that they delivered more aggression than they received. However, this was accompanied by an increase in overall activity. Although this non-specific effect could account for the increase in agonistic behavior, the fact that there were no increases in received

agonistic behaviors and such active behaviors as avoidance suggests that ERβ may, in fact, increase agonistic behaviors specifically in gonadally intact females. This would be in agreement with previous studies, which found a decrease in interfemale aggression following testosterone administration in gonadally intact female βERKO mice, and increased aggression toward ovx same-sex intruders in gonadally intact αERKO female mice (Ogawa et al., 1998a, 2004). Other ethopharmacological studies on aggression in rats, involving serotonin receptor agonists, also found analogous effects on activity and agonistic behavior, while leaving other behaviors unaffected (e.g., Olivier and Mos, 1992). These results further highlight the advantages of ethological assessments of animal behaviors, as they can help determining the underlying effects on specific behaviors that may drive observed increases or decreases in generalized activity.

The specificity of WAY-200070 effects on non-attack agonistic behaviors suggest that ERβ may be involved in the mediation of aspects of social agonistic interactions that are aimed at asserting dominance (Alleva, 1993; Miczek et al., 2001, 2007; Pietropaolo et al., 2004; Branchi et al., 2006). In our study, there was typically a clear predominance of agonistic behavior delivered, rather than received, by the resident, which suggests that the resident would have established dominance over the same-sex intruder had our study involved long-term pairing and observation of the mice. Dominance hierarchies, in which dominant females gain preferential access to space and resources, and possibly even exclusive breeding rights, have been observed in wild-descended female mice (Hurst, 1987). More long-term studies with comprehensive behavioral analyses in males as well as females are needed for a better understanding of ERβ mediation of different facets of agonistic behaviors.

The overall increase in the duration of all active behaviors by WAY-200070 observed in the gonadally intact CD-1 females was also observed in the frequency measures, reflecting the fact that these mice switched between behaviors more frequently ("behavioral shifting"). This is in agreement with the known facilitatory effects of estrogens on running wheel activity in several strains of female mice (e.g., Garey et al., 2001; Morgan and Pfaff, 2001, 2002). Conversely, gonadally intact αERKO and βERKO females displayed increased activity in response to social stimuli (Choleris et al., 2006) suggesting that, as for male aggression, female activity in a social context may be differentially affected by the timing of the manipulation of ERβ. Alternatively, ERβ involvement in activity may differ in social and non-social contexts.

In both male and female gonadex CD-1 mice, WAY-200070 increased the amount of behavioral shifting, social investigation, and individual behaviors, although it did not affect agonistic behavior. These results suggest that activation of ERβ in adult CD-1 mice with extremely low levels of endogenous sex hormones enhances their overall activity and arousal (Pfaff et al., 2008) without affecting agonistic behaviors.

4.2. Sex and gonadectomy differences

In addition to the WAY-200070 effects, we observed a number of sex differences. In agreement with the literature on aggression in various strains of mice (e.g., Anton, 1969; Edwards, 1970), in the present study the gonadally intact male CD-1 mice attacked the same-sex, gonadex intruder more and made more reciprocal attacks than the gonadally intact females. This supports the notion that the aggression (i.e., attacks) commonly studied in the mouse resident—intruder test is typical only for males; females instead tend to use aggressive postures and other non-attack agonistic behaviors, such as aggressive allogrooming or pushing down the intruder (Grant and Mackintosh, 1963; Clipperton et al., 2008). When looking at both types of aggression, we found that in both sexes, the resident mouse delivered more and received less agonistic behavior than the same-sex intruder. This occurred in males through attacks and in females through other agonistic behaviors. When looking at the total amount of time allocated to agonistic behaviors, female CD-1 mice were not different from males in their response to a gonadex, same-sex intruder. Hence, the overall levels of intrasexual agonistic behavior are similar in male and female CD-1 mice. However, the functional outcome of these interactions is likely to be different. In males, fighting may allow for the establishment of territories and regulation of space use (Scott and Fredericson, 1951), as male attacks tend to be in short bursts followed by periods when they retreat to the opposite sides of the cage (Ginsburg and Allee, 1942; Miczek et al., 2001). In females, the extended sessions of dominant agonistic behavior performed by the resident could lead to the establishment of a dominance hierarchy which, in turn, would allow them to share the same territory with other females.

In gonadally intact CD-1 mice, we found males showed more behavioral shifting and a shorter latency to social investigation than females. This may reflect greater general arousal induced by the social challenge represented by the presence of a same-sex intruder in gonadally intact, territorial males (Branchi et al., 2006; Pfaff et al., 2008), as castrated males, which are less territorial, also display a longer latency to social investigation and approach the intruder less than gonadally intact males. Thus, the observed sex differences in the type of agonistic behaviors displayed may be due to different underlying motivational responses to an unknown intruder in males and females.

In agreement with previous findings that castration affects a number of behaviors in many strains of mice (e.g., Uhrich, 1938; Beeman, 1947; Luttge, 1972; De Catanzaro, 1987), in the uninjected CD-1 mice in the present study, some differences were noted between the gonadally intact and gonadex males. Castrated CD-1 males exhibited reduced agonistic behavior when compared to intact males, not only by showing the well-established absence of attacks (e.g., Uhrich, 1938; Beeman, 1947; Luttge, 1972), but also by displaying an increase in submissive behavior, similar to that seen in the gonadally intact CD-1 females.

Ovariectomized CD-1 females, overall, appeared to be less afraid of the same-sex intruder than gonadally intact females, as indicated by fewer stretched approaches (Blanchard et al., 1993; Choleris et al., 2001) and more investigations directed towards the facial area. This is consistent with the well-documented involvement of estrogens in the mediation of anxiety and anxiety-like behaviors in rodents and other species (reviewed in Choleris et al., 2008).

4.3. Note on vehicle effects

When compared to the uninjected mice used in this study, the mice that had received sesame oil (a common vehicle for

gonadal hormones) showed some behavioral differences. In intact CD-1 males, the sesame oil vehicle increased aggressive postures and decreased the amount of received agonistic behavior, while in intact CD-1 females, oil increased social behavior, and decreased some non-social active behaviors. Gonadex CD-1 mice showed a number of vehicle effects as well, with sesame oil decreasing activity. Sesame oil also decreased social investigation in castrated males, while increasing non-social behaviors in general. This is in general agreement with studies showing that IP injection of sesame oil has been shown to produce partner preferences in prairie voles (Curtis and Wang, 2005) and oral ingestion of sesame oil by CD-1 mice reduced norepinephrine levels (Anton et al., 1974). Thus, sesame oil may affect social behavior by increasing overall arousal (Pfaff et al., 2008). Alternatively, it is possible that the effects found in the present study are due to metabolic effects of the sesame oil, which have been observed with SC injections in rats (Colafranceschi et al., 2007), although why they would be seen 72 h after administration is unclear. For the same reason, it is unlikely that these effects are due to the stress of the injection. An intriguing speculative hypothesis is that sesame oil may have as yet undescribed light hormonal effects.

Most research on the effects of gonadal hormones does not include an uninjected control group, and thus there may be vehicle effects that are overlooked, especially when a vehicle other than physiological saline is used. In the few studies in which an uninjected control group was used, or the effects of sesame oil were directly examined, vehicle or sesame oil effects were found in various species and with different modes of administration (oral, IP, SC; Anton et al., 1974; Curtis and Wang, 2005; Colafranceschi et al., 2007). Given the implications this may have for endocrinological studies, further investigations into these effects are warranted.

4.4. Conclusions

The ethological analysis employed allowed a complete assessment of the role of ERβ in agonistic interactions in male and female CD-1 mice and allowed us to establish not only generalized effects of the ERβ agonist WAY2000-70 on social and non-social behaviors, but also, and more importantly, drug effects on specific aspects of the mice's social responses in the intruder test. Our results suggest that in both sexes, acute stimulation of ERβ can mediate dominance-related agonistic behaviors, while leaving overt attacks largely unaffected. These results of acute ERβ agonist treatment in normally developed adult mice suggest that at least part of the effects of gene knockout of ERβ on aggression (Ogawa et al., 1999; Nomura et al., 2002) may be due to an activational, rather than a developmental, role of ERβ and further emphasize the need for ethological assessments of behavior in order to achieve a full understanding of the results of neurobiological studies.

Our ethological assessment of female interactions showed that CD-1 females spend as much time performing agonistic behaviors as males do, but use behaviors other than attacks in what is likely an attempt to establish dominance over a same-sex conspecific. We also showed that, similar to males, these agonistic behaviors are affected by acute activation of ERβ. However, more studies with females are needed in order to elucidate the respective roles of ERα and ERβ in intrasexual aggression and agonistic behaviors, as previous research with αERKO and βERKO females tended to focus on attacks and/or maternal aggression (Ogawa et al., 1999, 2004), and potential effects of the genetic manipulation on other agonistic behaviors would not have been observed. Additionally, further research with different strains and with intruders of the opposite sex are needed to determine if these results generalize to all strains of mice and intruders. By gaining a more complete picture of the behavior of male and female mice in the resident—intruder paradigm, we would be better able to understand this important aspect of behavior, as well as the roles played by ERα and ERβ in functionally different types of aggression.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen.2010.01.002.

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