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Opposing hormonal mechanisms of aggression revealed through short-lived testosterone manipulations and multiple winning experiences

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Abstract

Territorial aggression is influenced by many social and environmental factors. Since aggression is a costly behavior, individuals should account for multiple factors such as population density or reproductive status before engaging in aggression. Previous work has shown that male California mice (*Peromyscus californicus*) respond to winning aggressive encounters by initiating aggression more quickly in future encounters, and we investigated the physiological basis for this effect. We found that injections that produced a transient increase in testosterone (T) following an aggressive encounter caused males to behave more aggressively in an encounter the following day. Experience alone was not enough to change aggression, as males treated with saline injections showed no change in aggression. The effect of T injections on aggression was androgen-based, as the inhibition of aromatase did not block the T injections from increasing aggression. Aromatase inhibition did, however, increase aggression in the initial aggression tests (before application of T or saline injections), and aromatase activity in the bed nucleus of the stria terminalis (BNST) was negatively correlated with aggression. A previous study suggested that aromatase activity in the BNST decreases after males become fathers. Thus, distinct neuroendocrine mechanisms allow male California mice to adjust aggressive behavior in response to changes in social and reproductive status.
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Keywords: Aromatase; Estrogen; Social experience; Testosterone; Winner effect

Introduction

Although aggressive defense of a territory often provides the holder with increased access to food or mates, engaging in aggressive encounters comes at a cost of increased risk of injury (Michener and McLean, 1996) and/or increased energy expenditure (Marler et al., 1995). Thus, an individual should be able to account for multiple social and environmental factors before engaging in risky fighting behavior. The neuroendocrine system responds to social experiences, and thus is well suited to regulate aggression. For example, male prairie voles become more aggressive toward intruders after pair bonding with a female (Insel et al., 1995), a behavioral transition mediated by vasopressin (Winslow et al., 1993). In a wide variety of vertebrates, winning aggressive or competitive encounters is followed by an increase in male testosterone (T). This so-called challenge effect has been observed in birds (Wingfield et al., 1990), fish (Oliveira et al., 2002), humans (Mazur and Booth, 1998), non-human primates (Rose et al., 1971), and rodents (Oyegbile and Marler, unpublished data). Whether this increase in T regulates subsequent aggression is unknown, although other studies have shown that individuals that have won encounters are more aggressive in future encounters (Chase et al., 1994; Kudryavtseva, 2000).

The effects of T on aggression have been studied for over a century (Berthold, 1849/1994). Most studies manipulate T via castration, the administration of exogenous T via implants, or with injections of long-lasting T propionate. This approach provides insight on how baseline T regulates behavior, but does not address how transient hormonal changes influence aggression. An additional complicating factor is that many of the effects of T on behavior occur after T is converted to estradiol (E_2) by the aromatase enzyme (Baum, 2002). Estrogens can have important effects on

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aggression (Simon et al., 1993), although the direction of the effects may depend on which estrogen receptor subtype is activated (Ogawa et al., 1998, 2000). While many studies have found that castration reduces aggression in male mice and rats (Albert et al., 1992), several recent studies have found that in several species, castration does not reduce aggression (prairie vole, Demas et al., 1999; Syrian hamster, Jasnow et al., 2000; California mouse, Trainor and Marler, 2001). However, it has not been tested whether short-term increases in T influence aggression in these species.

We examined how the neuroendocrine system integrates winning experiences in aggressive encounters to influence aggression in subsequent encounters. In a previous study, we found that intact male California mice (*Peromyscus californicus*) with prior experience attacking intruders in resident–intruder aggression tests exhibited shorter attack latencies in subsequent tests but that castrated males, while attacking intruders, showed no such change in attack latencies (Trainor and Marler, 2001). Testosterone levels increase in intact residents after winning an aggressive encounter if they have previously won aggressive encounters (Oyegbile and Marler, unpublished data), indicating that repelling an intruder leads to an increase in T. We test the hypothesis that an increase in T after winning an encounter leads to changes in aggression in future encounters.

Methods

Subjects

We used virgin male California mice reared in a laboratory colony at the University of Wisconsin, Madison. Subjects were housed in standard cages and were fed Purina 5001 mouse chow and water ad libitum. Colony rooms were kept under a 14 h L/10 h D light cycle with lights on at 0500. Behavioral observations were conducted during the dark phase between 1900 and 2100 under dim red light. Animals were maintained in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Effects of testosterone and aromatase on aggression

To determine if the increased T observed after an aggressive encounter affects aggression in subsequent encounters, we tested castrated males in three aggression tests. We gave each male a silastic implant to provide baseline T levels, but since the males were castrated, their ability to increase T levels in response to social stimuli was greatly reduced. In one group, we gave T injections after each of the first two aggression tests to mimic the normal increase in T observed in intact males that win aggressive encounters. In a second group, we gave saline injections after each of the first two aggression tests to control for handling. In a third group, we gave T injections coupled with an aromatase inhibitor (fadrozole) to determine if aromatase mediated any effects of the T injection. In the first aggression tests for all three groups, we compared aggression levels in the fadrozole group with the other two non-fadrozole-treated groups to observe general effects of estrogen production on aggression, independent of prior experience. Thus, any observed effects of aromatase inhibition would be independent of T or saline injections, which were not applied until after the first aggression test.

We used sexually inexperienced males that had no prior experience in aggression tests. We castrated males and inserted silastic implants (i.d. 0.04 in, o.d. 0.085 in; containing 1 mm of T) subcutaneously. These implants produced a baseline T level of 0.81 ± 0.05 ng/ml, similar to the mean T level observed in sexually inexperienced male California mice (Marler et al., 2003). Males assigned to the aromatase inhibitor group were treated with subcutaneous osmotic implants (Alzet model 2002) containing fadrozole (FAD, 0.25 mg/kg) at the time of castration. This specific dose and delivery method of FAD has been shown to reduce brain aromatase activity in rodents (Clancy and Michael, 1994) and primates (Zumpe et al., 1993). This dose reduces estrogen-dependent paternal behavior and does not alter circulating T levels in male California mice (Trainor and Marler, 2002). We treated the T injection group with saline osmotic implants.

After a 10-day recovery period, each male was placed in a large Plexiglas observation cage for 2 days. Each cage contained one smaller chamber (22 × 29 × 30 cm) and one larger chamber (30 × 29 × 30 cm) that contained a running wheel, food, and a water bottle. On the 12th day after surgery, we conducted resident–intruder aggression tests by placing an unfamiliar, sexually inexperienced, intact adult male into each observation cage. We terminated each test after the resident attacked by biting the intruder. Previous studies have shown that this paradigm results in decreased attack latency in subsequent tests (Trainor and Marler, 2001). Attack latencies were later confirmed on videotape by a single observer blind to treatment groups. Thirty minutes after removing the intruder, each resident received either a subcutaneous saline or T (36 μg/kg T–cyclodextrin inclusion complex) injection. We used the T–cyclodextrin inclusion complex because it rapidly delivers T to the blood and is rapidly metabolized, resulting in a short-lived increase in T (Taylor et al., 1989). The next day, we conducted a second round of aggression tests and injection treatment with a different set of intruders (day 13). The final aggression test was conducted the next day (day 14). To observe the effects of aromatase inhibition on aggression, we compared attack latencies from the three treatment groups on the first trial only. This is important because the injection treatments were not applied until 30 min after the first trial. Thus, any behavioral effects of FAD observed in the first trial cannot be attributed to T or saline injection treatment.

In a separate pilot experiment, we gave T injections to castrated males implanted with T implants and collected

retro-orbital blood samples at 5, 10, 15, 30, 45, or 60 min post-injection using a between-subjects design. Details of hormone assays are described below. We found that the 36 μg/kg T injection produces a transient increase in plasma T levels (Fig. 1a) that approximates the peak T level observed in intact California mice after winning an aggressive encounter (Oyegbile and Marler, in review).

After examining $Q-Q$ plots to assess the assumptions of parametric statistics, we found that log transformation of attack latencies produced normal distributions for statistical analyses (Zar, 1996). We also checked cell variances to verify homogeneity of variances. To test for effects of injection treatments on attack latency, we used repeated measures ANOVAs. To test for effects of the aromatase inhibitor, we used an independent contrast to compare the mean attack latency of fadrozole-treated males with the non-fadrozole-treated males for the first test, when none of the males had any prior experience in aggressive encounters or injection treatment.

Effects of aggression on aromatase and plasma steroids

To test whether different experiences in aggressive encounters lead to long-term changes in neural aromatase activity or baseline steroid hormones, sexually inexperienced males were randomly assigned to one of four experimental groups: resident, intruder, control, or handle. Three 10-min resident–intruder aggression tests were conducted by transferring each intruder to a resident's home cage. Controls were treated like residents, but were not exposed to intruders; handles were treated like intruders but were not exposed to residents.

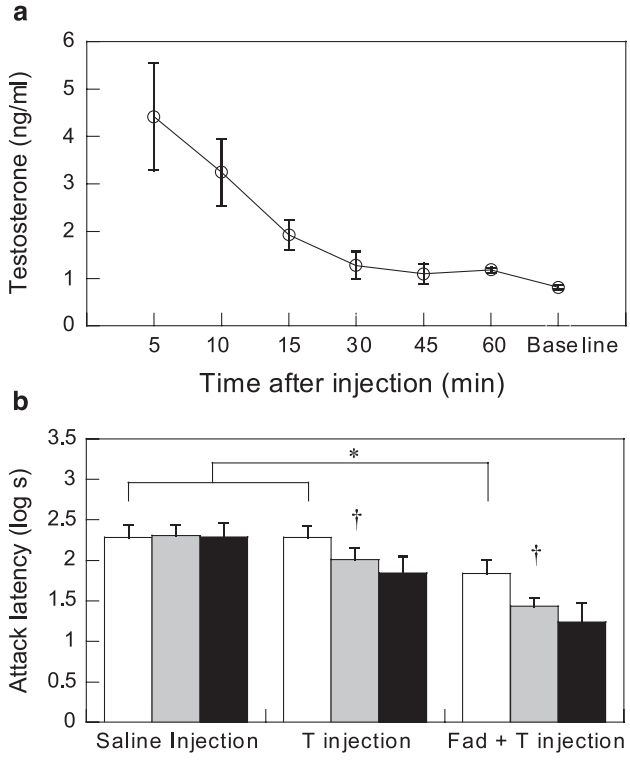

Fig. 1. The effects of a transient increase in T on aggression. (a) Time course of plasma T levels (mean ± SE) after T injection. (b) Mean (± SE) attack latencies for trial 1 (open bars), trial 2 (gray bars), and trial 3 (black bars). † Within treatment group repeated measures ANOVA, $P < 0.05$, * independent contrast for trial one $P < 0.05$.

For aggression and handling tests, each male was transferred to large Plexiglas observation cages as described above. After a 2-day acclimation period, each intruder was transferred from its home cage and introduced into the cage of an unfamiliar resident. We simultaneously transferred males assigned to the handle group to observation cages that had been washed and filled with fresh bedding, while we opened and closed the observation cages of control males to simulate an aggression test. We shaved a small patch of fur on the flanks of intruders and handled mice to facilitate the identification of intruders on videotapes. After 10 min, males in the intruder and handle groups were transferred back to their home observation cage. The 10-min aggression tests were repeated 2 and 4 days after the initial test. Each intruder faced the same resident for all three encounters (called trial 1, 2, and 3). Videotapes were scored by a single observer. Aromatase activity and hormone levels were measured after the behavioral scoring, so the observer was not aware of these physiological parameters. We measured the number of bites, chases, and bouts of wrestling exhibited by each resident. We also measured attack latency of the resident and time spent freezing by the intruder. All males were lightly anesthetized with isoflurane and rapidly decapitated to collect plasma and brain samples for aromatase assays 12–14 h after the final test.

Brain nuclei were dissected using the Palkovitz punch technique and frozen for aromatase assays (Trainor et al., 2003). The distribution of aromatase in the mammalian brain is limited primarily to hypothalamic and limbic brain areas (Roselli and Resko, 1989; Roselli et al., 1985, 1996), including the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), ventromedial hypothalamus (VMH), and medial amygdala (MA). Bilateral samples of the MPOA and BNST were collected with a 1000-μm punch and bilateral samples of the MA, VMH, and the CA1 region of the hippocampus (HPC) were collected with a 750-μm punch. We used the HPC as a control region since most studies on mammals have found little aromatase activity in this brain area. We measured aromatase activity using a water assay previously validated for use in California mice (Trainor et al., 2003). Briefly, punch samples were homogenized in a sucrose-phosphate buffer, sampled for protein content (Bradford protein assay), and frozen at −80 °C overnight. Homogenates were thawed and aliquoted, normalized for protein content. Samples were incubated with 1β-^{3}H androstenedione (200 nm) in the presence of a NADH-NADPH-generating system and incubated at 37 °C for 4 h. Radiolabeled steroids were removed by extraction with chloroform and any remaining steroids stripped with a charcoal/dextran wash. The aqueous phase was removed and radioactivity

determined in a liquid scintillation counter. Aromatase activity is reported as femtomoles (fmol) of product per minute per milligram protein. An aliquot of a frozen California mouse brain tissue homogenate pool was included in each assay. The inter-assay coefficient of variation for this pool was 7.19%.

Details of hormone assays procedures conducted at the Wisconsin Primate Research Center and assay validation information for the measurement of T (Trainor and Marler, 2001), progesterone (P_4, Davis and Marler, 2004), and corticosterone (B, Bester-Meredith and Marler, 2001) in California mice have been previously described. Intra-assay coefficients of variation for T, P_4, and B were 4.22%, 3.84%, and 7.29%. Inter-assay coefficients of variation were 14.9%, 6.4%, 7.3%. We used independent contrasts to compare aromatase activity and steroid hormone levels between residents and controls, and between intruders and handles.

After examining Q–Q plots to assess the assumptions of parametric statistics, we square root transformed all measurements of aromatase activity, chases, bites, and bouts of wrestling. We log transformed attack latencies and steroid hormone levels for statistical analyses. We checked cell variances of all variables to confirm the homogeneity of variance. To test for the effects of experience in aggressive encounters on aromatase activity and steroid hormone levels, we used one-way ANOVAs and independent contrasts to compare residents with control males and intruders with handled males. To test whether aggressive behavior changed across the three aggression tests, we used repeated measures ANOVA. We also correlated aromatase activity and steroid hormone measurements with aggressive behavior measured in each of the three aggression tests.

Results

Effects of testosterone and aromatase on aggression

Castrated males treated with baseline T implants and saline injections showed no change in attack latency (Fig. 1b), indicating that the experience of winning alone is not enough to facilitate increased aggression. When castrated males treated with baseline T implants were treated with T injections, attack latency decreased across the three aggression tests (Fig. 1b; $F_{2,18} = 4.16$, $P = 0.03$). This effect persisted when T injections were combined with FAD treatment (Fig. 1b; $F_{2,18} = 5.89$, $P = 0.01$), indicating that this increase in aggression is androgen-based.

An independent contrast indicated that in the first trial, males treated with FAD exhibited lower attack latencies compared to males in the two groups that were not treated with FAD (Fig. 1b; $F_{1,25} = 5.15$, $P = 0.03$). This effect is due to the inhibition of aromatase because during the first trial, none of the males had prior experiences in encounters and injections were not given until 30 min after the first trial.

Effects of aggression on steroids and aromatase

All residents attacked intruders during aggression tests. Repeated measures ANOVA indicated that residents attacked intruders faster (shorter attack latency) after the initial test ($F_{1,18} = 4.29$, $P = 0.03$). There were no significant changes in the residents' frequency of bites, chases, or bouts of wrestling across the three trials (all P's > 0.43). We detected no differences in the amount of time the intruders spent freezing across the three trials.

We did not detect any differences in aromatase activity in microdissected brain areas between residents and controls (Fig. 2, all independent contrast P's > 0.38), or between intruders and handled males (Fig. 2, all independent contrast P's > 0.28). This is consistent with the previous finding that winning increases aggression via an androgen-based mechanism. However, when we examined correlations between aromatase activity and behavior, we found that aromatase activity in the BNST was positively correlated with attack latency (Fig. 3). Interestingly, this correlation persisted in trial 1 ($r = 0.66$, $P = 0.04$), trial 2 ($r = 0.75$, $P = 0.01$), and trial 3 ($r = 0.66$, $P = 0.04$). Thus, even though residents exhibited shorter attack latencies after the initial trial, in each trial, residents with more aromatase activity in the BNST exhibited longer attack latencies. Aromatase activity in other brain areas was not significantly correlated with attack latency or any other aggressive behavior measured.

Independent contrasts indicated that residents did not differ significantly from controls in plasma T, P_4, or B (Table 1). Baseline T was not correlated with any aggressive behavior. Intruders had higher P_4 levels than handled males

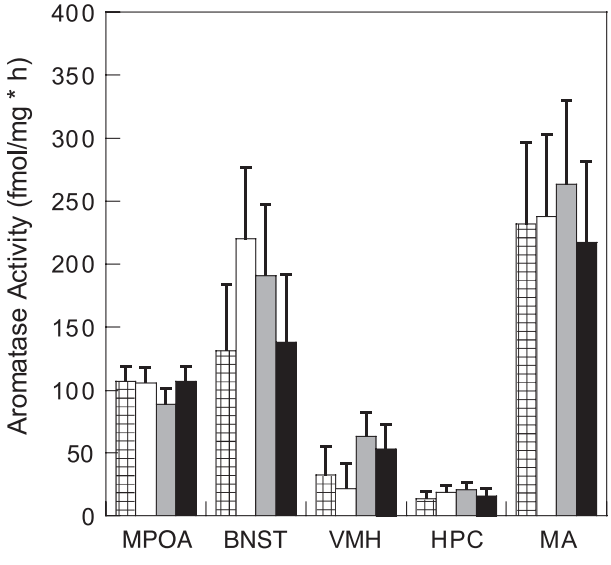

Fig. 2. Aromatase activity of microdissected brain nuclei. Mean levels (± SE) of aromatase activity in handles ($n = 10$, striped bars), intruders ($n = 10$, open bars), controls ($n = 8$, gray bars), and residents ($n = 10$, black bars). There were no significant differences in aromatase activity in the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), ventromedial hypothalamus (VMH), hippocampus (HPC), or medial amygdala (MA).

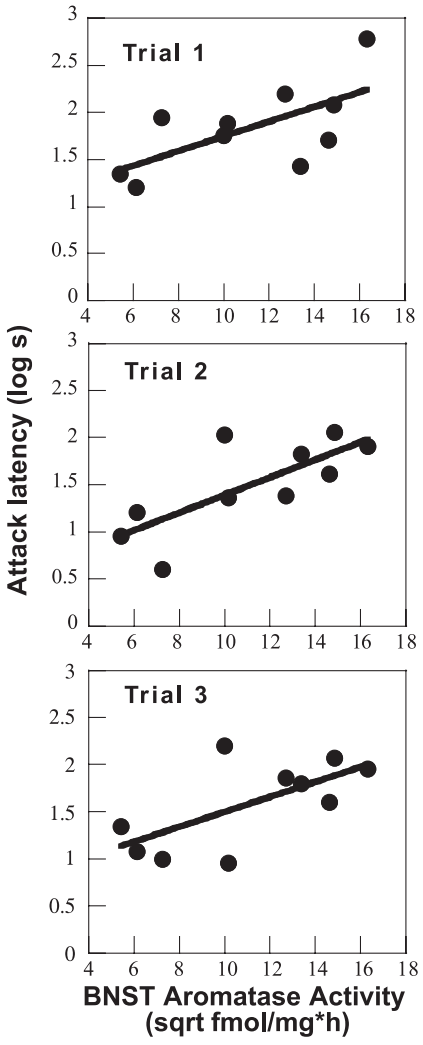

Fig. 3. Relationship between aromatase activity in the BNST and attack latency in trial 1 ($r = 0.66$, $P = 0.04$), trial 2 ($r = 0.75$, $P = 0.01$), and trial 3 ($r = 0.66$, $P = 0.04$).

($P = 0.02$), but did not differ in T ($P = 0.26$) or B ($P = 0.99$) levels.

Discussion

We found that transient increases in T following aggressive encounters are necessary for winning experiences to cause changes in aggression in subsequent encounters. Winning alone was not sufficient. These results suggest that increased T following social challenges, observed in a wide variety of vertebrate taxa, may function to adjust aggressive behavior in future encounters. Furthermore, we found that T acted through an androgen-based mechanism to affect aggression, since aromatase inhibition did not interfere with the effects of T injections. In contrast, aromatase inhibition decreased attack latency on the first trial. This is an important result because T injections were not applied until 30 min after the first trial. Thus, the increased aggression caused by aromatase inhibition occurs independently of increased T. Measurements of aromatase activity suggest that the BNST may mediate this effect, since this was the only brain region in which aromatase activity was correlated with attack latency. Interestingly, even though residents exhibited a decrease in attack latency across three trials (due to transient changes in T), the positive relationship between aromatase activity in the BNST and attack latency was unchanged in each trial. Attack latency was the only aggressive behavior that was significantly correlated with aromatase activity in the BNST. These observations strongly suggest that two distinct neuroendocrine mechanisms regulate aggression in the California mouse, and that they respond differently to environmental stimuli. The presence of multiple neuroendocrine mechanisms may allow individuals to adjust their behavior to multiple environmental variables simultaneously.

Many studies have documented that males become more aggressive after defeating intruders (Albert et al., 1988; Bevan et al., 1960; Kudryavtseva, 2000; Parmigiani and Brain, 1983), and in some cases, it has been documented that winners are more likely to win future encounters, independent of intrinsic competitive ability (Chase et al., 1994; Oyegbile and Marler, unpublished data). Our results suggest that the previously observed increase in T in California mice forms part of the mechanistic basis for the so-called winner effect (Oyegbile and Marler, in review). Testosterone must cause a long-term change in the brain because the change in aggression occurs long after the elevated T has been cleared from the blood (Fig. 2a). One possible pathway is modulation of dopaminergic function, as winning aggressive encounters increases dopamine release (Kudryavtseva, 2000), and transient T injections facilitate the expression of dopamine-dependent conditioned place preferences (Packard et al., 1998). Furthermore, rats tested as residents in multiple resident–intruder tests exhibited a conditioned increase in extracellular dopamine release in the nucleus accumbens immediately before an aggression encounter (Ferrari et al., 2003).

A previous study found that male California mice had higher T levels 45 min after winning an aggressive encounter (Oyegbile and Marler, unpublished data), but it was unclear whether winning led to longer term changes in T levels, such as the increase in T observed in mated California mice (Trainor et al., 2003). We found no difference

Table 1
Steroid hormones levels of intact males

Treatment	Testosterone (log ng/ml)	Progesterone (log ng/ml)	Corticosterone (log ng/ml)
Resident ($n = 10$)	0.21 ± 0.17	0.24 ± 0.17	1.85 ± 0.12
Control ($n = 8$)	0.19 ± 0.06	0.20 ± 0.05	1.93 ± 0.10
Intruder ($n = 10$)	0.22 ± 0.04	0.34 ± 0.05*	2.10 ± 0.12
Handle ($n = 10$)	0.16 ± 0.03	0.18 ± 0.02	2.10 ± 0.09

Independent contrasts compare residents to controls and intruders to handles. Results are means ± SE in each column, * ($P < 0.05$).

in T levels, or any other steroid, between residents that won encounters and control males when blood samples were collected 12–14 h after the last trial. This suggests that the increase in T after an aggressive encounter is transient. In contrast, intruders had significantly higher P_4 levels than handled males, indicating that losing aggressive encounters results in an increase in baseline P_4. Losing aggressive encounters decreases aggression in several species (e.g., Siegfried et al., 1984), and P_4 has been found to inhibit aggression (Erpino and Chapelle, 1971; Meisel et al., 1988). In contrast, female residents exposed to intruders exhibit a decrease in P_4 levels, suggesting that decreased P_4 may facilitate future increases in aggression (Davis and Marler, 2004).

While we found that aromatase inhibition increases aggression in male California mice, we found no evidence that this estrogen-based pathway responded to experience in aggressive encounters. In a previous study, we found a strong non-significant trend ($P = 0.05$) for decreased aromatase activity in the BNST of males that had fathered pups compared to sexually inexperienced males (Trainor et al., 2003). In biparental prairie voles, males are more aggressive after pair-bonding with females (Insel et al., 1995; Winslow et al., 1993). Thus, the results of the present study support the hypothesis that reproductive experience increases aggression, at least in part, by decreasing aromatase activity in the BNST. Aromatase activity in the BNST was positively correlated with attack latency in male California mice (Fig. 3). Other studies have implicated the BNST as a brain region that controls agonistic behavior (Irvin et al., 1990), and developmental manipulations that affect aggression alter vasopressin in the BNST (Bester-Meredith and Marler, 2001). The effect of estrogen on aggression likely depends on the subtype of receptor that is activated. Estrogen receptor α knock-out mice show greatly reduced aggression levels (Ogawa et al., 1998) while estrogen receptor β knock-out mice show heightened aggression levels (Nomura et al., 2002; Ogawa et al., 2000). These studies suggest that the two estrogen receptors have opposite effects on aggression in much the same way that they have opposite effects on cell proliferation in the female uterus (Weihua et al., 2000).

Our finding that aromatase inhibition increased aggression appears to conflict with results from a previous study (Trainor and Marler, 2001), in which we found that castration did not increase aggression. One possible explanation for this result is that there is an alternative source of aromatizable androgen. The androgen precursor dehydroepiandrosterone (DHEA) can be produced in the adrenal cortex or de novo in the brain (Young et al., 1996). Interestingly, DHEA promotes aggressive song in song sparrows during the non-breeding season (Soma et al., 2002), a period when castration does not influence aggression (Wingfield, 1994) and aromatase inhibitors decrease aggression (Soma et al., 2000a,b). In the present study, our use of fadrozole blocked estrogen production globally, regardless of the source of androgen substrate. Currently, it is unknown if male California mice express the necessary enzymes (3beta-hydroxysteroid dehydrogenase and/or 17beta-hydroxysteroid dehydrogenase) to produce aromatizable androgens from DHEA within the brain.

Our results demonstrate that different physiological mechanisms can act simultaneously to control aggression in different social situations. Our data support the hypothesis that at least one function of the challenge effect is to regulate aggression by integrating winning experiences (Oyegbile and Marler, unpublished data). In contrast, aromatase inhibits aggressive behavior and may be more responsive to changes in reproductive experience. Territorial defense is influenced by many social and environmental factors such as the distribution of food, population density, and social system (Ostfeld, 1990). The challenge effect has been observed in a wide variety of vertebrate taxa, suggesting that short-term changes in T could have similar effects on aggression in other species, including humans.

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