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Nongenomic effects of estradiol on aggression under short day photoperiods

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ABSTRACT

In several vertebrate species, the effects of estrogens on male aggressive behavior can be modulated by environmental cues. In song sparrows and rodents, estrogens modulate aggression in the nonbreeding season or winter-like short days, respectively. The behavioral effects of estrogens are rapid, which generally is considered indicative of nongenomic processes. The current study further examined the hypothesis that estradiol acts nongenomically under short days by utilizing a protein synthesis inhibitor, cycloheximide (CX). Mice were housed in either short or long day photoperiods, and treated with an aromatase inhibitor. One hour before resident–intruder testing mice were injected with either CX or saline vehicle, and 30 min later were treated orally with either cyclodextrin conjugated estradiol or vehicle. Under short days, mice treated with estradiol showed a rapid decrease in aggressive behavior, independent of CX administration. CX alone had no effect on aggression. These results show that protein synthesis is not required for the rapid effects of estradiol on aggression, strongly suggesting that these effects are mediated by nongenomic processes. We also showed that estradiol suppressed c-fos immunoreactivity in the caudal bed nucleus of the stria terminalis under short days. No effects of estradiol on behavior or c-fos expression were observed in mice housed under long days. Previously we had also demonstrated that cage bedding influenced the directional effects of estrogens on aggression. Here, we show that the phenomenon of rapid action of estradiol on aggression under short days is a robust result that generalizes to different bedding conditions.

Introduction

Estrogen receptors act as transcription factors migrating to the nucleus of the cell and altering gene expression by binding to promoters such as estrogen response elements (EREs) or cyclic AMP response elements (McDevitt et al., 2008; Nilsson et al., 2001). This form of cellular response is usually referred to as a genomic action. Considerable interest has reemerged, however, with regard to the nongenomic actions of estrogens, which gained recognition in the mid-20th century (i.e. Aizawa and Mueller, 1961; Means and Hamilton, 1966). One mechanism for nongenomic actions may be through estrogen receptors located at extranuclear sites within a cell (Blaustein et al., 1992; Milner et al., 2001; Revankar et al., 2005; Vasudevan and Pfaff, 2008). When activated, estrogen receptors at extranuclear sites can rapidly alter membrane conductance and activation of cyclic adenosine monophosphate (cAMP) (Aronica et al., 1994; Razandi et al., 1999; Tesarik and Mendoza, 1995). These actions are typically referred to as nongenomic since they do not regulate gene transcription directly. While changes in protein expression mediated by genomic processes can take several hours to manifest (Shughrue et al., 1997), cellular changes mediated by nongenomic mechanisms can act rapidly within seconds to minutes of activation (Szego and Davis, 1967; Taziaux et al., 2007).

The rapid effects of estrogens have been shown to occur in a variety of behavioral and environmental contexts (Laredo and Trainor, 2012). Sexual behavior in vertebrate species is especially sensitive to rapid effects of estrogens. Both appetitive and consumatory sexual behaviors are rapidly altered by exogenous estradiol injections or aromatase inhibition in male quail (Balthazart et al., 2006) with similar results shown in rats (Cross and Roselli, 1999). Aggressive behaviors are also mediated by the rapid effects of estradiol in song sparrows (*Melospiza melodia morphna*) (Soma et al., 2000), old-field mice (*Peromyscus polionotus*) (Trainor et al., 2007a) and California mice (*Peromyscus californicus*) (Trainor et al., 2008). Soma et al. (2000) showed that following an acute administration of the aromatase inhibitor fadrozole, aggression was reduced only in the

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nonbreeding seasons (autumn and winter). This demonstrates that photoperiod may be a potent environmental cue mediating the rapid effects of estrogens on aggression. Similarly, in *Peromyscus* aggressive behavior is rapidly regulated by estradiol only under short-day, winter-like photoperiods (Trainor et al., 2007a; Trainor et al., 2008), further supporting the hypothesis that nongenomic effects of estrogens are regulated by seasonal cues in the environment.

Photoperiod is not the only environmental cue that can mediate the effects of estrogens on behavior. Phytoestrogens are compounds found in plant material that can significantly alter estrogen-mediated behaviors (Jefferson et al., 2012). Previous studies investigating the rapid effects of estrogens on aggression in *Peromyscus* (i.e. Trainor et al., 2007a; Trainor et al., 2007b; Trainor et al., 2008) utilized corncob bedding as cage substrate, which contains phytoestrogens (Markaverich et al., 2002). Recently it was determined that the effects of estrogens on aggressive behavior in *Peromyscus* depend on the type of bedding used in cages (Villalon Landeros et al., 2012). Treatment with an aromatase inhibitor alone increased aggression in California mice housed on a cardboard-based bedding but not in mice housed on corncob bedding. California mice housed on corncob bedding had elevated levels of tetrahydrofurandiols (THF-diols, Villalon Landeros et al., 2012), which have estrogenic properties and disrupt sexual behavior in male rats (Mani et al., 2005).

Given the strong interaction between bedding and behavior, we tested whether photoperiodic modulation of the rapid effects of estrogens generalized to cardboard-based bedding. We further tested the hypothesis that rapid effects of estradiol are nongenomic using the protein synthesis inhibitor cylcoheximide (CX) (Mikics et al., 2004). We hypothesized that mice housed in short day photoperiods, but not long day photoperiods, would demonstrate decreased aggression in response to estradiol treatments, and that this effect would occur in the presence of CX. It has also been reported that CX can inhibit vasopressin (AVP) release (Ivell et al., 1992; Song et al., 2001) — a nonapeptide associated with aggression (Albers, 2012). AVP increases aggressive behavior in California mice housed in long day photoperiods (Bester-Meredith et al., 2005). We hypothesized that c-fos/AVP colocalizations in the bed nucleus of the stria terminalis (BNST) would be associated with increased aggression, whereas increased c-fos/AVP colocalizations in the paraventricular nucleus of the hypothalamus (PVN) would be associated with subordination (Ho et al., 2010).

Methods

Animals

California mice were bred in our colony or purchased from the *Peromyscus* Stock Center (Columbia, SC). All mice were housed in polypropylene cages on Carefresh bedding (Absorption Corp., Ferndale, WA) with up to two same-sex cage mates. Food (2016 Harlan Teklad, Madison, WI) and water were provided ad libitum. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee.

Experiment 1: drug validation

Estradiol validation. Twenty male mice were housed on short day photoperiods (8 h light: 16 h dark). Oral administration of estradiol was chosen over a second injection to avoid administering multiple injections (Ryabinin et al., 1999). Mice were habituated with pieces of Froot Loop cereal (Kellogg's, Battle Creek, MI) three days prior to testing, after which all mice rapidly consumed this palatable food item. On the day of testing, each mouse received a piece of cereal containing one of three concentrations of cyclodextrin-conjugated estradiol (Sigma-Aldrich, St. Louis, MO; 100 μg/kg, 500 μg/kg or 1000 μg/kg). Mice were randomly assigned for retroorbital blood sample collection at either 15 or 30 min following consumption of the Froot Loop. Blood samples were centrifuged and plasma was stored at −40 °C. Estradiol was analyzed using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) previously validated for California mice (Silva et al., 2010). The sensitivity of this assay was 6.60 pg/mL and the intra-assay coefficient of variation was 9.60%. We found that when mice were given a FrootLoop containing 500 μg/kg of estradiol, plasma levels of estradiol mimicked the plasma levels observed 15 min following a subcutaneous (s.c.) 100 μg/kg injection of estradiol (Trainor et al., 2008, Fig. 1). For all mice receiving estradiol, we therefore used the 500 μg/kg dose of estradiol on Froot Loop presentations.

Cycloheximide validation. Here we tested whether different doses of CX (Sigma-Aldrich, St. Louis, MO) blocked the expression of c-fos, an indirect protein marker for neuronal activity. Twelve female mice were housed under long day photoperiods and randomly assigned to be injected with either vehicle (3% ethanol in saline), or CX (2.5, 25, or 50 mg/kg) dissolved in 3% ethanol in saline. Thirty minutes following CX or vehicle injection, females were exposed to soiled male bedding. This stimulus creates a robust c-fos response in the BNST, which is an area of considerable interest in the current study (Veyrac et al., 2011). As controls, three females received vehicle injections and were exposed to clean bedding. One hour following bedding exposure, mice were anesthetized with isoflurane and rapidly decapitated. Brain tissue was collected and fixed in 5% acrolein in phosphate buffered saline (PBS) for 24 h at 4 °C. Brains were transferred to 20% sucrose in PBS overnight and frozen at −40 °C. Brain tissue was cut on a cryostat in 40 μm sections and stored at −20 °C in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v polyvinylpyrrolidone, and 30% v/v ethylene glycol). Sections

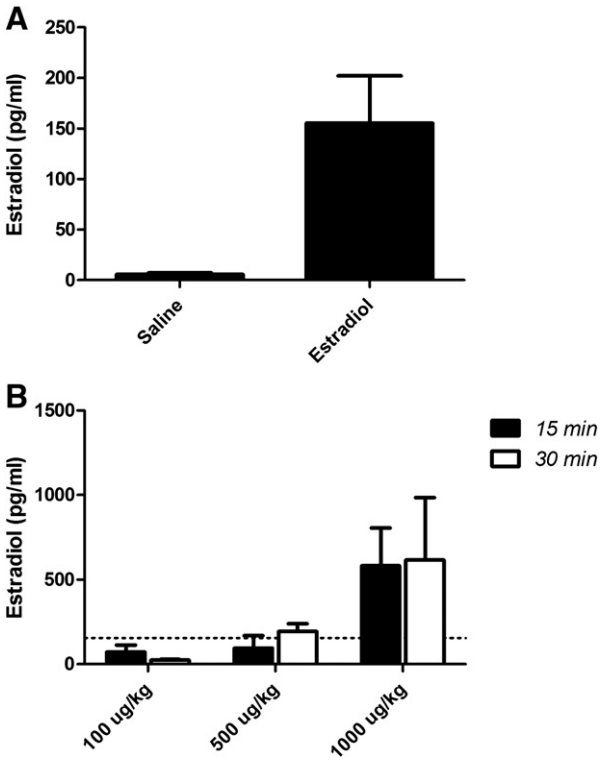

Fig. 1. Plasma estradiol levels (A) 15 min following a 100 μg/kg injection of estradiol (n = 8) or 100 μL saline (n = 4) and (B) 15 and 30 min following oral administration of estradiol. Doses of oral administration were 100 μg/kg (15 min: n = 3; 30 min: n = 3), 500 μg/kg (15 min: n = 4; 30 min: n = 3), and 1000 μg/kg (15 min: n = 4; 30 min: n = 3). The dotted line represents plasma levels of estradiol following a 100 μg/kg injection of estradiol. Estradiol levels 30 min following a 500 μg/kg oral dose of estradiol are comparable to plasma levels 15 min following a 100 μg/kg s.c. injection.

were washed 3 times for 5 min each in PBS, and then washed in 0.1 M sodium borohydride in PBS for 10 min. Sections were subsequently blocked for 30 min in 10% normal goat serum (NGS) in PBS and then incubated in a 1:15000 dilution of c-fos primary antibody (PC38T, Calbiochem, La Jolla, CA, USA) in 2% NGS and 0.5% triton X (TX) in PBS overnight. The following day, sections were washed three times for 5 min each in PBS, and subsequently incubated in diaminobenzadine (Vector Labs) for approximately 2 min. Photomicrographs were taken using a Zeiss Axioimager utilizing an Axiocam MRC camera. The caudal posteriomedial BNST (cBNST, equivalent to mouse bregma −0.46 through −0.58) was analyzed as stated in experiment 2a.

Data analysis. Immunoreactive (ir) c-fos cells were analyzed using a one-way ANOVA, employing LSD post-hoc tests to examine treatment comparisons.

Experiment 2a: the effect of short day photoperiods on estrogen dependent aggression

Hormone manipulation. Male mice were housed in short day photoperiods for 6 weeks. Each mouse was castrated and implanted s.c. with an osmotic mini-pump (Durect, Cupertino, CA) containing 0.25 mg/kg of fadrozole (Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline. Ten days following castration surgery, mice received an intraperitoneal (i.p.) injection with 100 μL of either 25 mg/kg of CX dissolved in 3% ethanol in saline, or 3% ethanol in saline. Thirty minutes following the CX injection, each mouse received a piece of cereal containing 0.5 mg/kg of cyclodextrin conjugated estradiol dissolved in saline, or 0.5 mg/kg of cyclodextrin (Sigma-Aldrich, St. Louis, MO) dissolved in saline. It took an average of 2.65 ± 0.51 min for mice to consume the piece of cereal. Thirty minutes following estradiol or cyclodextrin administration, all mice were tested in a resident-intruder aggression test that was digitally recorded. A novel, age-matched, same-sex intruder mouse was placed in the cage of the focal mouse. Each aggression test was 7 min long and was performed during the dark phase under dim red light. Tests were scored for number of bites administered to the intruder and latency to attack the intruder by an observer who was blind to the study groups. One hour following the resident-intruder test, mice were anesthetized with isoflurane and rapidly decapitated. Brains were collected and fixed as described above.

C-fos and vasopressin immunohistochemistry. Sections were washed and blocked as stated in experiment 1. Sections were then incubated in a 1:2500 dilution of c-fos primary antibody (PC38, EMD Chemicals, Inc., Gibbstown, NJ, USA) in 2% NGS and 0.5% triton X (TX) in PBS overnight. The following day, sections were washed three times for 5 min each in PBS, and subsequently incubated in a 1:500 dilution of goat anti-rabbit Alexofluor 555 secondary antibody (A-31572, Invitrogen, Grand Island, NY, USA) in 2% NGS and PBS-TX for 2 h, and then incubated in a 1:2500 dilution of vasopressin (AVP) primary antibody in 2% NGS and PBS-TX (T-5048.0050, Bachem, Torrance, CA, USA) for 48 h. On the final day, sections were incubated in a 1:500 dilution of goat anti-guinea pig DyLight 488 secondary antibody (106-485-003, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in 2% NGS and PBS-TX for 2 h. Sections were mounted on Superfrost Plus microscope slides (Fisher, Pittsburgh, PA, USA) and coverslipped using VectaShield mounting medium (Vector Labs, Burlingame, CA).

Image analysis. Photomicrographs were taken from areas closely associated with aggression (Nelson and Trainor, 2007; Trainor et al., 2010), including the rostral posteriomedial bed nucleus of the stria terminalis (rBNST, equivalent to mouse bregma −0.22 through −0.34), the cBNST, and the paraventricular nucleus of the hypothalamus (PVN, equivalent to mouse bregma −0.34 through −1.06) (Paxinos and Franklin, 2002). ImageJ (NIH, Bethesda, MD, USA) was utilized to count the number of c-fos-ir and AVP-ir cells, as well as AVP/c-fos colocalizations. Colocalizations were characterized by the percentage of AVP-ir cells demonstrating an overlap with a c-fos-ir cell (Figs. 2A and B). Each immunoreactive perikaryon or colocalization was counted using the "cell counter" plug-in in ImageJ. The box size used to frame the area of analysis remained uniform for each area analyzed. Box sizes for the BNST were approximately 0.40 × 0.40 mm and box sizes for the PVN were approximately 0.50 × 0.50 mm.

Data analysis. Behavioral data was analyzed using a one-way ANOVA and independent contrasts that differentiated between genomic and nongenomic hypotheses. If estradiol acts via genomic mechanisms, then mice treated with estradiol should be less aggressive and mice treated with saline or estradiol + CX should be more aggressive. In contrast, if estradiol acts via nongenomic mechanisms, then mice with estradiol or estradiol + CX should be less aggressive, and mice treated with saline should be more aggressive. C-fos-ir and AVP-ir cells were also analyzed with a one-way ANOVA and independent contrasts differentiating between genomic and nongenomic hypotheses. C-fos cell counts were log transformed due to heterogeneous variance between groups. The percentage of AVP/c-fos colocalizations was transformed using a square root transformation followed by an arcsin transformation (Zar, 1999). Correlations between AVP cell counts and behavior were analyzed using Spearman correlations.

Experiment 2b: the effect of cycloheximide on behavior and protein expression in short day photoperiods

In this study we tested the hypothesis that CX alone could affect aggressive behavior under short days. All mice were castrated and treated with fadrozole as described in experiment 2a. After recovery, one group of mice was treated with CX (as in experiment 2a) and the

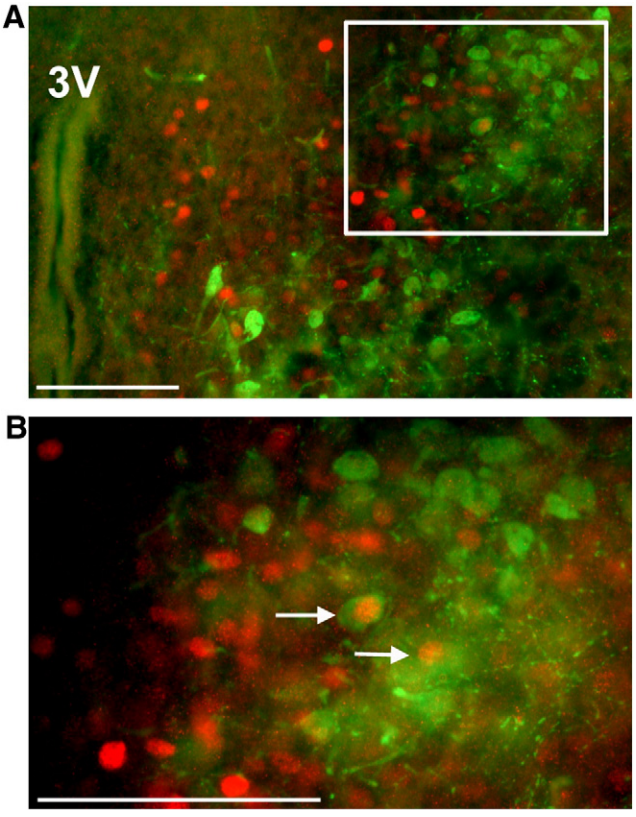

Fig. 2. Photomicrographs indicating AVP/c-fos colocalizations in the PVN at low (A) and high (B) magnification. The red nuclear staining indicates c-fos activity, while the green cytoplasmic staining indicates AVP. 3V = third ventricle. Bar = 100 um.

second group of mice injected with 3% ethanol vehicle followed by oral administration of 0.5 mg/kg of cyclodextrin. All timelines, behavioral procedures, and tissue analyses were followed as mentioned in experiment 2a.

Experiment 3a: the effect of long day photoperiods on estrogen dependent aggression

In this study, we tested whether the effects of estradiol and CX extended to mice housed on long days. Male mice were housed in long day photoperiods (16 h light: 8 h dark) for 6 weeks. All timelines, behavioral procedures, and tissue analyses were followed as mentioned in experiment 2a.

Experiment 3b: the effect of cycloheximide on behavior and protein expression in long day photoperiods

As in experiment 2b, we tested whether CX treatment alone affected aggressive behavior. All procedures were performed as described in experiment 2b except that mice were housed under long day photoperiods.

Results

Experiment 1: drug validation

Cycloheximide validation

There was a significant main effect of treatment on the number of c-fos-ir cells in the cBNST ($F_{2,9} = 4.62$, $p < 0.05$) (Fig. 3). Mice receiving vehicle injections showed significantly more c-fos-ir cells in the cBNST as compared to control mice exposed to clean bedding ($p < 0.05$). Females receiving 25 or 50 mg/kg of CX showed significantly reduced levels of c-fos-ir cells compared to mice receiving vehicle injections or mice receiving 2.5 mg/kg of CX ($p < 0.05$), indicating that 25 mg/kg of CX is sufficient for inhibiting protein synthesis.

Experiment 2a: the effect of short day photoperiods on estrogen dependent aggression

For mice housed in short day photoperiods, there were significant main effects of treatment for latency to attack ($F_{2,27} = 4.96$, $p < 0.05$) and number of bites ($F_{2,27} = 3.87$, $p < 0.05$) (Figs. 4A and C). Planned contrasts indicated that mice receiving estradiol alone or estradiol and CX demonstrated higher attack latencies ($p < 0.05$) and fewer bites ($p < 0.05$) than mice receiving cyclodextrin. There were no main effects of treatment on c-fos cell counts in the rBNST ($F_{2,20} = 1.30$, $p > 0.05$), cBNST ($F_{2,20} = 2.47$, $p > 0.05$) or PVN ($F_{2,22} = 0.54$, $p > 0.05$) (Table 1). Planned contrasts, however, demonstrated that mice receiving estradiol or estradiol and CX had significantly less c-fos in the cBNST than mice receiving cyclodextrin ($p < 0.05$) (Fig. 5). There were significant main effects of treatment for AVP cell counts in the cBNST ($F_{2,16} = 3.85$, $p < 0.05$) but not the rBNST ($F_{2,16} = 1.06$, $p > 0.05$) or PVN ($F_{2,19} = 1.13$, $p > 0.05$). Mice receiving estradiol and CX had significantly more AVP in the cBNST than mice receiving estradiol alone, or cyclodextrin ($p < 0.05$). There were no significant main effects of treatment or of planned contrasts for colocalizations in any brain region (all p's > 0.05). The number of AVP cells was not significantly correlated with number of bites or latency to attack (all p's > 0.05).

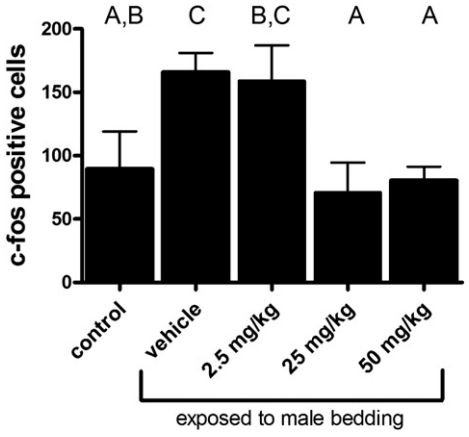

Fig. 3. The number of c-fos-ir cells in the cBNST following exposure to clean bedding (control) or soiled male bedding (vehicle and drug conditions) following CX administration. Mice received either 3% ethanol in saline (control and vehicle), 2.5 mg/kg CX, 25 mg/kg CX, or 50 mg/kg CX. n = 3 for all groups. Bars with different letters are significantly different according to LSD post hoc tests ($p < 0.05$).

Experiment 2b: the effect of cycloheximide on behavior and protein expression in short day photoperiods

In short day mice, CX had no effect on latency to attack (Fig. 4A, $t(14) = -0.47$, $p > 0.05$) or number of bites (Fig. 4C, $t(14) = -0.07$, $p > 0.05$). There were no significant main effects of treatment on c-fos-ir cells in any brain region examined (all p's > 0.05, Table 2). There was a significant main effect for the number of AVP-ir cells in the rBNST ($t(14) = -2.22$, $p < 0.05$) and cBNST ($t(14) = -2.58$, $p < 0.05$), where mice receiving CX had significantly more AVP-ir cells than mice receiving saline. Furthermore, there was a non-significant trend towards an increased number of AVP-ir cells in the PVN of mice receiving CX as compared to mice receiving saline ($t(14) = -1.95$, $p < 0.10$). There were no significant correlations between AVP-ir cells in the rBNST or cBNST and number of bites or latency to attack (all p's > 0.05). There was a significant correlation between AVP-ir cells in the PVN and latency to attack ($r = 0.59$, $p < 0.05$, Fig. 6). Upon visual inspection of the data, it was apparent that this correlation was driven by aggressive mice expressing fewer AVP-ir cells in the PVN. Mice were therefore split into aggressive mice (latency < 210 s) or non-aggressive mice (latency > 210 s). Indeed, aggressive mice had significantly fewer AVP-ir cells in the PVN than non-aggressive mice ($t(14) = -2.56$, $p < 0.05$). There were no significant main effects for the percentage of colocalizations in any brain region examined (all p's > 0.05).

Experiment 3a: the effect of long day photoperiods on estrogen dependent aggression

There were no significant main effects of treatment or of planned contrasts for latency to attack ($F_{2,26} = 0.90$, $p > 0.05$) or bites ($F_{2,26} = 1.17$, $p > 0.05$) (Figs. 4B and D). There were no significant main effects of treatment for c-fos-ir cells, AVP-ir cells or percentage of colocalizations in any brain region examined (all p's > 0.05, Table 1). The number of AVP cells was not significantly correlated with number of bites or latency to attack (all p's > 0.05).

Experiment 3b: the effect of cycloheximide on behavior and protein expression in long day photoperiods

Long day mice receiving CX did not significantly differ in latency to attack ($t(14) = -0.64$, $p > 0.05$) or number of bites ($t(14) = 0.26$, $p > 0.05$) as compared to mice receiving vehicle (Figs. 4B and D). There was a non-significant trend towards a decrease in c-fos-ir cells in the PVN of mice receiving CX as compared to mice receiving saline ($t(12) = 2.10$, $p < 0.10$, Table 2). There were no significant main effects of c-fos in the rBNST ($t(13) = 0.23$, $p > 0.05$) or cBNST ($t(12) = 0.24$, $p > 0.05$). AVP-ir cells in the cBNST were significantly positively correlated with number of bites ($r = 0.52$, $p < 0.05$, Fig. 6),

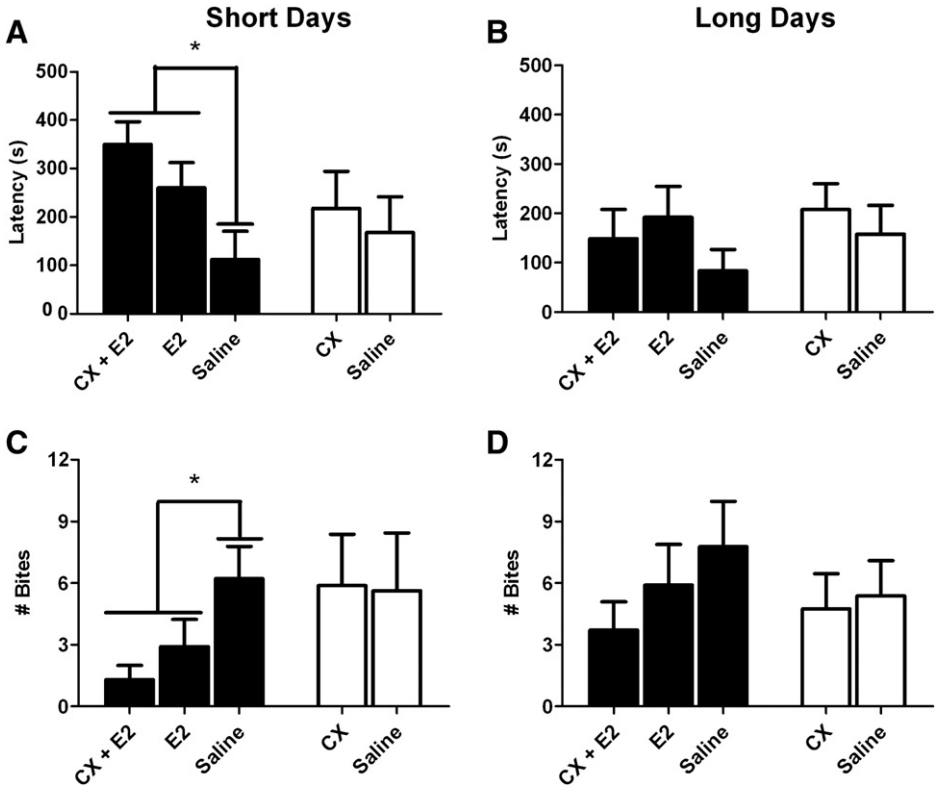

Fig. 4. Latency to attack in (A) short days and (B) long days, and number of bites given in (C) short days and (D) long days following a resident-intruder test. Black bars represent data from experiments 2a and 3a testing the combined effect of estradiol and CX on aggressive behavior. White bars represent data from experiments 2b and 3b testing the effects of CX alone on aggressive behavior. In short days, estradiol increased attack latency (A) and decreased bite frequency (C), and this effect was not blocked by CX. In contrast estradiol had no effect on aggression in long days (B & D). CX treatment alone had no effect on aggression whether mice were housed on short days (A& C) or long days (B & D). *Planned comparison, $p < 0.05$. $n = 8–11$ per group.

but there were no significant effects of AVP-ir cells or the percentage of colocalizations in any brain region examined under long day photoperiods (all p's > 0.05).

Discussion

These studies confirm and extend previous findings on the relationship between the environment and rapid actions of estrogens. Previously we showed that estrogens acted rapidly to increase aggression under short day photoperiods but not long day photoperiods. Rapid action of steroids is generally considered to be mediated by nongenomic processes. We confirmed that on Carefresh bedding, rapid action of estradiol on aggression persisted in the presence of a protein synthesis inhibitor, while the protein synthesis inhibitor itself did not alter aggressive behavior. The biological activity of CX was confirmed, because CX increased the number of AVP-ir cells. This is consistent with previous reports that CX interferes with AVP release (Ivell et al., 1992; Song et al., 2001). We recently reported that cage bedding, and in particular corncob bedding, determined the directional effect of estrogens on aggression. Here, we demonstrated that estradiol's nongenomic regulation of aggression is a robust phenomenon, generalizable to different laboratory conditions.

Nongenomic effects of estradiol on aggression

Our results indicating rapid effects of estradiol on aggressive behavior under short day photoperiods is in accordance with previous studies investigating the interaction of aggressive behavior and photoperiod. Previous studies in *Peromyscus* (conducted on corncob bedding) showed that when mice were injected with estradiol and rapidly tested in a resident-intruder paradigm, only mice housed in short days exhibited increased aggression (Trainor et al., 2007a; Trainor et al., 2008). A similar relationship between estradiol and aggressive behavior is observed in songbirds. When song sparrows were exposed to a simulated territorial intrusion (STI), it was shown that acute treatment with fadrozole minimized aggression in short day photoperiods, indicating that estrogens may be acting rapidly to affect territorial behavior during the nonbreeding season (Soma et al., 2000). Similarly, it was found that estradiol levels were rapidly suppressed in the BNST, hippocampus, and the ventromedial nucleus of the hypothalamus of male white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) following an STI (Charlier et al., 2011). Together, these studies show a strong relationship between the rapid regulation of estrogens and aggression which occurs under specific environmental conditions (short days or the nonbreeding season). In these studies nongenomic action was inferred via rapid action (Shughrue et al., 1997), but the role of protein synthesis was not directly examined.

An important consequence of gene transcription is the regulation of protein, so we used pretreatment with CX to test the hypothesis that estradiol regulates aggression via nongenomic mechanisms. Any behavioral effects observed following estradiol administration in the presence of CX should be a result of the nongenomic actions of estradiol. In short days we observed that aggressive behavior was rapidly suppressed following estradiol injections regardless of whether CX was administered concurrently. We also showed that CX alone has no effect on aggression. Together, these data indicate that even in the absence of protein synthesis, estradiol rapidly regulates aggression. This reinforces the inference that nongenomic pathways were activated under these conditions. This effect was not observed in long days, however, demonstrating that estradiol may mediate aggression via nongenomic mechanisms in short day photoperiods

Table 1
C-fos, AVP and AVP/c-fos colocalization cell counts in short and long day mice receiving saline, estradiol, or estradiol + CX treatments for rBNST, cBNST and PVN brain areas. Data shown as mean ± SE. Mice in short day photoperiods receiving estradiol or estradiol + CX had significantly fewer c-fos-ir cells in the cBNST as compared to mice receiving saline. Mice in short days receiving estradiol + CX had significantly more AVP-ir cells in the cBNST as compared to mice receiving saline or estradiol alone.

Region	Treatment	Protein	Short day	n	Long day	n
rBNST	Saline	c-fos	9.95 ± 2.68	5	64.53 ± 30.47	9
	Estradiol		6.85 ± 1.84	10	39.12 ± 17.97	9
	Estradiol + CX		8.53 ± 1.46	8	38.00 ± 23.45	10
cBNST	Saline		11.88 ± 3.65	5	24.06 ± 10.74	9
	Estradiol		4.88 ± 2.26*	10	15.6706 ± 7.01	9
	Estradiol + CX		4.92 ± 1.72*	8	12.13 ± 6.12	10
PVN	Saline		44.98 ± 11.85	8	146.94 ± 57.94	9
	Estradiol		29.92 ± 12.90	8	101.87 ± 41.19	10
	Estradiol + CX		31.39 ± 8.51	9	105.51 ± 44.98	10
rBNST	Saline	AVP	0.90 ± 0.49	5	0.86 ± 0.41	9
	Estradiol		1.86 ± 0.82	7	0.45 ± 0.18	9
	Estradiol + CX		2.75 ± 1.01	7	0.18 ± 0.11	10
cBNST	Saline		2.12 ± 0.87	5	1.65 ± 0.49	9
	Estradiol		1.82 ± 0.82	7	2.17 ± 0.94	9
	Estradiol + CX		5.29 ± 1.19*	7	0.89 ± 0.43	10
PVN	Saline		27.14 ± 3.79	8	16.58 ± 4.06	9
	Estradiol		18.54 ± 4.15	6	17.43 ± 5.15	10
	Estradiol + CX		23.26 ± 3.80	8	14.28 ± 3.89	10
rBNST	Saline	AVP/c-fos colocalizations	0.00 ± 0.00	5	8.33 ± 5.89	9
	Estradiol		0.00 ± 0.00	7	7.41 ± 5.63	9
	Estradiol + CX		1.53 ± 1.06	7	0.00 ± 0.00	10
cBNST	Saline		3.83 ± 2.34	5	13.45 ± 7.54	9
	Estradiol		1.39 ± 1.39	7	3.09 ± 3.09	9
	Estradiol + CX		4.14 ± 2.20	7	2.50 ± 2.50	10
PVN	Saline		7.18 ± 1.49	8	16.89 ± 6.77	9
	Estradiol		5.19 ± 3.28	6	6.03 ± 3.04	10
	Estradiol + CX		7.06 ± 4.04	8	10.38 ± 4.30	10

* $p < 0.05$.

only. Based on previous results (Trainor et al., 2007a; Trainor et al., 2007b; Trainor et al., 2008), it is conceivable that estradiol acts nongenomically to affect aggression in mice housed on corncob bedding, but this hypothesis should be directly examined in future studies.

Estradiol-mediated c-fos expression

Several in vivo studies have reported that estradiol induces phosphorylation of cAMP response element-binding protein (CREB) (Abraham et al., 2004; Szego et al., 2006; Zhou et al., 1996), a

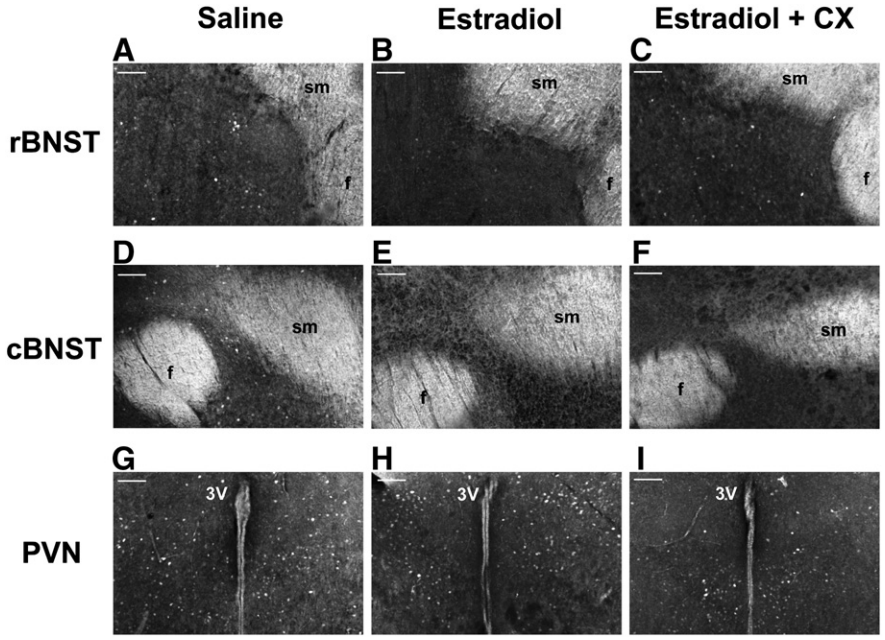

Fig. 5. Photomicrographs from short day mice of c-fos immunoreactivity in the rBNST (A–C), cBNST (D–F) and PVN (G–I) over three treatment conditions: saline (A, D & G), estradiol (B, E & H) and estradiol + CX (C, F & I). Mice treated with saline had significantly more c-fos-ir cells in the cBNST than mice receiving estradiol or estradiol + CX. rBNST and PVN cell counts were not significantly different between treatments. sm = stria medullaris, f = fornix, 3V = third ventricle. Bar = 100 um.

Table 2
C-fos, AVP and AVP/c-fos colocalization cell counts in short and long day mice receiving saline or CX treatments for rBNST, cBNST, and PVN brain areas. Data shown as mean ± SE. Mice in short day photoperiods demonstrated significantly more AVP-ir cells in the cBNST following CX treatment.

Region	Treatment	Protein	Short day	n	Long day	n
rBNST	Saline	c-fos	39.03 ± 7.98	8	17.91 ± 4.60	8
	CX		24.97 ± 6.32	8	25.85 ± 5.41	7
cBNST	Saline		11.63 ± 2.59	8	17.33 ± 3.96	8
	CX		10.98 ± 2.18	8	11.42 ± 1.43	6
PVN	Saline		86.63 ± 21.60	8	97.70 ± 12.68	8
	CX		71.41 ± 13.94	8	64.65 ± 8.62‡	6
rBNST	Saline	AVP	0.03 ± 0.03	8	0.25 ± 0.16	8
	CX		0.31 ± 0.12*	8	0.45 ± 0.22	7
cBNST	Saline		0.13 ± 0.09	8	0.94 ± 0.43	8
	CX		0.83 ± 0.26*	8	1.17 ± 0.51	6
PVN	Saline		3.63 ± 1.52	8	6.85 ± 1.46	8
	CX		8.34 ± 1.88‡	8	9.75 ± 2.39	6
rBNST	Saline	AVP/c-fos colocalizations	0.00 ± 0.00	8	3.13 ± 3.13	8
	CX		3.13 ± 3.13	8	7.14 ± 4.61	7
cBNST	Saline		0.00 ± 0.00	8	0.00 ± 0.00	8
	CX		3.91 ± 3.11	8	0.00 ± 0.00	6
PVN	Saline		6.41 ± 2.97	8	3.11 ± 1.30	8
	CX		7.33 ± 2.64	8	2.70 ± 1.21	6

* $p < 0.05$.
‡ $p < 0.1$.

transcription factor that plays an important role in stimulating the transcription of c-fos(Sheng et al., 1990). Studies by Boulware et al. (2005), however, indicate that estradiol can have a bidirectional effect on pCREB signaling. Upon interacting with membrane estrogen receptors, estradiol can concurrently influence two signaling pathways regulated by G-protein coupled metabotropic glutamate receptors (mGluRs). An excitatory pathway stimulates phospholipase C and mitogen activated protein kinase, thereby inducing CREB phosphorylation. An inhibitory pathway inhibits adenylyl cyclase and protein kinase A (PKA), thereby suppressing CREB phosphorylation (Boulware et al., 2005; Grove-Strawser et al., 2010). This discovery may provide an explanation for the dichotomous literature describing the effects of estradiol on pCREB and c-fos activation. Our results show that estradiol suppressed c-fos expression is consistent with the activation of PKA. We also found that this effect of estradiol on c-fos was only observed under short day photoperiods. This is consistent with recent data reporting the effects of estradiol on pCREB immunostaining in song sparrows. Under winter-like photoperiods estradiol reduced the number of pCREB positive cells in multiple nuclei related to song control as

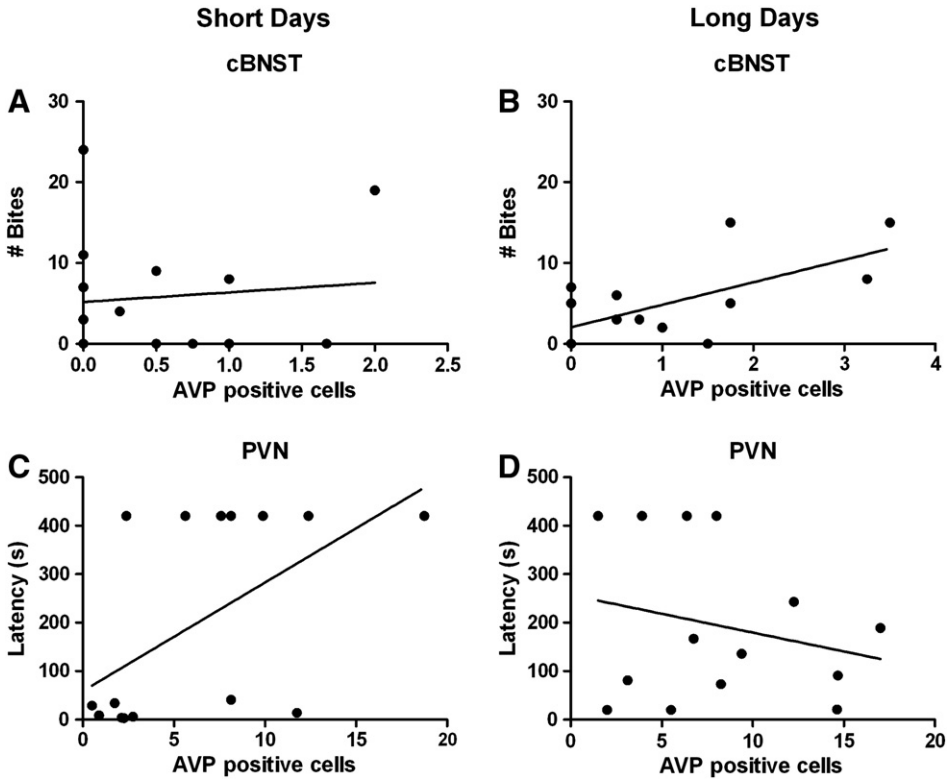

Fig. 6. Correlations between the number of bites and AVP-ir cells in the cBNST (A & B) and latency to attack and AVP-ir cells in the PVN (C & D). In mice housed in long days, there was a significant positive correlation between the number of bites and AVP-ir cells in the cBNST. In mice housed in short days, there was a significant positive correlation between latency to attack and AVP-ir cells in the PVN.

well as the medial preoptic nucleus (Heimovics et al., 2012). These data suggest that one possible mechanism for nongenomic estrogen regulation of aggression is through activation of PKA activity.

AVP and aggression

Previous work in songbirds and *Peromyscus* has indicated that AVP and its avian homolog, vasotocin (VT), are important for the expression of aggressive behavior (Bester-Meredith and Marler, 2001; Bester-Meredith et al., 1999; Goodson, 2008). California mice are more aggressive than white-footed mice (*Peromyscus leucopus*), and have more AVP-ir cells in the BNST and greater V1a receptor binding in the lateral septum than white-footed mice (Bester-Meredith et al., 1999). Furthermore, $V1_a$ receptor antagonists infused into the lateral ventricle reduced aggression in California mice but not in the white-footed mouse (Bester-Meredith et al., 2005). These studies were all conducted using long day photoperiods. Our data are consistent with these results, as we found that under long day photoperiods, the number of AVP-ir cells in the cBNST is positively correlated with the number of bites. In short days, however, this association was not present. These data suggest that vasopressin regulation of aggression may be more important in long days and less important in short days. There was, however, a significant positive correlation between the latency to attack and the number of AVP-ir cells in the PVN of short day mice which appears to be driven by non-aggressive mice demonstrating fewer AVP-ir cells in the PVN as compared to aggressive mice. Thus, there may be different AVP systems regulating aggression. Increased activity of AVP cell bodies in the BNST may promote aggressive behavior under long days, while increased activity of AVP neurons in the PVN may decrease aggression (Ho et al., 2010). If correct, this hypothesis could help explain why vasopression/vasotocin manipulations sometimes promote aggressive behavior but sometimes inhibit aggressive behavior (Goodson and Bass, 2001).

Conclusions

Previously, our lab demonstrated that there was a significant interaction between aggressive behavior and cage bedding (Villalon Landeros et al., 2012), with estradiol decreasing aggression on cardboard-based bedding and the reverse trend on corncob bedding. Previous studies investigating nongenomic effects of estradiol on aggression all utilized corncob bedding (Trainor et al., 2007a; Trainor et al., 2007b; Trainor et al., 2008), raising the question of whether these effects would generalize to an environment without phytoestrogen compounds. The most important finding in the current studies is that the rapid effects of estradiol on aggression are only observed under short day photoperiods. Thus, while bedding determines the directional effects of estrogens on aggression, photoperiod determines the mechanism of action. We extend previous findings by demonstrating that the rapid effects of estradiol occur independently of protein synthesis on Carefresh bedding, further supporting the hypothesis that estradiol acts nongenomically in short day photoperiods, but not long day photoperiods. Our immunostaining results suggest that these rapid effects of estradiol occur independently of vasopressin. When considered in the context of previous studies of vasopressin and aggression, it seems likely that vasopressin regulation of aggression may be more important under long day photoperiods.

In summary, we have shown that under short day photoperiods, estradiol rapidly suppresses aggressive behavior in California mice independent of protein synthesis. This strengthens the emerging theme that under both laboratory and field conditions seasonal cues play an important role in determining which molecular pathways are activated to regulate aggressive behavior.

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