



Nongenomic effects of estradiol on aggression under short day photoperiods



Sarah A. Laredo^{a,b,*}, Rosalina Villalon Landeros^{a,c}, James C. Dooley^{a,d}, Michael Q. Steinman^{a,e},
Veronica Orr^a, Andrea L. Silva^a, Katie K. Crean^a, Cindee F. Robles^a, Brian C. Trainor^{a,b,d,e}

^a Department of Psychology, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA

^b Animal Behavior Graduate Group, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA

^c Endocrinology and Reproductive Physiology Program, University of Wisconsin–Madison, 1025 West Johnson Street, Madison, WI 53706, USA

^d Center for Neuroscience, University of California Davis, 1544 Newton Court, Davis, CA 95616 USA

^e Molecular, Cellular and Integrative Physiology Graduate Group, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA

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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.

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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

* Corresponding author at: Animal Behavior Graduate Group, 135 Young Hall, 1 Shields Ave., University of California, Davis, CA 95616, USA.

E-mail address: salaredo@ucdavis.edu (S.A. Laredo).

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 tetrahydrofurandiol (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 cycloheximide (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

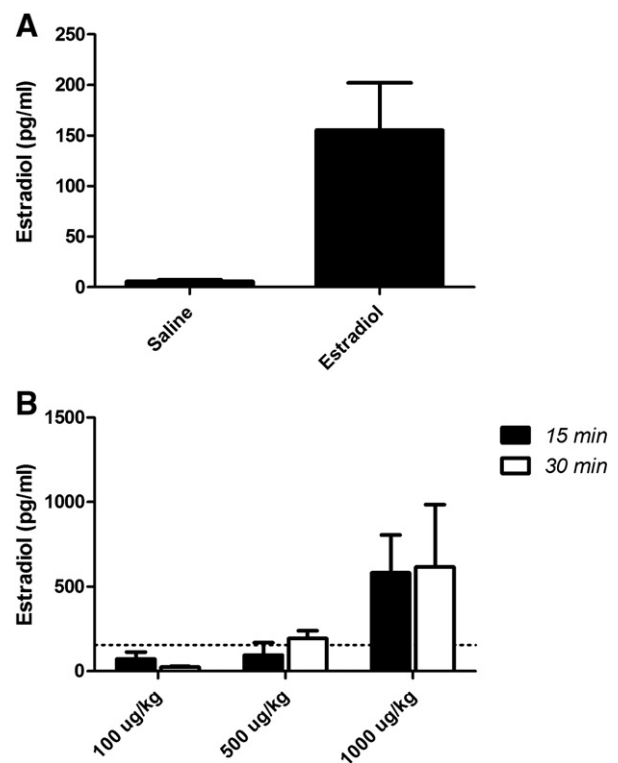


Fig. 1. Plasma estradiol levels (A) 15 min following a 100 µg/kg injection of estradiol ($n = 8$) or 100 µL saline ($n = 4$) and (B) 15 and 30 min following oral administration of estradiol. Doses of oral administration were 100 µg/kg (15 min: $n = 3$; 30 min: $n = 3$), 500 µg/kg (15 min: $n = 4$; 30 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.

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