Effects of photoperiod and experience on aggressive behavior in female California mice

Andrea L. Silva, William H.D. Fry, Colleen Sweeney, Brian C. Trainor

Department of Psychology, University of California, Davis, CA 95616, USA

UC Davis Cancer Center, 4645 2nd Avenue, Sacramento, CA 95817, USA

Article info

Article history:
Received 30 October 2009
Received in revised form 21 December 2009
Accepted 28 December 2009
Available online 7 January 2010

Keywords:
Aggression
Female
Amygdala
Hypothalamus

Abstract

Aggressive behavior among females is observed in many species, but the mechanisms of this behavior have historically been understudied. In many species of rodents, winter-like short day photoperiods induce increased aggression levels compared to summer-like long day photoperiods. Recent reports in hamsters show that short days also increase aggression in females. We examined the effects of photoperiod on aggression in female California mice, and for the first time compare brain activity of aggression-tested female rodents under different photoperiods. We observed that female California mice were more aggressive when housed in short days versus long days. Intriguingly, we also observed that under long days female attack latency decreases with repeated testing in resident–intruder tests. These data suggest that winner effects that have been described in males may also occur in females. We also used the expression of phosphorylated extracellular signal-regulated kinases (pERK) in the brain to estimate brain activity during aggression tests. pERK can alter neuronal activity in the short term and in the long term can act as a transcription factor. Using immunohistochemical analyses we observed that aggression-induced pERK expression in the female bed nucleus of the stria terminalis and medial amygdala occurs under both long and short days. Thus, the mechanisms controlling increased aggression under short days are still unclear and additional study is needed.

© 2010 Elsevier B.V. All rights reserved.
during winter-like short days. Female–female aggression in Syrian [24] and Siberian [25] hamsters is also increased under short days. In hamsters and Peromyscus, studies using c-fos as an indirect marker of neuronal activity found no differences in c-fos expression in several brain regions between long day and short day aggression-tested males [23,26], even though short day males were more aggressive. We recently used measurements of phosphorylated extracellular signal-regulated kinase (pERK, also known as mitogen activated protein kinase or pMAPK) as an alternative marker of extracellular signal-regulated kinase (pERK, also known as mitogen activated protein kinase or pMAPK) as an alternative marker of neuronal excitability [28] and over time can affect brain function acting as a transcription factor [29]. Following resident–intruder tests, pERK expression in the BNST increased when males were housed in short days but not long days [30]. This result suggested that pERK could be a useful marker for identifying effects of photoperiod on brain activity. Based on these results, we hypothesized that pERK might also be a useful marker for examining circuits of aggression in females.

In experiment 1, we used immunohistochemistry to identify the location of pERK positive cells in the social behavior network of female California mice housed in long days and short days. In experiment 2, we examined the effect of photoperiod on aggressive behaviors in females tested in three consecutive resident–intruder aggression tests. The rationale for this experiment was that multiple testing would allow for averaging of behavior across the estrous cycle. However, we also found that the effect of repeated testing differed across long day and short day females. Finally, in experiment 3, we examined the effect of photoperiod on aggressive behavior in females tested in a single resident–intruder aggression test. We then used immunobLOTS to quantify pERK expression in the BNST and MEA. Based on previous results in males, we hypothesized that aggression testing would increase pERK expression in the BNST and MEA in short day females but not long day females.

2. Methods

2.1. Animals and behavioral testing

Adult female California mice aged 3–6 months were bred in our laboratory colony. Mice were housed in clear polycarbonate cages provided with Carefresh bedding and cotton nestlets. Harlan Teklad 2016 food and water were provided ad libitum. All testing procedures were approved by the UC Davis Institutional Animal Care and Use Committee. Three days before resident–intruder aggression or control testing, each female was individually housed. In both long day and short day light cycles lights were turned off at 1400 Pacific Standard Time (PST). All behavioral tests were conducted under dim red light between 14:30 and 17:00 PST. Resident–intruder aggression tests were conducted by introducing an unfamiliar group-housed female intruder into the resident’s home cage. All intruders were unrelated to residents and housed in long days. It is possible that different results could have been obtained had we tested short day females with intruders housed in short days. Using an infrared camera, aggressive behavior was observed for 10 min after which the intruder was removed. In control tests the lid of the cage was removed and replaced to simulate the handling during a resident–intruder test.

Animals were maintained in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experiment 1: effect of photoperiod on diestrus females and immunohistochemical detection of pERK

A total of 34 virgin female mice were randomly assigned to be housed in long days (16L:8D) or short days (8L:16D) for 8 weeks. Females were group housed with 2–3 mice per cage. Estrous cycle stage was determined on the morning of testing (08:00–12:00 PST) by vaginal lavage [20] and c-fos expression was measured using immunohistochemistry. Immediately after aggression tests, each resident mouse was anesthetized with isoflurane and rapidly decapitated. Vaginal lavage was conducted as described above post mortem. Interestingly, similar to male California mice, females were not observed to be reproductively suppressed in short days and continued to cycle through proestrus and estrus (Table 1).

2.3. Experiment 2: effect of photoperiod across multiple resident–intruder tests

To avoid the stressful effects of handling (for vaginal lavage) prior to resident–intruder aggression tests, we conducted a second study in which females were tested 3 times on successive days. The rationale for testing on consecutive days is that this would ensure that our sample of aggressive behavior would cover multiple stages of the estrous cycle. A total of 18 virgin females were randomly assigned to be housed in long days or short days (2–3 mice per cage). After 8 weeks, each female was individually housed for three days, and resident–intruder aggression was conducted as described above. A different intruder was used for each resident during each of the three aggression tests. Immediately after each test, each female was anesthetized with isoflurane and rapidly decapitated. Vaginal lavage was conducted as described above post mortem. Interestingly, similar to male California mice, females were not observed to be reproductively suppressed in short days and continued to cycle through proestrus and estrus (Table 1).

2.4. Experiment 3: expression of phosphoERK and total ERK following resident–intruder tests

Twenty-five females were randomly assigned to long days or short days for 8 weeks. Each female was either tested in a single resident–intruder test or control test as described above. Immediately after testing, each mouse was anesthetized with isoflurane and rapidly decapitated. The brain was rapidly removed and a brain matrix was used to collect 2 mm coronal sections [32]. A slice starting at the optic chiasm and ending 2 mm anterior contained the BNST. A second slice starting at the optic chiasm and ending 2 mm posterior contained the MEA. Sections were cooled on a freezing plate and bilateral 1 mm punch samples of each brain area were collected and frozen on dry ice. Samples were stored at −80 °C until processed for western blot analyses.

2.5. Immunohistochemistry

In experiment 1, it appears that handling from vaginal lavage reduced the expression of aggressive behaviors, so only a small subset of brains were examined with immunohistochemistry to identify brain regions expressing pERK. Brains were sectioned at 40 μm on a microtome. Sections were stored in cryoprotectant (50% phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol) at −20 °C. Sections were then washed 3 times in PBS and incubated in 1% sodium borohydride in PBS for 10 min. Sections were then blocked in 10% normal goat serum and 0.3% hydrogen peroxide in PBS for 20 min. Sections were then incubated in primary pERK antibody (64370, Cell Signaling, Danvers, MA), concentration 1:250 dissolved in 2% normal goat serum and 0.5% triton X (TX) in PBS overnight at 4 °C on an orbital shaker. The specificity of this antibody in male Peromyscus is described by Trainor et al. [30]. The sections were then washed three times in PBS before transferring to biotinylated goat–rabbit antibody in 2% normal goat serum in PBS (TX) (Vector Laboratories, Burlingame, CA, 1:500) for 2 h. Sections were washed 3 times in PBS and incubated in avidin–biotin complex (ABC Elite Kit, Vector Laboratories) for 30 min. Sections were washed in PBS and developed in nickel-enhanced diaminobenzidine (Vector Laboratories) for 2 min. Sections were then mounted, dehydrated, and coverslipped. Slides were analyzed using a Zeiss Axiosmager equipped with an Axioscam MRc camera. Representative photomicrographs of each of the following brain areas were identified using a mouse brain atlas [33]: bed nucleus of the stria terminalis (bregma-0.02), basolateral amygdala (BLA, bregma-1.22), central nucleus of the amygdala (CEA, bregma-1.22), ventromedial hypothalamus (bregma-1.58) and medial amygdala (bregma-1.77).

2.6. Estradiol enzyme immunnoassay

We measured estradiol using an enzyme immunnoassay kit (Cayman Chemical, Ann Arbor, MI). When assay concentrations for serial dilutions of a California mouse plasma pool were compared with standards, computed regression lines did not differ in slope (p > 0.2). The sensitivity of this assay is 6.6 pg/mL and the intra-assay coefficient of variation was 14.3%. We measured estradiol in the 18 females tested in aggression tests for experiment 2 as well as 9 females tested in control tests (5 long day, 4 short day).

2.7. Western blot analyses of extracellular signal-regulated kinase (ERK) and pERK

Punch samples were homogenized in 150 μL of RIPA buffer containing 1 mM of phenylmethylsulphonylfluoride (PMSF). Total protein concentrations were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Estrous cycle stages in third aggression test of experiment 2.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diestrus</td>
</tr>
<tr>
<td>Long day</td>
<td>6</td>
</tr>
<tr>
<td>Short day</td>
<td>6</td>
</tr>
</tbody>
</table>
determined by Bradford assay and 20 μg of protein was separated by polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose membranes and blotted with pERK antibody (1:1000, #4370, Cell Signaling) then stripped and reblotted with total ERK antibody (1:1000, #4695, Cell Signaling). Antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Invitrogen) followed by development with SuperSignal West chemicals (Pierce, Rockford, IL, USA). An Alpha Innotech imaging station with FluorChem software was used to capture images. The p42 and p44 isoforms of both total ERK and pERK were detected with horseradish peroxidase-conjugated secondary antibodies (Invitrogen). Antibodies were quantified.

2.8. Statistical analyses

After inspecting behavior data with Q-Q plots to assess normality [34], the number of bites was square root transformed and attack latency was log transformed. In experiment 2, a mixed model repeated measures ANOVA was used to assess effects of photoperiod on number of bites and attack latency across the three aggression tests. We used the conservative Greenhouse-Geisser correction for all within-subject comparisons. Independent t-tests were used to test for effects of photoperiod within each of the three aggression tests. We also used a Chi-square test to determine whether photoperiod influenced the likelihood that females would be in different stages of the estrous cycle. Estradiol levels were log transformed and analyzed with three-way ANOVA testing for effects of photoperiod, aggression testing, and estrus cycle. In experiment 3, pERK (normalized to total ERK) expression levels as well as total ERK band intensities were analyzed transformed and analyzed with three-way ANOVA testing for effects of photoperiod, aggression testing, and estrus cycle. We also used a conservative Greenhouse-Geisser correction for all within-subject comparisons. Independent t-tests were used to test for effects of photoperiod within each of the three aggression tests. We also used a Chi-square test to determine whether photoperiod influenced the likelihood that females would be in different stages of the estrous cycle. Estradiol levels were log transformed and analyzed with three-way ANOVA testing for effects of photoperiod, aggression testing, and estrus cycle. In experiment 3, pERK (normalized to total ERK) expression levels as well as total ERK band intensities were analyzed transformed and analyzed with three-way ANOVA testing for effects of photoperiod, aggression testing, and estrus cycle.

3. Results

3.1. Experiment 1: pERK positive cells in the female brain following aggression tests

Although on average short day housed females bit intruders (mean ± s.e., 2.4 ± 0.9) twice as frequently as long day housed females (1.2 ± 0.66), this difference was not statistically significant (t_{19} = 1, ns). Similarly, although short day housed females had shorter mean attack latencies (360 ± 82 s) than long day housed females (433 ± 70 s), this difference was not significant (t_{19} = 1, ns). Based on our subsequent studies in which vaginal lavage was conducted post mortem, we believe that the low aggression levels observed in this study are most likely an effect of conducting lavage prior to testing (about 4 h). Immunohistochemistry analyses detected expression of pERK in several brain areas related to aggressive behaviors including the MEA, BNST, VMH, anterior hypothalamus, and basolateral amygdala (Fig. 1).

3.2. Experiment 2: effect of photoperiod across multiple resident–intruder tests

When females were tested in multiple aggression tests without vaginal lavage prior to testing, repeated measures ANOVA indicated that residents bit intruders significantly more in short days compared to long days (Fig. 2A, F_{1,19} = 5.3, p = 0.03). The number of bites did not change significantly across the three aggression tests and there was no test by photoperiod interaction (all ps > 0.6). Although repeated measures ANOVA indicated there was no significant overall effect of photoperiod on attack latency (F_{1,19} = 2.73, p = 0.11), there was a nonsignificant trend for test by photoperiod interaction (F_{1,1,49} = 2.93, p = 0.08). Repeated measures ANOVA of long day mice indicated a significant decrease in attack latency across the three tests (F_{1,1,24} = 4.09, p = 0.04), whereas the same analysis of short day mice showed no significant change in attack latency across the three tests (F_{1,1,16} = 0.67, p = 0.45). Independent t-tests showed that short day females had shorter attack latencies than long day females in the first test (Fig. 2B, t_{19} = 3.0, p = 0.01) but not the second (Fig. 2C, t_{19} = 1.5, p = 0.16) and third tests (Fig. 2D, t_{19} = 0.01, p = 0.99).

There was no evidence that females were more likely to be diestrus in short days compared to long days after the third aggression test (Table 1, χ2 = 0.15, ns). Estradiol levels varied significantly across the estrous cycle (Fig. 3, F_{1,16} = 4.0, p < 0.05) and were higher in proestrus females than in diestrus females (planned comparison, p = 0.02). There was a nonsignificant trend for estradiol levels to be higher in proestrus females compared to estrus females (planned comparison, p = 0.06). There was no significant effect of photoperiod (F_{1,21} = 1.2, p > 0.05) or aggression testing (F_{1,21} = 1.3, p > 0.05).

Fig. 1. Photomicrographs of phosphorylated extracellular signal-regulated kinase (pERK) immunoreactivity in the BNST (A), MEA (B), VMH (C), and BLA (D). Abbreviations: a.c., anterior commissure; f, fornix; o.t., optic tract; e.c. external capsule. Scale bars = 1 mm.
3.3. Experiment 3: expression of pERK following resident–intruder tests

When females were tested in a single aggression test without vaginal lavage prior to testing, females bit intruders more frequently (Fig. 4, $t_{15} = 3.87, p < 0.01$) and had shorter attack latencies (Fig. 4, $t_{15} = 3.79, p < 0.01$) when housed under short days versus long days. In punch samples of the BNST, aggression testing caused a significant increase in expression of the pERK p44 isoform (Figs. 5B and 6A, $F_{1,12} = 5.22, p < 0.05$) and there was a nonsignificant trend for increased expression of the pERK p42 isoform (Figs. 5A and 6A, $F_{1,12} = 4.53, p = 0.06$). There was no effect of photoperiod or interac-

Fig. 2. Short days increased the average number of bites across three resident–intruder tests (A). Repeated measures analyses of variance showed that attack latency significantly decreased across the three tests in long day mice but not short day mice (B, C, D). Note that individual dots represent individual data points and lines indicate means. (*) $p < 0.05$.

Fig. 3. Estradiol levels in females from experiment 2 ($n = 4–6$ per group). Estradiol levels were significantly higher in proestrus females than diestrus females. Among diestrus females, estradiol levels were higher in short days. (†) Effect of estrous cycle stage $p < 0.05$, (*) effect of photoperiod $p < 0.05$.

Fig. 4. Effects of photoperiod on aggression in a single aggression test ($n = 8–9$ per group). Females bit intruders more frequently and had shorter attack latencies when housed in short days. (*) $p < 0.05$.

Fig. 5. Effects of aggression testing on pERK activation (relative to total ERK activation) in the BNST (A and B) and MEA (C and D) ($n = 4$ per group). Aggression testing significantly increased expression of the p42 isoform in the BNST (A) and MEA (C). Aggression testing significantly increased expression of the p44 isoform in the MEA (D) whereas there was a nonsignificant trend in the BNST (B). (*) $p < 0.05$ effect of aggression testing and (+) $p = 0.055$ effect of aggression testing.
increased aggression phenotype observed under short days. We also observed that repeated aggression testing decreased attack latency in long day females but not short day females. Previous work in males has shown that winning aggressive encounters can increase aggressive behavior in males, including California mice [35]. These changes in behavior are thought to comprise part of the “winner effect”, in which the experience of winning aggressive encounters increases the probability of winning future encounters independent of intrinsic competitive ability [36]. Our results suggest that winner effects may also occur in females.

4. Discussion

Female California mice show a similar phenotype to males in that aggression levels in resident–intruder tests are increased under short days. However, in contrast to males, increased pERK expression was induced in the BNST and MEA following aggression tests under both long days and short days. In males, aggression-induced pERK expression in the BNST was observed only under short days. In contrast to males, increased pERK expression in the MEA following aggression tests was induced in the BNST and MEA following aggression tests under both long days and short days. However, in contrast to males, increased aggression phenotype observed under short days. We also observed that repeated aggression testing decreased attack latency in long day females but not short day females. Previous work in males has shown that winning aggressive encounters can increase aggressive behavior in males, including California mice [35]. These changes in behavior are thought to comprise part of the “winner effect”, in which the experience of winning aggressive encounters increases the probability of winning future encounters independent of intrinsic competitive ability [36]. Our results suggest that winner effects may also occur in females.

4.1. Photoperiod and circuits of aggression in female California mice

As has been reported for Syrian and Siberian hamsters, we have observed that both male and female California mice show higher aggression levels when housed in short day photoperiods. However, it appears that the mechanisms underlying this environmental modulation of aggression differ in males and females. In male California mice, aggression-induced pERK in the BNST is only observed in short day mice whereas pERK in the MEA is elevated under short days in both control and aggression-tested mice [30]. Based on these data, we hypothesized that only more aggressive short day female California mice would have increased aggression-induced pERK expression in the BNST and/or MEA. Our immunoblot analyses do not support this hypothesis, as aggression-induced pERK expression was observed under both long days and short days. In the BNST the p44 isoform was more consistently elevated by aggression tests whereas there was only a nonsignificant trend for an increase in the p42 isoform. In contrast, in the MEA resident–intruder testing caused a significant increase in both isoforms of pERK. It is possible that pERK in the BNST and MEA may have different effects on aggression in males and females, but we have not determined that pERK plays a causal role in regulating aggression. A related possibility is that there may be sex differences in the neurochemical pathways that regulate pERK expression. Recent data suggest there are important sex differences in the hormonal regulation of aggression in photoperiod sensitive rodents, which could affect pERK regulation.

In male California mice and old field mice (P. polionotus), estrogens act rapidly to increase aggression in short days but not long days ([37,23]). Rapid action of steroid hormones is usually assumed to be mediated by nongenomic pathways [38], which do not involve changes in transcription mediated by nuclear estrogen receptors. For example, estrogens act rapidly to phosphorylate ERK [39]. Although no study has tested whether estrogens act rapidly to regulate aggression in virgin female rodents, recent studies in hamsters suggest that the relationship between photoperiod and estrogen function is different in females. In Siberian hamsters ovariectomy does not significantly alter aggression whether females are housed in long days or short days [25]. Intriguingly, when ovariectomy was combined with exogenous estradiol implants, aggression levels were reduced. Similar results were reported in female Syrian hamsters [40]. Thus, while rapid estrogen action has been linked to increased male aggression in short days, it appears unlikely that estrogens facilitate increased aggression during short days in females. Our measurements of estradiol showed that similar to other rodents [31], estradiol levels increased during proestrus. Combined with our observations that females continue to cycle in short days, these data reinforce observations from males that reproduction in California mice is not inhibited in short days [23,41].

An important consideration is that to some extent the increased expression of pERK following aggression tests was caused by exposure to odors from intruders as opposed to engaging in aggressive behavior per se. For example, female odors have been shown to increase pERK expression in the vomeronasal system of male mice.
4.2. Effects of experience on aggression

In experiment 2 females showed a significant decrease in attack latency between the first and third aggression tests, but only if they were housed in long days. Increased aggression after winning aggressive encounters has been observed widely across the animal kingdom in males [44–46], but we are not aware of any previous reports showing a similar effect in females. This change in aggressive behavior is thought to be a component of the “winner effect”, which occurs when the experience of winning increases the probability that an individual wins an aggressive contest. In males, testosterone levels are often observed to be increased after winning aggressive encounters [35,47,48], and this increase is necessary for future changes in aggression to occur [49]. In females, less is known about the hormonal changes associated with winning aggressive encounters. Female California mice housed in long days exhibit decreased progesterone following aggressive encounters [14]. Interestingly, aggressive challenges in an African black coucal (a species of cuckoo) also resulted in a rapid decrease in progesterone levels in females [50]. Progesterone can affect behavior rapidly [51], raising the possibility that rapid changes in progesterone could mediate the effects of experience on aggression in females. We did not observe changes in aggression in short day mice, although this could be due to a ceiling effect given that short day mice had aggression levels that were already very high in initial aggression tests.

It could be argued that the effects of experience on aggression in long day females would make photoperiod differences irrelevant, because aggression levels in long day females would increase by winning fights. However, gaining a territory is an important challenge for California mice as females typically disperse from their natal home range [52]. Furthermore, the winner effect is only observed when a male is tested in the home cage [46]. Although it is not clear whether this effect is also present in females, it is likely that initial aggressive encounters for both males and females end in losses. Thus in the absence of winning experiences, photoperiod could exert critical effects on the outcome of aggressive encounters and whether an individual (male or female) gains a territory.

5. Conclusions

Like males, females show higher aggression levels when housed in short days versus long days. However, it appears that different mechanisms are used to achieve this phenotype. Our immunoblot analyses on females showed that aggression-induced pERK expression in the BNST is observed in both long and short days. In contrast, for males aggression-induced pERK expression in the BNST is observed only in short days. These results resemble findings on the mechanisms regulating parental behavior in prairie voles [53]. Both male and female prairie voles exhibit parental behaviors, yet males have more AVP mRNA than females after pups are born [54]. Furthermore, vasopressin promotes parental behavior in males but has no effect on females [55]. Thus, a sex difference in AVP function is associated with the expression of parental behavior in both males and females. Similarly, we observed that male and female California mice exhibit shorter attack latencies after winning aggressive encounters, yet previous studies show that females have a very different hormonal profile than males after winning aggressive encounters [14]. Future studies will be needed to determine the mechanisms used by females to translate information on photoperiod and winning experiences into changes in aggressive behavior.

Acknowledgements

The authors thank Katie Crean, Michael Steinman, and Julie Van-Westenhuzyen for technical help, and Cindy Clayton for animal care. This work supported by a President’s Undergraduate Fellowship award to A.L.S. and grants from the UC Davis Hellman Fellows Program and R01 MH85069 to B.C.T.