Published in final edited form as:

Neuroscience. 2010 January 20; 165(2): 325. doi:10.1016/j.neuroscience.2009.10.050.

Activation of extracellular signal-regulated kinases in social behavior circuits during resident-intruder aggression tests

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Abstract

Using a variety of experimental methods, a network of brain areas regulating aggressive behaviors has been outlined in several groups of vertebrates. However, aggressive behavior expressed in different contexts is associated with different patterns of activity across hypothalamic and limbic brain regions. Previous studies in rodents demonstrated that short day photoperiods reliably increase both male and female aggression versus long day photoperiods. Here we used immunohistochemistry and western blots to examine the effect of photoperiod on phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK) in male California mice (*Peromyscus californicus*) during resident-intruder tests. Phosphorylated ERK (pERK) can alter neuronal activity in the short term and in the long term acts as a transcription factor. In the posterior bed nucleus of the stria terminalis (BNST) males tested in aggression tests had more phosphorylated ERK (pERK) positive cells when housed in short days but not long days. This result was replicated in western blot analyses from microdissected BNST samples. In the medial amygdala, immunostaining and western analyses showed that pERK expression also was generally increased in short days. Immunostaining was also used to examine phosphorylation of cyclic AMP response element binding protein (CREB). CREB can be phosphorylated by pERK as well as other kinases and functions primarily as a transcription factor. Intriguingly, aggressive interactions reduced the number of cells stained positive for phosphorylated CREB in the infralimbic cortex, ventral lateral septum and MEA. This effect was observed in mice housed in long days but not short days. Overall, these data suggest that different (but overlapping) networks of aggressive behavior operate under different environmental conditions.

Keywords

basolateral amygdala; estrogens; androgens; mitogen activated kinase; social behavior; infralimbic cortex; stress

Introduction

Aggressive behavior is complex and is affected by a variety of physiological and environmental factors. There is growing evidence that there may be multiple neurobiological circuits that regulate aggressive behavior. For example, individuals diagnosed with depression or

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intermittent explosive disorder can be prone to exaggerated aggressive behavior, which is typically impulsive and occurs in a context of extreme arousal (Fava 1998; Blair 2004). In contrast, aggressive behavior observed in individuals diagnosed with antisocial personality disorder or conduct disorder typically engage in aggression in a state of hypoarousal (reviewed in (Haller and Kruk 2006)). Although the specific behaviors expressed during impulsive and instrumental aggression may sometimes appear superficially similar, different physiological mechanisms appear to be activated in these different contexts. These data suggest that it will be essential to understand the mechanistic bases of aggressive behavior in different contexts to develop successful behavioral or physiological interventions. Neural circuits regulating aggression have been identified in a variety of species (Nelson and Trainor 2007), but very little is known about how these circuits function in different environmental conditions.

A common technique for outlining neural circuits of aggression has been the measurement of immediate early genes (ieg) which are used as indirect markers of neuronal activation. The most widely used ieg to study male aggression has been c-fos, and consistent results have emerged across species and laboratories (Kollack-Walker and Newman 1995; Delville et al. 2000; Veenema et al. 2003; Gobrogge et al. 2007; Veenema et al. 2007). Male-male aggressive encounters reliably induce c-fos in the bed nucleus of the stria terminalis (BNST), ventromedial hypothalamus (VMH), and medial amygdala (MEA). In most species aggressive behavior induces c-fos in the anterior hypothalamus (AH), and reduced c-fos in the lateral septum (LS) has been observed in rats and mice. One limitation to measuring c-fos is that not every activated neuron produces c-fos, so important changes in neuronal activity could remain undetected (Hoffman and Lyo 2002). Studies in California mice (*Peromyscus californicus*) (Trainor et al. 2008b) and Siberian hamsters (*Phodopus sungorus*) (Jasnow et al. 2002) have demonstrated that male aggressive behavior is increased in short day photoperiods compared to long day photoperiods. However, when c-fos was examined following aggressive encounters in California mice (Trainor et al. 2008b) and hamsters (Kramer et al. 2008), no effect of photoperiod on c-fos positive cells was observed in any brain region examined. These results were perplexing because of the large differences in aggression behavior. Although it is possible that c-fos expression in brain areas that were not examined by these studies could show important differences, we hypothesized that alternative indicators of activity could provide a different perspective on the workings of neural circuits regulating aggression.

Specifically, we hypothesized that measurements of phosphorylated cyclic AMP response element binding protein (CREB) and extracellular signal-regulated kinases 1 and 2 (ERK) might be useful proteins for identifying differences in brain activity related to photoperiod. These proteins can be rapidly phosphorylated by estrogens (Abraham et al. 2004; Zsarnovszky et al. 2005; Szego et al. 2006), and we previously showed that estrogens act rapidly in male *Peromyscus* to increase aggression in short days but not long days (Trainor et al. 2007; Trainor et al. 2008b). Once phosphorylated, ERK can exert immediate effects by altering neuronal excitability (Selcher et al. 2003) and can exert long term effects on neuronal function by acting as a transcription factor (Valjent et al. 2001). CREB functions as transcription factor (Brindle et al. 1993) and is phosphorylated by ERK and other kinases such as protein kinase C (Roberson et al. 1999). Thus examining phosphorylated CREB (pCREB) provides an integrated estimate of cellular activity. Previous studies have used immunostaining for pCREB as a marker of neuronal activity (Gammie and Nelson 2001; Nichols and DeBello 2008), but the use of pERK is less prevalent (Gerrits et al. 2006). Because our previous study showed that estradiol acts rapidly to increase aggression in male California mice housed in short days (Trainor et al. 2008b), we hypothesized that aggression testing would increase pCREB and pERK immunostaining primarily in short days. In immunostaining experiments, we examined a wide range of brain nuclei on the chance that important changes in brain activity related to aggression may have escaped detection in studies using c-fos. We then used western blots, which are easier to quantify, to confirm patterns of ERK expression in the BNST, MEA, and VMH.

Materials and Methods

Adult male California mice were purchased from the *Peromyscus* Stock Center (University of South Carolina, Columbia, SC) and bred in our laboratory colony. Mice were housed in clear polypropylene 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. Animals were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Behavioral Experiments

Male mice were randomly assigned to be housed in long days (light:dark, 16:8) or short days (light:dark, 8:16) for 8 weeks. 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. In Experiment 1 we examined the effect of photoperiod and aggression testing on pERK and pCREB immunoreactivity. Mice housed in long days were either tested in resident-intruder aggression tests ($n = 10$) or in control tests ($n = 6$). Mice housed in short days were also tested in either aggression tests ($n = 12$) or control tests ($n = 6$). In Experiment 2 we examined the effect of photoperiod and aggression testing on ERK and pERK levels via western blot ($n = 3$ -4 mice per group). Our goal was to combine the anatomical resolution of immunostaining (Experiment 1) with the quantitative power of immunoblots (Experiment 2) to obtain an accurate picture of how photoperiod regulates the activation of ERK during aggression tests. When presenting data from immunostaining experiments we provide greater anatomical detail (e.g. posterodorsal MEA) on the region of interest. When presenting data from immunoblot experiments we refer to the entire nucleus (MEA) to reflect that multiple subregions are included in the micropunch samples.

In resident-intruder tests a group-housed sexually inexperienced male intruder was introduced into the home cage of each resident. Tests lasted for seven minutes after which the intruder was removed. Behavioral observations were digitally recorded and scored by an observer without knowledge of treatment assignments. In control tests the lid of the cage was removed and replaced to simulate the addition and removal of the intruder. Immediately after the aggression or control test, each resident was anesthetized with isoflurane gas for about 2 min and rapidly decapitated. Previous work in rats demonstrated that when quantifying pERK expression, isoflurane anesthesia followed by decapitation is a preferred method of euthanasia compared to sodium pentobarbital (Takamura et al. 2008). In Experiment 1, the brain was quickly removed and placed in 5% acrolein in 0.1 M phosphate saline buffer (PBS). Each brain was fixed in acrolein overnight at 4° C, transferred to 25% sucrose in PBS for 48 hrs at 4° C, and frozen on dry ice. Brains and plasma samples were stored at -40° C. In Experiment 2, each brain was quickly dissected with a brain matrix to generate coronal slices (Trainor et al. 2003). A slice containing the BNST and MPOA was collected by cutting at the optic chiasm and 2 mm anterior. A second slice containing the VMH and MEA began at the optic chiasm and ended 2 mm posterior. Each section was chilled on a freezing plate and bilateral samples for each nucleus were dissected using a 1 mm punch. Samples were snap frozen on dry ice and stored at -80° C. For one brain, we immersion fixed (5% acrolein) the remaining sections after punch samples were collected. After cutting 40 μ m sections on a cryostat and counterstaining these sections with eosin, we confirmed that our dissection protocol isolates the expected regions (Fig. 1F).

Immunohistochemistry

For immunostaining experiments we sectioned a subset of brains from the behavior tests described above (long day control, $n = 6$; long day aggression, $n = 8$; short day control, $n = 6$;

short day aggression, $n = 8$). Brains were sectioned at 40 μm on a microtome and stored in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v polyvinylpyrrolidone, 30% v/v 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 (#4370, Cell Signaling, Danvers, MA, concentration 1:250) or pCREB (#9198, Cell Signaling, concentration 1:100) antibodies dissolved in 2% normal goat serum and 0.5% triton X (TX) in PBS overnight at 4°C on an orbital shaker. The primary pERK antibody recognizes both ERK1 (p44) and ERK2 (p42). The sections were then washed three times in PBS before transferring to biotinylated goat anti-rabbit antibody in 2% normal goat serum in PBS TX (Vector Laboratories, Burlingame, CA, 1:500) for 2 hr. Sections were washed 3 times in PBS and incubated in avidin-biotin complex (ABC Elite Kit, Vector Laboratories) for 30 min. Sections were then washed 3 times in PBS and developed in nickel enhanced diaminobenzidine (Vector Laboratories) for 2 minutes. Sections were then rinsed in PBS and mounted onto plus slides (Fisher, Pittsburgh, PA). Slides were dehydrated in ethanol followed by Histoclear (National Diagnostics, Atlanta GA) and coverslipped with Permount (Fisher).

The primary antibodies listed above have been used previously in other rodent species to assess phosphorylation of ERK and CREB (McNulty et al. 1998; Abraham et al. 2004; Springell et al. 2005). In validation studies, omission of primary antibody resulted in no positive staining. We also found that pre-incubation with the immunizing peptide against pERK, but not total ERK, prevented positive pERK staining (Fig. 1A, B, C). Preincubation with pCREB immunizing peptide prevented positive pCREB staining (Figure 1D, E). Positive staining in California mouse brain samples appeared similar to reports in other species, with primarily nuclear staining for pCREB and cellular staining for pERK.

Image Analysis

We focused our analyses on brain areas that comprise a social behavior network that controls a variety of social behaviors (Newman 1999; Goodson 2005), including aggression (Delville et al. 2000; Nelson and Trainor 2007). Slides were analyzed using a Zeiss Axioimager equipped with an AxioCam MRC camera. Representative photomicrographs of each of the following brain areas were identified using a mouse brain atlas (Paxinos and Franklin 2002): infralimbic cortex (IFL, bregma 1.54), agranular insular cortex (AI, bregma 1.54) claustrum (CL, bregma 1.54), ventral lateral septum (VLS, bregma 0.26), posterior BNST (bregma 0.02), medial preoptic area (MPOA, bregma 0.02), anterior hypothalamus (AH, bregma -0.58), basolateral amygdala (BLA, bregma -1.22), central nucleus of the amygdala (CEA, bregma -1.82), lateral amygdala (LA, bregma -1.82), ventrolateral VMH (bregma -1.82), posterodorsal medial amygdala (MEA, bregma -1.82), CA1 region of hippocampus (CA1, bregma -1.82), dentate gyrus (DG, bregma -1.82), periaqueductal gray (bregma -4.84). The background for each image was normalized by adjusting the exposure time. The number of immunopositive cells in each brain area was counted in a frame of uniform size (Table 1) using Image J (NIH, Bethesda, MD) by an observer unaware of treatment assignments. A constant threshold of staining was set to determine positively stained cells, and the number of positive cells was counted using the “analyze particles” function of Image J. Analyses of pERK staining in the paraventricular nucleus will be described elsewhere (Trainor, Takahashi, Silva, Crean, and Hostetler submitted).

Western Blot Analyses

Punch samples were homogenized in 100 μl of RIPA buffer containing 1 mM of phenylmethanesulphonylfluoride (PMSF) and 50 mM sodium fluoride. Total protein concentrations were determined by Bradford assay and 20 μg of protein was separated by polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes.

Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 hr. Membranes were then incubated with pERK (1:1000, #4370, Cell Signaling), ERK (1:1000, #4695, Cell Signaling), or GAPDH (1:2000, #2118, Cell Signaling) antibodies in TBST overnight at 4° C. Membranes were then washed three times in TBST and incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000, Invitrogen) for 1 hrs. After washing 3 times in TBST, membranes were visualized with SuperSignal West chemicals (Pierce, Rockford, IL, USA). An Alpha Innotech imaging station with FluorChem software was used to capture images. Both ERK1 and ERK2 showed similar patterns of expression levels (for both pERK and total ERK), so we combined ERK1 and ERK2 expression levels for statistical analyses of pERK and total ERK.

Data Analysis

The effect of photoperiod on aggressive behavior was assessed with independent t-tests. Cell count and western blot data were analyzed with two-way ANOVA testing for effects of aggression testing, photoperiod, and the interaction. Planned comparisons were used to test the effect of aggression testing within each photoperiod group. Nonparametric Spearman correlations were used to examine relationships between immunostaining and behavior.

Results

Aggressive Behavior

Housing in short days increased aggressive behavior. Males housed in short days showed significantly shorter attack latencies (Fig. 2A, $t_{20}=2.185$, $p = 0.04$) and bit intruders more frequently (Fig. 2B, $t_{20}=2.7$, $p = 0.01$).

pERK Immunostaining

There was a significant photoperiod $\times$ aggression interaction on the number of pERK positive cells in the posterodorsal MEA (Fig. 3E-H, $F_{1,24} = 5.34$, $p < 0.05$), posterior BNST (Fig. 3A-D, $F_{1,24} = 4.46$, $p < 0.05$), and VLS (Table 1, $F_{1,24} = 8.81$, $p < 0.01$). Planned comparisons indicated that aggression testing increased the number of pERK cells in the posterodorsal MEA and posterior BNST of short day males but not long day males (Figure 3I, J). In the VLS aggression testing decreased the number of pERK cells in long day males but not short day males. There was also a nonsignificant trend for an effect of aggression testing in the AHA (Table 1, $F_{1,24} = 3.44$, $p = 0.07$). Planned comparisons indicated that aggression testing increased the number of pERK cells in short day males but not long day males. In the ventrolateral VMH, males tested in aggression tests had significantly more pERK positive cells than control males (Table 1, $F_{1,24} = 11.64$, $p < 0.01$), regardless of photoperiod. Short day males had significantly more pERK positive cells than long day males in the BLA (Fig. 4A-C, $F_{1,24} = 7.49$, $p = 0.01$). There were no significant effects of photoperiod, aggression, or the interaction on the number of pERK positive cells in the IFL, MPOA, AHA, CEA, DG, LA, CL, or CA1 (Table 1). However, planned comparisons indicated that aggression testing increased the number of pERK cells in the PAG of short day males but not long day males (Table 1). Also, aggression testing significantly reduced the number of pERK cells in the IFL of long day males (Table 1) but not short day males.

The number of pERK positive cells in the BNST was positively correlated (Fig. 3K, Spearman $\rho = 0.54$, $p < 0.05$) with the number of bites during aggression tests whereas the correlation between bites and pERK cells was positive but not significant in the posterodorsal MEA (Fig. 3L, Spearman $r = 0.45$, $p = 0.09$) or ventrolateral VMH (Spearman $r = 0.22$, $p = 0.28$). In the CEA ($\rho = -0.72$, $p < 0.01$), and BLA (Fig. 4D, $\rho = -0.80$, $p < 0.001$) the number of pERK positive cells was negatively correlated with attack latency. There were no other statistically

significant correlations between the number of pERK positive cells and behavior in other brain regions.

pCREB Immunostaining

There was a nonsignificant photoperiod $\times$ aggression interaction in the IFL ($F_{1,24} = 3.70$, $p < 0.06$). There were no significant effects of photoperiod, aggression, or the interaction on the number of pCREB positive cells in the VLS, AI, CL, posterior BST, posterodorsal MEA, ventrolateral VMH, DG, BLA, or PAG. However, planned comparisons indicated that aggression tests significantly reduced the number of pCREB positive cells in the IFL (Fig. 5A), AI, CL, VLS (Fig. 5B), MEA (Fig. 5C), and PAG when males were housed under long days but not short days (Table 2). In the AHA, males tested in aggression tests had significantly fewer pCREB positive cells than control males ($F_{1,24} = 4.71$, $p < 0.05$).

The number of bites was positively correlated with the number of pCREB positive cells in the AHA ($\rho = 0.64$, $p < 0.01$). There was a negative correlation between attack latency and then number of pCREB positive cells in the LA ($\rho = -0.66$, $p < 0.01$). There were no other statistically significant correlations between the number of pCREB positive cells and behavior in other brain regions.

Western Blot Analyses

In the BNST there was a nonsignificant trend for an effect of aggression testing on pERK expression ($F_{1,13} = 3.5$, $p = 0.08$). Planned comparisons indicated that aggression testing caused a significant increase in pERK expression in short day males but had no effect in long day males (Fig 6A, 7). In the MEA there was a significant effect of photoperiod on pERK expression (Fig. 6C, $F_{1,13} = 9.91$, $p < 0.01$), with short day mice having significantly higher expression than long day mice. There was no effect of aggression testing on pERK in the MEA. In the VMH there was a nonsignificant trend for aggression-tested mice to have increased pERK expression (Fig. 6E, $F_{1,13} = 2.89$, $p = 0.11$). There no effect of photoperiod and no interaction on pERK expression in the VMH. There were no significant differences in total ERK expression across treatment groups in either MEA, BST, or VMH (Fig. 6B, 6D, 6F). Analyses of pERK/ERK ratios yielded similar results. In the BST pERK/ERK was higher in aggression-tested mice in short days ($p = 0.05$) but not long days ($p > 0.25$) and in the MEA pERK/ERK was higher in short days versus long days ($p = 0.03$). No differences in pERK/ERK were detected in the VMH.

Discussion

Despite the striking effect of short days on male aggression in rodents, previous attempts to identify differences in brain activity using c-fos immunostaining have been unsuccessful (Kramer et al. 2008; Trainor et al. 2008b). Using both immunostaining and western analyses we found significant differences in pERK expression in several brain regions that are known to regulate aggressive behavior. Engaging in aggressive behavior induced increased expression of pERK in the BNST of short day animals but not long day animals, and there was a positive correlation between pERK positive cells and number of bites. Results in the MEA were not as consistent, but generally showed that pERK expression is increased in short days. Although ERK can be phosphorylated through multiple mechanisms, these results are consistent with our earlier findings that estradiol acts rapidly to increase aggression in short days but not long days (Trainor et al. 2007; Trainor et al. 2008b). A much different pattern was observed with pCREB immunostaining, which generally pointed towards aggressive behavior decreasing the number of pCREB positive cells when mice were housed in long days but not short days. The mechanistic implications of these pCREB results are unclear, but are consistent with the hypothesis that there are different neurochemical circuits regulating aggression in long days

and short days. Overall, these data indicate that photoperiod controls important functional plasticity in neural circuits of aggression.

Neural Circuits of Aggression

In the BNST, both immunostaining and western analyses showed that increased pERK activity was induced by aggression tests when males were housed in short days but not long days. Immunostaining data showed a similar pattern in the PAG. In addition, the number of pERK cells in the BNST was positively correlated with the number of bites during resident-intruder tests. Previous studies have suggested that the BNST is an important nexus where experience can impact levels of aggressive behavior. For example, California mice raised by less aggressive white-footed mice (*P. leucopus*) show lower levels of aggression and reduced vasopressin immunostaining in the BNST (Bester-Meredith and Marler 2001). Similarly, maternal separation in male rats causes increased aggression during play fighting as well as increased vasopressin mRNA in the BNST (Veenema and Neumann 2009). Male prairie voles that have been paired with a female show enhanced aggression in a resident-intruder aggression tests and subsequently have increased c-fos expression in the BNST compared to males that had not been paired with a female (Wang et al. 1997). Although it is likely that social experience and photoperiod work through different mechanisms to regulate aggression levels, these findings suggest that the BNST may be an important location where these neurochemical changes occur. Further study is needed to determine whether elevated ERK activation in the BNST plays a causal role in the increased aggression phenotype observed in short days.

The BNST receives projections from the MEA (Canteras et al. 1995), and in rodents it is thought that aggressive behavior is strongly driven by olfactory cues processed by the MEA (DaVanzo et al. 1983). Many studies indicate that aggressive behavior is associated with increased neuronal activity in the MEA (Kollack-Walker and Newman 1995; Delville et al. 2000). In our immunostaining study, short day males had an increased number of pERK positive cells only when tested in an aggression tests whereas our western analyses showed increase pERK expression in short days regardless of behavioral state. Technical issues may have contributed to the slightly different results. For example, the immunostaining experiment focused on the posterodorsal MEA, where activity is tightly correlated with aggression in male California mice (Trainor et al. 2008a), whereas the punch samples included a larger portion of the MEA including anterior regions of the MEA. Future studies will need to test more extensively whether increased pERK expression in the MEA under short days is constitutive or induced.

Previous studies examining the VMH and behavior suggest that the lateral, but not medial region of this nucleus is active during aggressive behavior (Kollack-Walker and Newman 1995; Delville et al. 2000). Our observations of aggression-induced pERK immunopositive cells are consistent with these reports, as most cells were located in the ventrolateral VMH. In western blot analyses there was a nonsignificant trend for increased pERK expression in aggression tested animals, which is generally consistent with the immunostaining analyses. Interestingly in both song sparrows (Goodson and Evans 2004) and tree lizards (Kabelik et al. 2008), aggressive interactions stimulate immediate early gene expression (ZENK) in the lateral but not medial aspects of the VMH. Although the VMH may be involved in regulating aggressive behaviors, it is also possible that the observed pERK responses are stimulated by exposure to intruders (see below).

Short day mice had more pERK positive cells in the BLA than long day mice, and there was a strong negative correlation between the number of pERK positive cells and attack latency. Several lines of evidence suggest that the BLA may have important effects on aggression. First, in Siberian hamsters short days reduce the expression of neuronal nitric oxide synthase (nNOS) in the BLA, and attack latency is positively correlated with the number of nNOS positive cells (Wen et al. 2004). Second, in rats c-fos is induced in the BLA by aggression tests but not sexual

experience (Veening et al. 2005). Finally, there appears to be a relationship between BLA size and abnormal aggression in dogs. Aggressive behaviors are normally preceded by threat displays, but some individuals exhibit pathological aggression in which threat displays are not utilized (Beata 2001). Stereological analyses showed that the BLA of dogs showing pathological aggression was significantly larger than dogs showing normal sequences of aggression behavior (Jacobs et al. 2006). Interestingly we observed that the number of pERK cells in the BLA was negatively correlated with attack latency but not number of bites. This raises the interesting possibility that short day mice might use fewer threat displays, possibly via a reduction in ultrasonic vocalizations (Kalcounis-Rueppell et al. 2006).

Surprisingly, we generally found that engaging in aggressive encounters decreased pCREB immunostaining in a number of brain areas (IFL, VLS, MEA) and that these differences were observed preferentially in long day mice. These data support the hypothesis that the neural circuitry used to regulate aggression in long days differs from that used in short days. There are conflicting data on the role of the lateral septum, with some studies reporting that lesions in this region increase aggression (Albert et al. 1978) whereas other studies report a decrease in aggression (Annen and Fujita 1985). It is possible that the effects of the lateral septum on aggression may be context dependent. Future studies will need to consider more carefully how environmental contexts affect behavioral outcomes. We also observed aggression induced decreases in pCREB immunostaining in the IFL and AIV, which are subregions of the frontal cortex. There is an interesting paradox between studies using lesions to examine the effects of frontal cortex on aggression versus studies using immediate early genes to estimate activity. In rats lesions to the frontal cortex are associated with increased male aggression (de Bruin et al. 1983). A study in Rhesus macaques also reported that frontal cortex lesions increased aggressive behavior, but this was only observed in dominant, not subordinate males (Machado and Bachevalier 2006). In contrast to these findings, studies in rats (Halász et al. 2006) and mice (Haller et al. 2006) showed aggression testing increased c-fos expression in several frontal cortex areas including IFL and AIV. These data suggested an increase in frontal cortex activity during aggression. One possible explanation for these apparently contrasting results is that long term deficits in frontal cortex function could have different consequences than a short term increase in activity during an aggressive encounter.

Overall, we had predicted that there would be increased pERK and pCREB immunostaining in short day aggression tested mice and little or no effect of aggression testing in long days. This pattern was observed in the BNST and MEA for pERK, whereas an aggression induced decrease in pCREB immunostaining was observed in long day mice. It is possible that pCREB immunostaining was affected by the time course in which the brains were collected. Previous studies have reported that CREB phosphorylation is facilitated by pERK (Wade and Dorsa 2003; Lee et al. 2004), so it is possible that a longer delay between behavior tests and brain collection may have produced different results. However, in the IFL and VLS the effects of aggression testing on pERK matched the pCREB results, with an aggression induced decrease in the number of pERK cells observed in long days but not short days. The correspondence between pERK and pCREB in these brain areas suggests that our results in long day animals are not an artifact. It is also notable that there were many more pCREB positive cells than pERK positive cells. This may be because CREB can be phosphorylated via several pathways including protein kinase A, calcium calmodulin-dependent kinase, DARPP-32, and ERK (Berke and Hyman 2000). It is possible that one or more of these pathways may have important effects on the regulation of aggressive behaviors. Together, these observations suggest that analyses of pERK identify a more distinct cellular population than pCREB in a given brain nucleus.

During aggression tests, the activation of ERK and CREB could be influenced by a number of processes. For example, it could be that exposure to intruders and not aggressive behavior *per*

se that is associated with activation of ERK. This may be the case in the ventrolateral VMH, where we observed more pERK positive cells in both long day and short day mice. Furthermore, neither bites nor attack latency was correlated with the number of pERK positive cells in the ventrolateral VMH. In contrast, our data suggest that ERK activation in the BNST may be more tightly linked to aggression versus intruder stimuli alone. In immunoblot analyses, increased pERK expression in the BNST was only observed in more aggressive short day mice whereas this increase was absent in less aggressive long day mice tested in aggression tests. In addition, the number of bites in aggression tests was positively correlated with the number of pERK positive cells in the posterior BNST. The most likely case is that increases in pERK during aggression tests are mediated by a combination of intruder stimuli and behavioral response. Future studies will be needed to separate out effect of aggressive behavior versus exposure in intruder cues.

Finally it is possible that increase pERK following aggression tests is due to stress, as a previous study showed that restraint stress can activate ERK (Kwon et al. 2006). Analyses of the stress hormones corticosterone and oxytocin suggest that ERK activation during aggression tests does not reflect a stress response. Aggression tests do not increase corticosterone or oxytocin in either long day or short day males, whereas females tested in aggression tests show increased corticosterone regardless of photoperiod (Trainor et al. submitted). Furthermore in males and females, aggression tests do not increase pERK expression in either the magnocellular or parvocellular regions of the paraventricular nucleus. Although it is possible more subtle stress responses could be involved, it appears that the hypothalamic-pituitary-adrenal axis is not activated in male California mice during resident intruder tests.

Potential Neuroendocrine Mechanisms

There is accumulating evidence that steroid hormones mediating the effect of photoperiod on aggression are not produced by the gonads (Wen et al. 2004; Scotti et al. 2008; Trainor et al. 2008b). In particular it appears that the source of behaviorally active steroids in short days is the adrenal gland, the brain, or possibly a combination. In Siberian hamsters, males housed in long days that are given melatonin injections administered 2 h before lights out show increased aggression levels similar to those seen in short days (Demas et al. 2004). This effect can be blocked by adrenalectomy but not adrenal demedullation, suggesting that hormones produced by the adrenal cortex play a key role in regulating aggression under short days. The adrenal cortex can affect behavior via secretion of either glucocorticoids or dehydroepiandrosterone (Soma et al. 2008). Dehydroepiandrosterone can be converted to androstenedione by 3 β -HSD, and AE can be further converted to testosterone or aromatized to estrone. Estrogens could affect behavior via estrogen receptors. There is also evidence that glucocorticoids have important effects on aggressive behavior. In particular, glucocorticoids may affect the integration of threat displays before fighting, although this relationship appears to be complex. In rats adrenalectomy results in a sharp increase in offensive attacks such as biting (Haller et al. 2001). Notably, the patterns of aggressive behavior are abnormal because adrenalectomized rats use fewer offensive threats such as upright displays or chasing (Haller et al. 2004). Intact rats that use more offensive attacks typically use more threat displays. An obvious contrast between these rat studies is that aggressive mice and hamsters in short days have increased glucocorticoid secretion. Further study is needed to determine whether short days affect threat displays in rodents and whether this is influenced by glucocorticoids. We did observe negative correlations between pERK positive cells and attack latency (but not total number of bites) in the BLA. Both of these nuclei express glucocorticoid receptors (Ahima and Harlan 1990; Morimoto et al. 1996), raising the possibility these brain areas could mediate effects of glucocorticoids on a decision to engage in aggressive behavior.

We previously demonstrated that estradiol acts rapidly to increase aggression in short days but not long days (Trainor et al. 2007; Trainor et al. 2008b), and others have shown that estradiol can rapidly phosphorylate ERK (Abraham et al. 2004). These data raise the hypothesis that estradiol increases aggression in short days via phosphorylation of ERK. Given its cellular distribution, ERK is well positioned to alter neuronal activity or neurotransmitter release (Selcher et al. 2003). There is evidence that both classical estrogen receptors (Abraham et al. 2004) and membrane estrogen receptors (Revankar et al. 2005) can phosphorylate ERK. Our knowledge of the distribution of membrane receptors in the rodent brain is still limited but rapidly growing (Milner et al. 2005).

Summary

In a wide range of rodent species, short days increase aggressive behavior in males. Using measurements of pERK and pCREB we demonstrated for the first time that there are differences in brain activity in males housed in short days. In particular, immunostaining and western analyses showed that an increase in pERK expression in the BNST was induced by aggression tests under short days but not long days. These data suggest that the BNST should be a focal point for future studies examining the role of ERK in regulating intensity of aggression. Other brain regions that may be of interest include the MEA and BLA, because these regions exhibited increased pERK under short days. These data add to a growing literature that different neurochemical circuits are used to regulate aggression under different contexts (Trainor et al. 2008a; Kabelik et al. 2009).

Acknowledgments

The authors thank Andrea Silva, Michael Steinman, and Julie VanWesterhuyzen for technical help, and Jim Trimmer for immunostaining advice. This work supported by NIH R01 MH85069 and UC Davis Hellman Fellows Program (B.C.T.).

Abbreviations

IFL	infralimbic cortex
AI	agranular insular cortex
VLS	ventral lateral septum
BNST	bed nucleus of the stria terminalis
MPOA	medial preoptic area
CG2	area 2 of cingulate cortex
AH	anterior hypothalamus
BLA	basolateral amygdala
CEA	central nucleus of the amygdala
vVMH	ventromedial hypothalamus
MEA	medial amygdala
CA1	CA1 region of hippocampus
DG	dentate gyrus
PAG	periaqueductal gray

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Figure 1.

Immunostaining of phosphorylated ERK and CREB in *P. californicus*. Positive staining for pERK (A) was eliminated by pre-incubation with the pERK immunizing peptide (B) but not with a nonphosphorylated ERK peptide (C). Positive staining for pCREB (D) was eliminated by omission of the primary pCREB antibody (E). Histology for a 1 mm diameter micropunch sample of the bed nucleus of the stria terminalis (F). This section is stained for pCREB (blue black) and counterstained with eosin (pink). Abbreviations: lv, lateral ventricle; cc, corpus callosum; f, fornix; ac, anterior commissure; bed nucleus of the stria terminalis, bnst. Scale bar in panels A-E 20 μm. Scale bar in panel F = 0.5 mm.

Figure 2. Effect of photoperiod on number of bites and attack latency in resident intruder tests. * $p < 0.05$, ** $p < 0.01$.

Figure 3.

Photomicrographs of phosphorylated extracellular-related kinase (pERK) immunoreactivity in the posterior BNST (A-D) and posterodorsal MEA (E-F) from California mice tested in control or resident intruder tests. In both the posterior BNST (I) and posterodorsal MEA (J), aggression testing caused a significant increase in the number of pERK positive cells in short day mice but not long day mice. For animals tested in aggression tests, there is a significant positive correlation between the number of pERK cells in the posterior BNST and bites during aggression tests (K, $p < 0.05$) and a nonsignificant trend in the posterodorsal MEA (L, $p = 0.09$). Abbreviations: f, fornix; ac, anterior commissure; ot, optic tract. * $P < 0.05$ planned comparison vs control. Scale bars = 0.2 mm.

Figure 4.

Photomicrographs of phosphorylated extracellular related kinase (pERK) immunoreactive cells the BLA from California mice housed in long days (A) or short days (B). Short day mice had more pERK positive cells than long day mice (C). The number of pERK positive cells was negatively correlated with attack latency (D). * $P < 0.05$ effect of photoperiod. Abbreviations: ec external capsule. Scale bars = 0.2 mm.

Figure 5. Photomicrographs of phosphorylated CREB immunoreactive cells in the IFL (A), VLS (B), and posterodorsal MEA (C). Abbreviations: cc, corpus callosum; lv lateral ventricle; ac, anterior commissure; ot, optic tract. Scale bars = 0.2 mm.

Figure 6.

Western blot analyses of pERK and total ERK from punch samples of the BNST (A,B), MEA (C,D), and VMH (E, F). In the BNST aggression testing increased pERK expression in short day mice but not long day mice (A). In the MEA pERK expression was increased in short day mice compared to long day mice (C). In the VMH there was a nonsignificant trend ($p = 0.1$) for increased pERK expression in aggression-tested mice (E). There was no effect of photoperiod or aggression testing on total ERK expression in any brain area (B, D, F). * $P < 0.05$ planned comparison vs. control, † $P < 0.05$ effect of photoperiod.

Figure 7.
Representative images from western blot analysis of protein extracted from BNST punch samples. A = aggression, C = control.

Table 1

Effects of photoperiod and aggression testing on pERK positive cells, n =6-8 per group * effect of aggression within photoperiod $p < 0.05$, † overall effect of photoperiod $p < 0.05$ # overall effect of aggression testing

	Box Size (mm)	control	Long Day aggression	control	Short Day aggression
IFL	0.4×0.4	53±15	41±11*	40±13	41±12
CL	0.75×0.3	40±9	29±5	36±3	27±6
VLS	0.28×0.44	40±6	22±5*	18±5	30±5
MPOA	0.36×0.6	86±14	100±21	63±17	95±16
posterior BNST	0.34×0.34	13±3.1	9±3	7±2	24±7*
AHA	0.4×0.5	35±5	45±13	17±4	40±9*
ventrolateral VMH	0.24×0.34	12±4	27±9#	9±1	34±6#
posterodorsal MEA	0.34×0.6	55±13	45±8	48±7	82±9*
CEA	0.48×0.65	60±26	31±12	42±17	43±15
LA	0.37×0.29	11±4	12±4	16±6	27±6
BLA	0.71×0.29	9±2	13±4	19±4†	23±4†
CA1	0.9×0.2	10±4	9±4	15±6	9±3
DG	0.6×0.3	7±3	6±2	7±3	7±2
PAG	0.3×0.4	6±2	9±4	2±0.5	9±4*

Table 2

Effects of photoperiod and aggression testing on pCREB positive cells, n =6-8 per group * effect of aggression within photoperiod $p < 0.05$, † overall effect of photoperiod $p < 0.05$ # overall effect of aggression testing

	Box Size (mm)	control	Long Day aggression	control	Short Day aggression
IFL	0.4×0.4	193±78	54±25*	54±25	117±57
AI	0.9×0.5	347±125	169±83*	280±68	231±82
CL	0.75×0.3	90±35	39±15*	66±22	88±25
VLS	0.28×0.44	114±15	66±14*	98±21	105±17
MPOA	0.36×0.6	475±26	431±27	454±20	423±30
posterior BNST	0.34×0.34	145±22	150±14	137±18	132±11
AHA	0.4×0.5	306±22	217±34#	308±24	279±21#
ventrolateral VMH	0.24×0.34	211±14	221±8	194±25	201±15
posterodorsal MEA	0.34×0.6	325±43	254±28*	238±29	283±37
LA	0.37×0.29	119±26	126±28	155±23†	188±15†
BLA	0.71×0.29	192±52	161±43	205±34	244±30
DG	0.6×0.3	76±8	72±7	83±10	65±8*
PAG	0.3×0.4	54±17	28±7*	26±12	26±10