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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 identified 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 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 (MEA), 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.

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Abbreviations: AH, anterior hypothalamus; AI, agranular insular cortex; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1, CA1 region of hippocampus; CEA, central nucleus of the amygdala; CG2, area 2 of cingulate cortex; CREB, cyclic AMP, response element binding protein; DG, dentate gyrus; ERK, extracellular signal-regulated kinases 1 and 2; IFL, infralimbic cortex; MEA, medial amygdala; MPOA, medial preoptic area; PAG, periaqueductal gray; PBS, phosphate saline buffer; pCREB, phosphorylated CREB; pERK, phosphorylated ERK; VLS, ventral lateral septum; vlVMH, ventromedial hypothalamus.

Key words: basolateral amygdala, estrogens, mitogen activated kinase, social behavior, infralimbic cortex, stress.

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 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, 2007; Gobrogge 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 follow-

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ing 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 aggressive 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, 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). 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.

EXPERIMENTAL PROCEDURES

Adult male California mice were purchased from the *Peromyscus* Stock Center (University of South Carolina, Columbia, SC, USA) 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 7 min 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 (Sigma, St. Louis, MO, USA) 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 h 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 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 μm sections on a cryostat and counterstaining these sections with Eosin, we confirmed that our dissection protocol isolated 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 three 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, USA 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,