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Deletion of Corticotropin-releasing Factor Binding Protein Selectively Impairs Maternal, but not Intermale Aggression

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Abstract

Corticotropin-releasing factor (CRF) binding protein (CRF-BP) is a secreted protein that acts to bind and limit the activity of the neuropeptides, CRF and urocortin (Ucn) 1. We previously selected for high maternal defense (protection of offspring) in mice and found CRF-BP to be elevated in the CNS of selected mice. We also previously determined that both CRF and Ucn 1 are potent inhibitors of offspring protection when administered centrally. Thus, elevated CRF-BP could promote defense by limiting endogenous actions of CRF or Ucn 1. To test this hypothesis, we crossed the deletion for CRF-BP into the mice selected for high maternal defense and evaluated offspring protection and other maternal behaviors. CRF-BP knockout (KO) mice exhibited significant deficits in maternal aggression relative to wild-type (WT) mice in three different measures. Other maternal features were almost identical between groups, including dam and pup weight, litter size, nursing time, and pup retrieval. Both groups performed similarly in a forced swim stress test and aggression in both groups was reduced following the swim test. Virgin KO female mice exhibited higher levels of anxiety-like behavior in terms of decreased time in the light portion of the light/dark box test. For males, no differences in light/dark box or swim test were found. However, increased anxiety-like behavior in male KO mice was identified in terms of contact and approach to a novel object both with and without previous exposure to the swim test. No differences in isolation induced resident intruder male aggression were found between groups. Together, these results indicate that loss of CRF-BP selectively impairs maternal, but not intermale aggression and that loss of the gene induces anxiety-like behavior in males and females, but there are sex differences in terms of how that anxiety is revealed.

Keywords

anxiety; maternal aggression; maternal defense; lactation; mice

Offspring protection, also termed maternal defense or maternal aggression, is a critical components of maternal care for mammals raising vulnerable offspring (Wolff, 1985, Agrell et al., 1998, Wolff and Peterson, 1998). Maternal aggression is highly conserved among

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mammals and involves a dramatic change in how females respond to stressors and to social cues (Gammie and Lonstein, 2006, Gammie et al., 2008, Slattery and Neumann, 2008). We recently selected for high levels of maternal defense in mice (Gammie et al., 2006) and then used gene arrays and real-time PCR to examine gene expression changes in the CNS with high defense (Gammie et al., 2007). We found corticotropin releasing factor (CRF) binding protein (CRF-BP) to be significantly elevated in selected mice, suggesting elevated CRF-BP may promote the emergence of high maternal aggression.

CRF-BP is a 37 kDa secreted protein that can act in the CNS by binding either CRF or its related peptide, urocortin (Ucn) 1 (Behan et al., 1989, Potter et al., 1991, Cortright et al., 1995, Westphal and Seasholtz, 2006). CRF-BP is expressed in the CNS in a number of cortical and subcortical regions, including hypothalamus, and in the pituitary gland (Potter et al., 1992, Timofeeva et al., 1999, Speert et al., 2002, Henry et al., 2005). Lateral septum (LS) produces high levels of CRF-BP and this region has been implicated in offspring protection (Flannelly et al., 1986, Gammie et al., 2004, D'Anna et al., 2005, D'Anna and Gammie, 2006, Lee and Gammie, 2007). Numerous studies have shown that the secreted CRF-BP acts extracellularly to modulate neuronal and endocrine activity by binding CRF or Ucn 1, decreasing the normal interactions of CRF or Ucn 1 to either CRF receptor 1 (CRF1) or CRF receptor 2 (CRF2), and thereby reducing CRF receptor activation (Potter et al., 1992, Cortright et al., 1995, Westphal and Seasholtz, 2006). Consistent with this hypothesis, CRF-BP-deficient mice show heightened anxiety-like behavior (Karolyi et al., 1999), which likely reflects a decreased ability to regulate CRF and/or Ucn 1 resulting in higher “free” peptide levels that have been shown to be anxiogenic.

In previous studies, we found that icv injections of either CRF (Gammie et al., 2004) or Ucn 1 (D'Anna et al., 2005) significantly impair offspring protection. Our finding that mice selected for high maternal aggression have elevated CRF-BP levels is consistent with the framework that CRF and related peptides are endogenous negative regulators of offspring protection. By elevating CRF-BP, the selected mice may promote high aggression by minimizing any negative effects of CRF or Ucn 1 on offspring protection. Interestingly, prior work indicated that reactivity of the CNS to CRF is blunted during lactation, including within LS (da Costa et al., 1997), but the mechanisms are not known. One possibility is that CRF-BP expression is elevated during lactation and this blunts CRF action and provides a mechanism for promoting offspring protection.

In this study, we used knockout mice deficient for CRF-BP to test the hypothesis that a loss of CRF-BP would decrease maternal defense due to a lowered ability to modulate CRF and Ucn 1. For this study, we crossed the deletion into our line of mice bred for high maternal defense. One rationale for using selected mice is that they exhibit excellent maternal profiles relative to inbred mouse strains that often have reduced litter sizes and reproductive problems. Thus, were able to examine the effect of loss of gene on maternal defense in mice with a reliable maternal profile. We also examined anxiety-like behavior and other measures of maternal behavior to establish an overview of the range of effects of loss of this gene. Aggression occurs in a number of forms and the neural mechanisms underlying these forms can differ (Gammie and Lonstein, 2006, Grimes et al., 2006, Miczek and Fish, 2006). In this study, we also examined isolation induced intermale aggression to determine whether CRF-BP plays a similar role in males and females.

Experimental Procedures

Mice

CRF-BP knockout mice (Karolyi et al., 1999) on a C57BL/6J background (14 generations) were mated with high maternal defense mice that we had selectively bred for high maternal

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aggression (Gammie et al., 2006); these constituted the first generation of mice. Heterozygote offspring (50% selected background) were then mated to selected mice and constituted the second generation of mice. Offspring from these pairings were genotyped and were the third generation (now 75% selected background). Only heterozygotes (males and females of the third generation) were mated and produced fourth generation wild-type (WT), knockout (KO), and heterozygote mice within the same litters (still ~75% selected background). All WT and KO male and female mice examined came from 25 litters that produced 28% KO, 28% WT, and 44% heterozygote mice. Only two litters contained KO, but not WT mice and only one litter contained WT, but not KO mice. All litters contained heterozygote mice, which provided a common rearing environment. Per litter, the average number of WT mice was 2.92 and the average number of KO mice was 2.88. The maximum number of KOs in a given litter was 6 (out of 13 total) and the maximum number of WTs in a given litter was 7 (out of 13 total). Female WT (N = 28) and KO (N = 28) mice were then examined as adults for maternal defense, other maternal behaviors, and anxiety. Male WT (N = 29) and KO (N = 28) mice were examined as adults for anxiety and intermale aggression. For focal female WT and KO mice, they were housed with breeder males for 1 week and then housed individually for the remainder of the study. The rationale for using outbred breeder males was that this would provide a common breeding experience among mice and increase the genetic similarity of offspring from WT and KO mothers, which would decrease possibilities of offspring effect on maternal profiles. Mice were given ad lib access to tap water and to Breeder Chow (Harlan, Madison, WI) for females or regular chow for males. Just prior to parturition, female mice were given precut nesting material. Litter size and pup weight were measured on postpartum Days 0 and 10. On Day 1, litters were culled to a maximum of 11. For the 56 total litters, only 5 contained fewer than 10 pups (WT = 2 and KO = 3); the smallest litter size was 4. Polypropylene cages were cleaned weekly prior to parturition, but afterwards cages were not changed for the duration of the experiment. Sexually naïve male mice of the outbred hsd:ICR strain (Harlan) were used as intruders during maternal and intermale aggression tests. Intruder males were group housed (4 mice/cage) and never used more than once per day and not for more than 3 total tests each. Over the course of testing, each male and female mouse was exposed to different intruder males and due to the design of the study (see below), males with varying experience were equally distributed over the different days and for the two genotypes. All mice were housed on a 14:10 light/dark cycle with lights on at 0600 CST. All testing was performed between 1000 and 1500 h. All procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of the University of Wisconsin.

Genotyping

Mice were genotyped by PCR using a Multiplex PCR analysis with three primers: primer c, (5′-TGG ACC CTC GTC ATT GCC ACG C-3′), primer f, (5′-AGA CTA GTG AGA CGT GCT ACT TCC ATT TGT-3′) and primer d, (5′-CCC GTC GGT ACG GCT GCT CCT CTG CCA GGT-3′). Reactions were run with purified DNA from ear snips and analyzed as previously described (Karolyi et al., 1999). The deletion within the CRF-BP gene removes the translated portion of Exon 1 and Exons 2-5 (Karolyi et al., 1999). WT PCR fragment is 700 bp and CRH-BP deficient mouse band is 440 bp. Previous work indicates that neither CRF-BP RNA nor protein is detected in CRF-BP deficient mice as assayed via in situ hybridization or ^{125}I-CRH crosslinking assays. Because PCR outcome reliably indicates loss of gene (Karolyi et al., 1999), no additional tests to confirm loss of gene were made.

Overview of testing procedures

Both males and females were first tested for anxiety (light/dark box and/or novel object) and then tested for anxiety in close association with aggression testing. The forced swim stress test provided swim data, but also served as a stressor to test the stress effects on anxiety and/or

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aggression. Females were examined for general maternal behaviors on postpartum Day 3 and then examined for aggression on postpartum Days 5-7. Females and males were ~4 months old at onset of testing. The maximum age difference among females was 9 days; the same maximum difference was true for males as well.

Light/dark box testing

Mice were placed in the dark portion of the light/dark box to initiate the 5 min test session. The length and width of the box were 26 cm × 26 cm, with a dark box covering half of the square. The height of all elements of the box was 31 cm. The height and width of the opening from dark to light box were 3.8 cm. Time spent in the light and dark portions of the box were recorded with time in light portion of the box defined as entry of all four paws into this region. Female mice were examined as adults once as virgins and for three tests during lactation. All behaviors were recorded on videotape and subsequently analyzed off-line. The light/dark box was used as a tool for examining levels of anxiety (Bouwknecht and Paylor, 2002, Gimenez-Llort et al., 2002, Henry et al., 2006).

Maternal behavior observations

On postpartum Day 3, females were observed undisturbed in their home cages and home room. Observations were made between 0900 and 1000 by individuals blind to experimental conditions. Every 30 sec, the behavior of each mouse was recorded. Measures noted included nursing, licking and grooming of pups, and nest building. Whether the mouse was on nest, off nest, or eating or drinking was also noted.

Maternal aggression and pup retrieval testing

Females were moved into the testing room, where they were tested in their home cages. Previously, females had not entered the testing room, but had resided in their home cages since pairing. Just prior to testing pups were removed from the home cage; removal of pups from the home cage of a dam before an aggression test does not diminish the expression of maternal aggression in mice (Svare et al., 1981). Maternal aggression testing involved placing a male intruder into the female's home cage for 5 min. Initially, males are placed in the quadrant opposite from the females. A typically initial behavior by the male is to slowly explore the new cage until approached by the female. Males from the same cage were evenly distributed among WT and KO mice and females were exposed to different males each day. Females were tested for aggression on postpartum Days 5, 6, and 7. On Days 5 and 7, light/dark box testing occurred immediately following the aggression test. On Day 6, the light/dark test preceded aggression testing. The rationale for altering test order between aggression and anxiety testing is that it was possible that one test might affect outcome of the other test. By reversing the orders, we were able to analyze both aggression and anxiety without the confound of an immediate prior testing event. Pup retrieval testing involved evenly scattering pups away from the nest and examining retrieval of the pups by the mother for 2 min. Pup retrieval was always the last test, so on Days 5 and 7, it immediately followed the light/dark test and on Day 6, it followed the aggression test. Each test session was recorded on videotape and subsequently analyzed off-line to quantify behaviors by individuals blind to testing conditions. For quantification of maternal aggression the following features were measured: latency to first attack, number of attacks, and total duration of attacks (Gammie and Nelson, 1999, Gammie et al., 2004, D'Anna and Gammie, 2006). Pup retrieval was quantified by measuring the time elapsed to retrieval of first and fourth pup (D'Anna et al., 2005, D'Anna and Gammie, 2006).

Forced swim stress test

For the swim stress test, mice were placed in a glass cylinder (30 cm tall, 15 cm diameter) that was half filled with room temperature water for 2 min. After the swim exposure, mice were

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lightly patted dry and returned to their home cage. All sessions were videotaped and analyzed off-line by individuals blind to genotype. Latency to float and total time floating were evaluated. After 15 min, mice were tested for aggression, anxiety, and pup retrieval. Females were exposed to the swim test on postpartum Day 7.

Novel object test and light/dark box test for males

Prior to isolation, group housed WT and KO male mice were weighed and examined for approach to a novel object for three days. Each test lasted 5 min and began with the male placed along the wall of an open field test apparatus (40 X 40 X 40 cm). The bottom was divided into 16 square grids. A 5 cm wood ball was place in the center of the open field. All sessions were videotaped and analyzed off-line by individuals blind to experimental conditions. The time to first touch the object, the number of touches, and the total time in the vicinity of the novel object (within the middle four square grids) were examined. For the first two days of testing, males were also examined for light/dark box testing as described above. On day 1 the light dark/box test followed the novel object test and on the second day the order was reversed. The rationale for the order was that either test might have some effect on the second test and using this approach we were able to evaluate each measure first. Prior to the 3rd test, mice were exposed to the forced swim stress test for 2 min and then given the novel object test 15 min after the swim test.

Intermale aggression testing

Following four weeks of isolation, each male was tested for intermale aggression for 3 consecutive days for 5 min each between 0900 and 1500 h. An intruder (hsd:ICR strain) male mouse was placed in the resident's home cage and each test session was recorded on videotape. Just prior to the third test, the males were again examined behaviorally in the light/dark box. Analysis of intermale aggression was conducted exactly as for maternal aggression described above.

Data analysis

Data were analyzed with SigmaStat 3.0 (SPSS Inc, Chicago, IL). If data were not normally distributed, then attempts were made to transform data to achieve normality. If attempts failed, then data were analyzed using non-parametric tests. One-way ANOVAs were used to test differences between WT and KO mice. For any cases where a significant effect was found, post hoc tests were conducted. For parametric data, the Holm-Sidak post hoc method was used. For non-parametric data, the Kruskal-Wallis ANOVA on Ranks along with the Dunn's method post hoc test was used. Final significance was based only on post hoc tests. Additionally, to test the effect of swim stress on behaviors within each group, a one-way repeated measures ANOVA was used because this allowed us to directly compare individual performance with and without the stressor. In the cases where the data were not normally distributed, a non-parametric Friedman RM ANOVA on Ranks test was performed. In the case of latency to first attack, if an animal was non-aggressive, a time of 300 s (the maximum time of the aggression test) was assigned. Likewise, if an animal did not retrieve the first or fourth pup within a time of 120 seconds, then a time of 120 s was assigned as performed previously (D'Anna et al., 2005). The standard p-value cutoff of 0.05 was used to evaluate the significance of the behavioral data.

Results

Maternal defense

Offspring protection was significantly decreased in KO relative to WT mice on postpartum Day 5 in terms of total duration of attacks ($H(1,55) = 6.13$; $p = 0.013$ ANOVA on Ranks;

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Q=2.4, p<0.05 post hoc) (Fig. 1A), number of attacks (F(1,55) = 5.17; p = 0.027) (Fig. 1B), and latency to attack (H(1,55) = 4.40; p = 0.036 ANOVA on Ranks; Q=2.0, p<0.05 post hoc) (Fig. 1C). Similarly, maternal defense was impaired in KO mice on postpartum Day 6 in terms of total duration of attacks (H(1,55) = 6.47; p = 0.011 ANOVA on Ranks; Q=2.5, p<0.05 post hoc) (Fig. 1A), number of attacks (H(1,55) = 6.47; p = 0.011 ANOVA on Ranks; Q=2.5, p<0.05 post hoc) (Fig. 1B), and latency to attack (H(1,55) = 4.27; p = 0.039 ANOVA on Ranks; Q=2.0, p<0.05 post hoc) (Fig. 1C). Following the swim stress on Day 7, a trend towards differences between groups was found, but even when the overall model indicated significant differences, these were not found following post hoc tests as seen in terms of total duration of attacks (H(1,55) = 4.13; p = 0.011 ANOVA on Ranks; Q=1.9, p>0.05 post hoc) (Fig. 1A), number of attacks (H(1,55) = 3.64; p = 0.056 ANOVA on Ranks) (Fig. 1B), and latency to attack (H(1,55) = 4.27; p = 0.046 ANOVA on Ranks; Q=1.8, p>0.05 post hoc) (Fig. 1C).

A repeated measures analysis within WT mice revealed a significant decrease in time aggressive and number of attacks following the swim stress relative to the previous two tests (p<0.05, Dunn's posthoc). A repeated measures analysis within KO mice revealed a significant decrease in time aggressive and number of attacks following the swim stress relative to Day 5 levels (p<0.05, Dunn's posthoc).

Forced swim test in females

Female WT and KO mice performed similarly on the forced swim test in terms of time to first float (H(1,55) = 0.07; p = 0.786 ANOVA on Ranks) and total time floating (F(1,55) = 0.82; p = 0.367). For latency to first float, WT = 63.8 ± 5.1 sec and KO = 68.3 ± 4.6 sec. For total time floating, WT = 30.0 ± 3.5 sec and KO = 34.8 ± 3.8 sec.

Light/dark box test in females

When examined as virgins, KO mice showed increased indices of anxiety in the light/dark box test in terms of time in light (F(1,55) = 6.72; p = 0.012) (Fig. 2A) and number of entries to the light portion of the box (F(1,55) = 7.4; p = 0.009) (Fig. 2B), but not in terms of latency to enter the light (H(1,55) = 3.43; p = 0.064 ANOVA on Ranks) (Fig. 2C). However, on Day 5 of lactation, no differences between groups were found in terms of time in light (F(1,55) = 2.69; p = 0.107) (Fig. 2A), number of entries to the light portion of the box (F(1,55) = 1.24; p = 0.264) (Fig. 2B), and latency to enter the light (H(1,55) = 2.91; p = 0.088 ANOVA on Ranks) (Fig. 2C). Similarly, no differences between groups were found on Day 6 in terms of time in light (F(1,55) = 1.20; p = 0.277) (Fig. 2A), number of entries to the light portion of the box (F(1,55) = 1.58; p = 0.213) (Fig. 2B), and latency to enter the light (H(1,55) = 1.30; p = 0.254 ANOVA on Ranks) (Fig. 2C). Following the stress on Day 7, no differences between groups were found in terms of time in light (H(1,55) = 0.04; p = 0.837 ANOVA on Ranks (Fig. 2A), number of entries to the light portion of the box (H(1,55) = 0.09; p = 0.757 ANOVA on Ranks) (Fig. 2B), and latency to enter the light (H(1,55) = 0.118; p = 0.731 ANOVA on Ranks) (Fig. 2C).

A repeated measures analysis within WT mice revealed a significant increase in latency to enter light and a significant decrease in entries to and time in light following the swim stress relative to the previous two tests (p<0.05, posthoc). A repeated measures analysis within KO mice revealed a significant increase in latency to enter light and a decrease in time in light following the swim test relative to the previous test (p<0.05, posthoc). A decrease in number of entries to the light was found relative to both previous tests for KO mice (p<0.05, posthoc).

Maternal and pup profiles

Fertility rate was high in both groups. Out of 30 KO females mated with males, only two did not give birth. Only one WT female (out of 29) did not give birth. No litters were lost after

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birth. The average latency to give birth after pairing with a male was similar between groups (WT = 22.6 ± 0.5 days; KO = 23.0 ± 0.5 days). In terms of body weight, WT and KO mice were almost identical when evaluated as virgins ($F(1,55) = 1.91$; $p = 0.172$) (Fig. 3A), on postpartum Day 1 ($F(1,55) = 0.49$; $p = 0.485$) (Fig. 3A), or on Day 10 ($F(1,55) = 0.05$; $p = 0.817$) (Fig. 3A). Litter weight was also almost identical between the two groups when examined on Day 0 ($F(1,55) = 0.82$; $p = 0.368$) (Fig. 3B) and Day 10 ($H(1,55) = 0.84$; $p = 0.359$ ANOVA on Ranks) (Fig. 3B). Also, in terms of litters size, groups were very similar on Day 0 ($F(1,55) = 0.16$; $p = 0.688$) (Fig. 3C) and Day 10 ($H(1,55) = 0.01$; $p = 0.938$) (Fig. 3C). Note that on Day 1, litter size was culled to a maximum of 11.

General maternal behaviors were observed for one hour on postpartum Day 3 and in no measures were differences found between groups. Proportion of time nursing was similar between groups (WT = 0.52 ± 0.05; KO = 0.59 ± 0.04) ($F(1,49) = 1.3$; $p = 0.254$), as was proportion of time licking and grooming offspring (WT = 0.16 ± 0.01; KO = 0.17 ± 0.01) ($F(1,49) = 0.00$; $p = 0.949$) and proportion of time nest building (WT = 0.03 ± 0.01; KO = 0.03 ± 0.00) ($H(1,49) = 0.03$; $p = 0.857$). In terms of proportion of time eating or drinking and time on or off nest, no differences were found between groups (data not shown).

Pup retrieval did not differ between groups in terms of time to retrieve first pup on Day 5 ($H(1,55) = 0.31$; $p = 0.576$ ANOVA on Ranks) (Fig. 3D), Day 6 ($H(1,55) = 1.37$; $p = 0.242$ ANOVA on Ranks) (Fig. 3D), or Day 7 following the swim stress ($H(1,55) = 1.81$; $p = 0.177$ ANOVA on Ranks) (Fig. 3D). Time to retrieve the fourth pup also did not differ between groups on Day 5 ($H(1,55) = 0.70$; $p = 0.401$ ANOVA on Ranks) (Fig. 3E), Day 6 ($H(1,55) = 1.23$; $p = 0.266$ ANOVA on Ranks) (Fig. 3E), or Day 7 following the swim stress ($H(1,55) = 2.13$; $p = 0.144$ ANOVA on Ranks) (Fig. 3E).

Male weight and light/dark box test

When examined around four months of age, weight between male groups was almost identical (WT = 44.2 ± 0.8 grams, KO = 44.7 ± 1.5 grams) ($H(1,56) = 0.00$; $p = 0.994$ ANOVA on Ranks). When examined while group housed and prior to isolation or following isolation, no differences were found between WT and KO male mice in any aspect of light/dark performance. In terms of time in light, groups did not differ on the first ($H(1,56) = 0.37$; $p = 0.538$ ANOVA on Ranks) or second test pre-isolation ($F(1,56) = 1.8$; $p = 0.176$), or following isolation ($F(1,56) = 0.56$; $p = 0.455$) (Fig. 4A). In terms of number of entries to the light, groups were similar on the first ($F(1,56) = 0.60$; $p = 0.439$) and second test pre-isolation ($F(1,56) = 1.8$; $p = 0.176$), and following isolation ($F(1,56) = 0.00$; $p = 0.971$) (Fig. 4B). In terms of time to light, no differences were found on the first ($H(1,56) = 0.37$; $p = 0.538$ ANOVA on Ranks) or second test pre-isolation ($F(1,56) = 1.8$; $p = 0.176$), or following isolation ($H(1,56) = 0.123$; $p = 0.720$ ANOVA on Ranks) (Fig. 4C).

Male novel object performance

When examined while group housed and prior to isolation, male KO mice exhibited heightened anxiety in terms of performance with a novel object in some tests. In terms of latency to first touch, KO mice took significantly longer on the first test ($F(1,56) = 4.4$; $p = 0.040$; ANOVA on power 0.3 transformed data) and third test, which followed the swim test ($H(1,56) = 4.9$; $p = 0.026$, $Q=2.2$,$p<0.05$, ANOVA on Ranks), but not on the second test ($H(1,56) = 0.25$; $p = 0.617$) (Fig. 5A). A lower number of touches occurred for KO males only on the third test ($H(1,56) = 11.1$; $p < 0.001$, $Q=3.3$, $p<0.05$) and not on the first ($F(1,56) = 3.6$; $p = 0.06$) or second test ($F(1,56) = 2.2$; $p = 0.132$) (Fig. 5B). In terms of total time in the vicinity of the novel object, KO male mice exhibited significantly lower levels following the third test ($H(1,56) = 4.3$; $p = 0.038$, $Q=2.0$, $p<0.05$, ANOVA on Ranks), but not on the first ($F(1,56) = 1.4$; $p = 0.232$) or second test ($F(1,56) = 0.79$; $p = 0.37$) (Fig. 5C).

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A repeated measures analysis within both WT and KO mice revealed a significant decrease in number of touches and time in area of novel object following the swim stress relative to the previous two tests ($p<0.05$). For KO mice, an increase in latency to first touch object was found following the swim test relative to the previous test ($p<0.05$), but levels did not reach significance for WT mice.

Forced swim test in males

Male WT and KO mice performed similarly on the forced swim test in terms of time to first float ($H(1,56) = 0.06$; $p = 0.806$ ANOVA on Ranks) and total time floating ($F(1,56) = 0.15$; $p = 0.695$). For latency to first float, WT = 64.5 ± 3.5 sec and KO = 66.8 ± 3.8 sec. For total time floating, WT = 32.7 ± 3.2 sec and KO = 34.8 ± 4.1 sec.

Intermale aggression

Intermale aggression did not differ between WT (N=28) and KO (N=29) mice in any measure on either the first, second, or third day of testing. For the first test day, performance was equivalent in terms of total duration of attacks ($H(1,56) = 2.0$; $p = 0.153$ ANOVA on Ranks) (Fig. 1A), number of attacks ($F(1,56) = 1.46$; $p = 0.226$) (Fig. 1B), and latency to attack ($H(1,56) = 1.04$; $p = 0.306$ ANOVA on Ranks) (Fig. 1C). Similarly, no differences were found on the second test day in terms of total duration of attacks ($H(1,56) = 1.40$; $p = 0.236$ ANOVA on Ranks) (Fig. 1A), number of attacks ($H(1,56) = 1.10$; $p = 0.293$ ANOVA on Ranks) (Fig. 1B), and latency to attack ($H(1,56) = 1.48$; $p = 0.224$ ANOVA on Ranks) (Fig. 1C). For the third test, no differences between groups were found in terms of total duration of attacks ($H(1,56) = 0.16$; $p = 0.690$ ANOVA on Ranks) (Fig. 1A), number of attacks ($H(1,56) = 0.37$; $p = 0.534$ ANOVA on Ranks) (Fig. 1B), and latency to attack ($H(1,56) = 0.03$; $p = 0.848$ ANOVA on Ranks) (Fig. 1C).

Discussion

In the current study, we find that loss of the gene for CRF-BP results in a specific impairment of offspring protection, but no other maternal or offspring measures are found to differ. The results provide new evidence that down regulation of CRF and/or Ucn 1 (the activity of which is suppressed by CRF-BP) is important in allowing the full emergence of offspring protection.

We previously have found that both CRF and Ucn 1 are potent inhibitors of maternal defense and the results presented here suggest that CRF-BP-mediated regulation of CRF and Ucn 1 activity in normal mice promotes maternal aggression. The results are also consistent with our initial findings that mice selected for high maternal defense have elevated levels of CRF-BP (Gammie et al., 2007). It is possible that the selected mice exhibit elevated aggression in part because of an additional dampening of endogenous CRF and/or Ucn 1 activity. We have recent evidence that CRF and Ucn 1 can impair offspring protection by acting in LS (S.C. Gammie, K.L. D'Anna, unpublished results). Given that LS exhibits a decreased reactivity to CRF during lactation (da Costa et al., 1997), CRF-BP may mitigate CRF or Ucn 1 action on LS during lactation and promote maternal defense. However, as the CRF-BP KO model used in this study is lacking CRF-BP in all sites of expression, additional studies will be needed to determine where CRF-BP may be acting in the CNS to alter CRF/Ucn 1 activity to promote aggression.

As previous studies have suggested that CRF-BP binds 65-90% of total CRF in many regions of the CNS (Behan et al., 1997), the loss of CRF-BP would be predicted to result in a significant increase in free CRH and Ucn 1 levels. While this has not been directly tested in this KO model, it is consistent with the increased anxiety-like behavior as would be predicted by the anxiogenic properties of free CRF. Changes in free CRF or Ucn 1 levels could also cause alterations in various types of cognitive function, including learning, memory, attention, perception or

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integration of sensory information, and locomotor activity that could affect maternal defense. In terms of total locomotor activity in an open field, no differences in the KO mice were observed in a previous study (Karolyi et al., 1999). In this study, we observed almost identical levels of swimming among genotypes, suggesting normal motor abilities of KO mice in this test. CRF can modulate learning and in two studies that used CRF fragments thought to trigger the release of CRF or Ucn 1 from CRF-BP, aspects of learning, including navigation, were enhanced (Zorrilla et al., 2001). In our mouse KO model, it is possible that elevated CRF or Ucn 1 from deletion of CRF-BP also modulates olfactory processing, learning, or motivation and these alter offspring protection. Because there is an important role for olfaction in both mating and maternal care (Numan and Insel, 2003), our finding of no deficits in mating or maternal behaviors, including nursing, nest building, and pup grooming, in KO mice suggests no overt olfactory deficiencies. However, we have not directly examined possible relationships between olfactory, learning, or motivation and offspring protection in these mice. It must also be noted that deletion of a gene may have a developmental or compensatory effect that alters phenotype (Nelson, 1997), and these compensatory changes may or may not match what occurs in a normal population. While we are not able to exclude these possibilities, our findings that only maternal, but not intermale aggression was altered by CRF-BP deletion, supports the idea that offspring protection suppression was a specific response to gene deletion.

Although our studies are consistent with an increase in free CRF and/or Ucn 1 with loss of the CRF-BP gene, a recent study suggested that CRF-BP was required to promote CRF/CRF receptor-mediated potentiation of NMDA receptors in the ventral tegmental area (VTA) (Ungless et al., 2003). In this scenario, the KO mice would exhibit an impaired ability to potentiate VTA and the decrease in offspring protection might result from changes in NMDA activity in VTA. In one study, reduction of dopamine in VTA did not alter maternal defense (Hansen et al., 1991), but other studies have suggested a possible role for mesolimbic dopamine in this behavior (Sorenson and Gordon, 1975, Yoshimura and Ogawa, 1991, Johns et al., 1998). Thus, a role for VTA cannot be excluded and our current analysis does not allow us to distinguish between the different possible mechanisms for change in phenotype.

While an important role for VTA and dopamine in maternal behaviors has been documented (Numan and Insel, 2003), our finding of unaltered pup retrieval in KO mice indicates this one aspect of maternal care is unaltered by gene deletion. Our finding of similar levels of nursing, licking and grooming of pups, and nest building on postpartum Day 3 suggests these aspects of maternal care are also intact. Pup weight was almost identical at birth and at postpartum Day 10 between groups, suggesting that cumulative nursing was similar among groups, supporting our observed nursing data. Given that we only observed general maternal behaviors once and for only one hour, it is possible that additional measures would have indicated maternal care differences between groups. As indicated in the Methods section, we used outbred breeder males for WT and KO mice in order to minimize possible effect of offspring on maternal profiles. We avoided KO mice raising KO offspring, but because WT mice raised WT offspring and KO mice raised heterozygote offspring, we cannot exclude the possibility of some offspring effects on maternal outcome. The similar litter size, birth weight, and weight gain, though, suggest no overt differences among offspring. However, additional maternal behavior information along with cross-fostering would be needed to address these issues.

KO female mice exhibited significantly higher anxiety than WT mice when examined as virgins, but only a trend towards higher anxiety was seen during lactation when aggression was tested. Previous studies have suggested a decrease in anxiety supports offspring protection, but counter-examples to this link exist, for reviews, see (Lonstein and Gammie, 2002, Gammie et al., 2008). When comparing the virgin to lactating state within either mouse group, no differences in anxiety were observed. Because other studies have found a decrease in anxiety with lactation, our results indicate that either these mice do not exhibit this change in anxiety

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state, or that our methods for testing anxiety were not appropriate to capture this change. Other measures of anxiety, such as elevated plus maze or novel object test, may have revealed different results. The composite neural circuits for both anxiety-like responses and maternal defense are unique, but may include some overlap of signaling pathways, including CRF or Ucn 1, that could link the two behaviors. The heightened anxiety in the KO mice appears loosely linked with decreased aggression and this may reflect common roles for CRF and Ucn 1 in both anxiety and maternal defense regulation.

In contrast to the negative effect of loss of CRF-BP on offspring protection, no effect of gene deletion was observed in any measure of intermale aggression. In this study, we used isolation-induced aggression (assayed via the resident intruder test), which mirrored the resident-intruder test used on lactating females. Other tests and other forms of intermale aggression exist, including dominance aggression, sexual aggression, and stress-induced aggression (Blanchard and Blanchard, 2006, Wingfield et al., 2006). It is not known whether removal of CRF-BP would affect these other forms of aggression. At the very least, though, we find a sex difference in terms of how the presence of CRF-BP alters aggression in males and females in at least one comparison. One explanation for this difference is that the maternal aggression circuit is more sensitive to the proposed elevation of CRF or Ucn 1 seen in the KO mice than is the intermale circuit. If altered VTA activity underlies the phenotype, then it is possible that maternal aggression circuitry is more responsive to VTA activity than intermale circuitry. Given that the underlying neuronal basis of maternal and this form of intermale aggression are thought to differ (Gammie and Lonstein, 2006), it is not surprising that here we see a differential effect of gene deletion. In previous studies of knockout mice, different effects were seen on maternal versus intermale aggression with the loss of neuronal nitric oxide synthase (Nelson et al., 1995, Gammie and Nelson, 1999), endothelial nitric oxide synthase (Demas et al., 1999, Gammie et al., 2000), CRF receptor 2 (Gammie et al., 2005), and a subset of genomically linked vomeronasal receptors that affect pheromonal perception (Del Punta et al., 2002).

The finding that virgin female CRF-BP KO mice show elevated anxiety as seen in the light dark box is consistent with the previously published study that found male KO mice also had elevated anxiety in terms of elevated plus maze and defensive withdrawal performance (Karolyi et al., 1999). Previous work indicates that estrous cycle length is not significantly different between genotypes (N.J. Westphal and A.F. Seasholtz, unpublished observations), but estrous cycle state itself can influence anxiety in rodents (Frye et al., 2000). Because we did not measure estrous state, we cannot exclude the possibility that differences in the estrous state among individuals contributed to anxiety differences observed between genotypes. In terms of anxiety measured in the light/dark box test, no differences in male groups were found. However, higher anxiety was found in the KO males tested here when examined in the novel object test. The elevated anxiety in this measure was also pronounced when examined following a brief forced swim stress test. Interestingly, we found no differences in swim performance between genotypes. We did not test females on the novel object test, so comparisons cannot be made. These results suggest anxiety was increased in KO males, but it was only manifested in one of two tests.

We saw no weight differences between groups in males or females. A lack of effect on weight was previously seen for females, but lower KO male weight was observed as mice aged beyond 10 weeks (Karolyi et al., 1999). In this study, we examined male weight only once around 4 months of age. Our finding of lack of weight differences between groups suggests that in this background, weight loss in males is not a phenotype of the gene deletion. Without additional timepoints for male weight, though, this result should be treated cautiously.

In summary, we previously identified elevated expression of CRF-BP in mice that were selected for high maternal defense and here we see that loss of CRF-BP in these mice

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significantly impairs offspring protection. We do not think elevated expression of CRF-BP is the only means for elevating aggression because aggression was reduced by only 50% (but not completely) with the deletion. Thus, KO females were still able protect offspring (at a reduced level), so some important protective mechanisms remain intact, but how the deletion impinges on those circuits needs to be clarified. Also, we originally identified a number of other genes with altered expression due to selection, and we would predict that a range of genes will work together to regulate this important social behavior.

How selection resulted in elevated expression of CRF-BP is not known. CRF-BP is positively regulated by stress, cAMP, and interleukin-6 and differentially regulated by estradiol and glucocorticoids (McClennen et al., 1998, Lombardo et al., 2001, Speert et al., 2002, Herringa et al., 2004, Westphal and Seasholtz, 2006) The CRF-BP promoter also includes CRE, ERE, and AP-1 binding sites (Behan et al., 1993, Cortright et al., 1997, van de Stolpe et al., 2004). Thus, altered levels or activation of transcription factors is one possible mechanism for increased CRF-BP expression. There are currently two transgenic models of CRF-BP overexpression in mice (Burrows et al., 1998, Lovejoy et al., 1998, Seasholtz et al., 2001). It would be interesting to determine whether offspring protection levels were elevated in these transgenic mice. Finally, a truncated version of CRF (CRF 6-33) can act to dissociate CRF from CRF-BP and thus is thought to act as a pharmacological inhibitor of CRF-BP (Chan et al., 2000, Heinrichs and Joppa, 2001). Based on our findings, it would be expected that the CRF-BP inhibitor would suppress offspring protection, but this inhibitor has not yet been tested.

Acknowledgements

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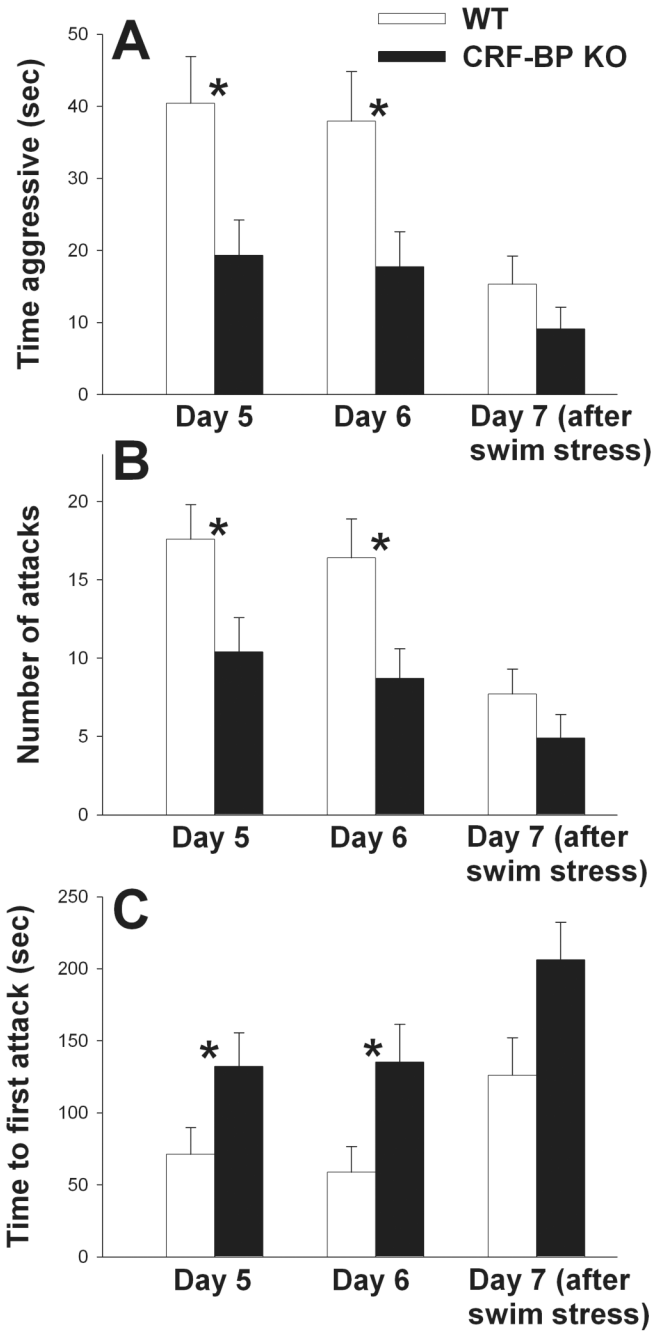

Fig. 1.

Analysis of maternal aggression in WT and CRF-BP KO mice. Using a resident-intruder paradigm, KO females showed an impaired ability to express maternal aggression in terms of total duration of attacks (A), number of attacks (B), and latency to first attack (C) on both Days 5 and 6 postpartum. Following the swim stress test on day 7, aggression decreased for both groups (see Results section) and no differences between groups were found (A-C). Bars represent means+SE. White bars indicate WT mice and black bars indicate KO mice. * = $p<0.05$.

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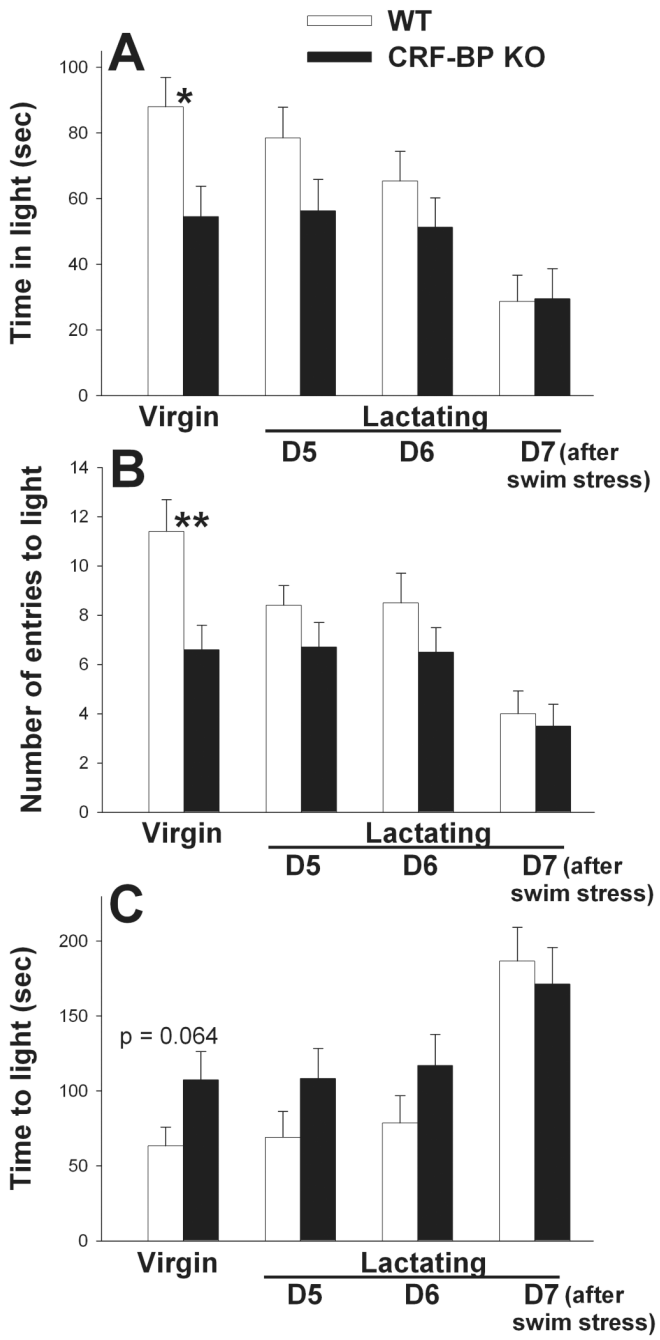

Fig 2.

Analysis of anxiety in WT and CRF-BP KO female mice. When examined as virgins, KO females exhibited heightened anxiety in terms of decreased time in light portion of the light/dark box (A) and decreased number of entries to the light portion of the light/dark box (B). The latency to first enter the light portion of the box was longer in KO mice, but this did not reach significance ($p = 0.064$). When examined during lactation on Days 5 and 6, no differences between groups in any measure were found (A-C). Following a swim stress test on day 7, no differences between groups were found (A-C). Bars represent means + SE. White bars indicate WT mice and black bars indicate KO mice. * = $p<0.05$; ** = $p<0.01$.

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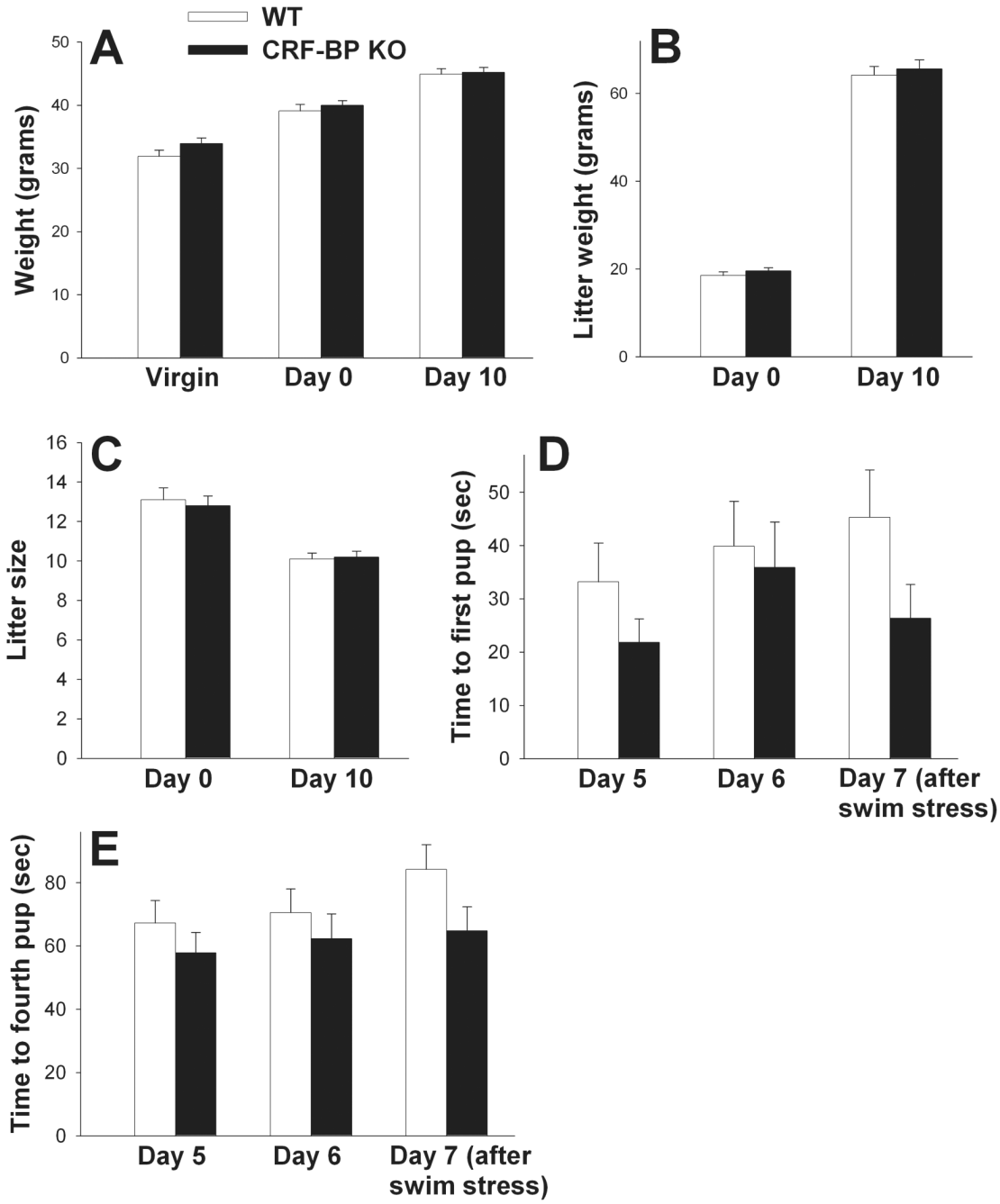

Fig 3.

Maternal and pup profiles of WT and CRF-BP KO female mice. In terms of body weight both as virgins and on postpartum Days 0 and 10, no differences between groups were found (A). Litter weight did not differ between groups when examined on Day 0 and Day 10 (B). Litter size was similar on Day 0 and Day 10 (C). Note that on Day 1 litters were culled to 11, so almost no pup loss was seen in either group. In terms of pup retrieval, no difference were found between groups on Days 5 or 6 or after a brief swim stress on Day 7 (D, 1st pup) and (E, 4th pup). Bars represent means + SE. White bars indicate WT mice and black bars indicate KO mice.

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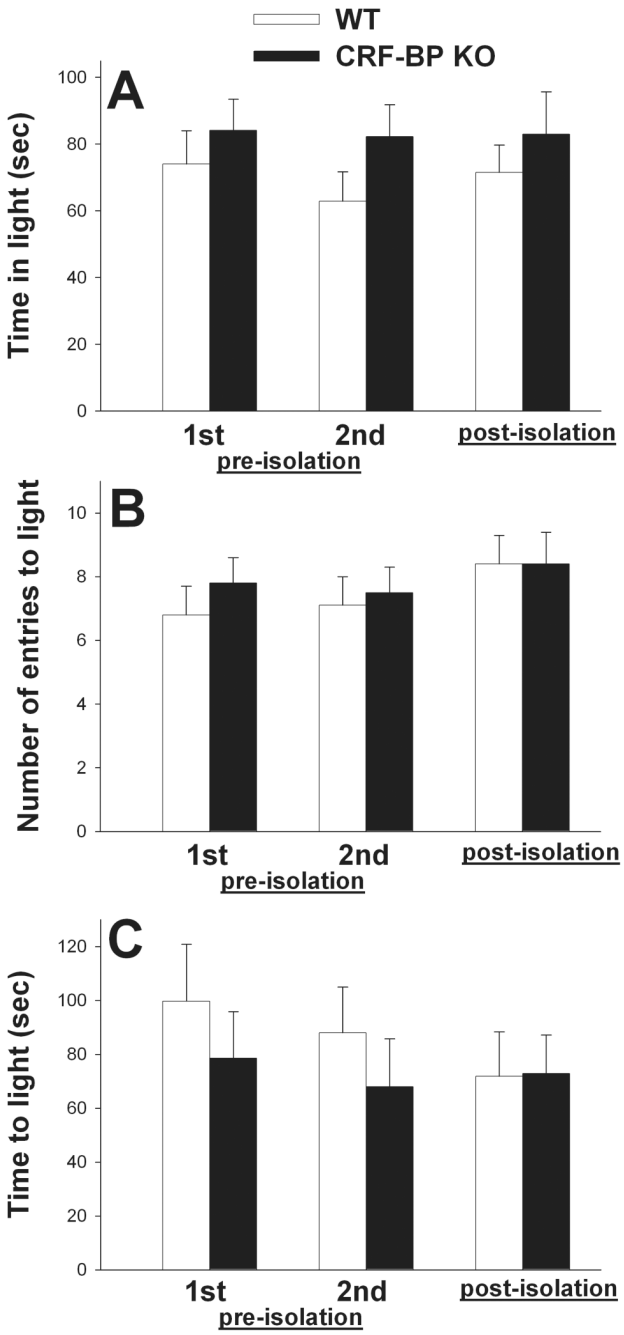

Fig. 4.

Analysis of anxiety in WT and CRF-BP KO males using the light/dark test. When examined as group-housed adults and following isolation, no differences were found between groups in terms of either time in light (A), number of entries to the light (B), or latency to enter light portion of the light/dark box. Bars represent means + SE. White bars indicate WT mice and black bars indicate KO mice.

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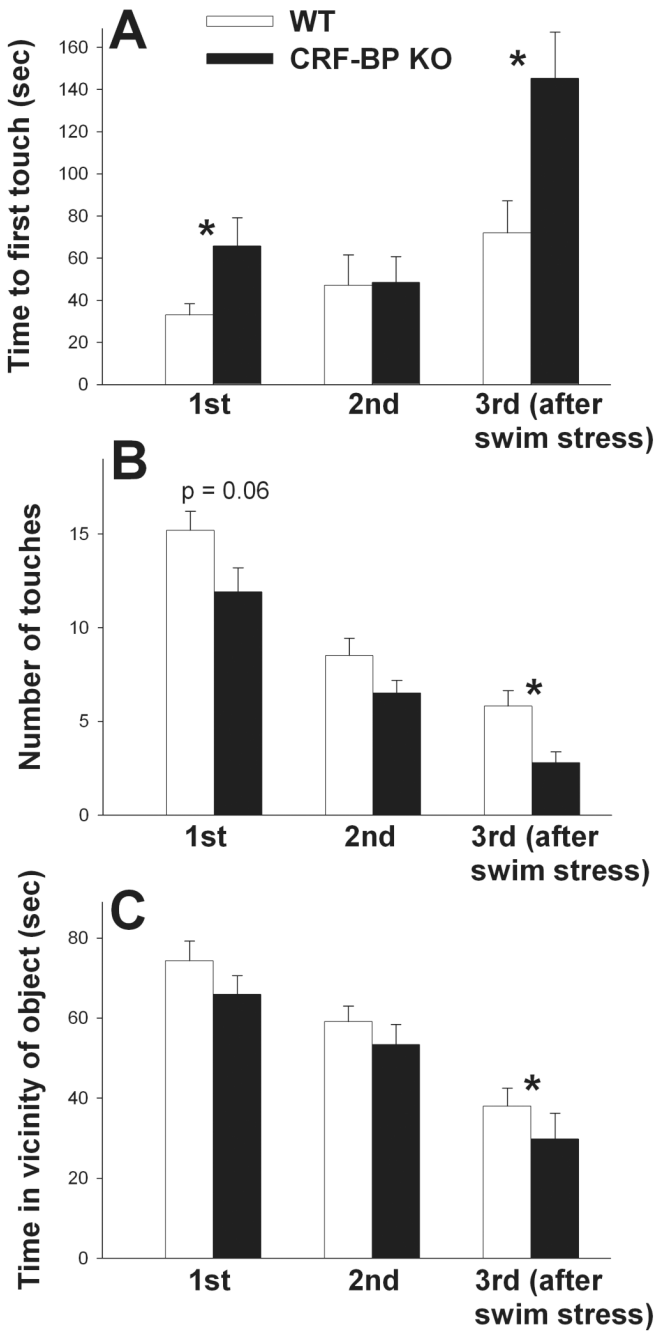

Fig. 5.

Analysis of anxiety in WT and CRF-BP KO males using the novel object test. When examined as group-housed adults prior to isolation, KO males exhibited heightened anxiety in terms of prolonged latency to first touch the novel object on the first test and third test, which followed a forced swim test (A). A decreased number of touches was observed for KO mice on the third test; differences approached significance on the first test (B). The amount of time in the vicinity of the novel object was significantly decreased in KO relative to WT mice for the third test, but not the other tests (C). Bars represent means + SE. White bars indicate WT mice and black bars indicate KO mice. * = $p<0.05$.

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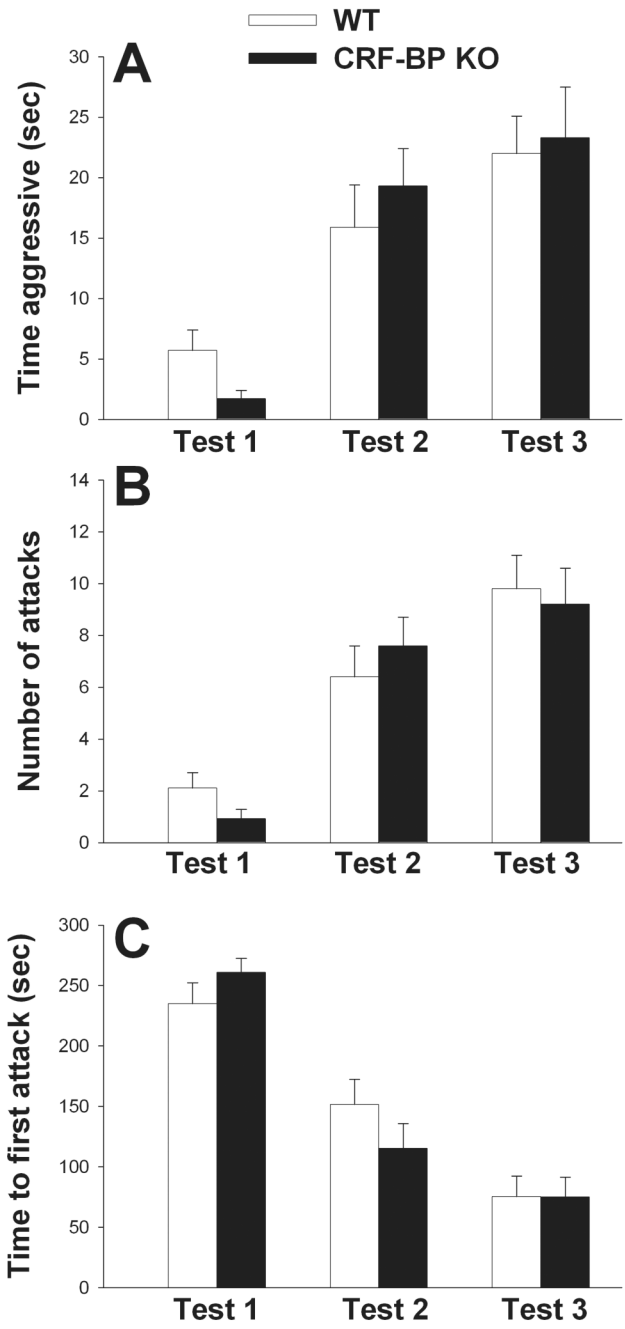

Fig. 6.

Analysis of intermale aggression in WT and CRF-BP KO mice. Using a resident-intruder paradigm on males isolated for 30 days, no differences in aggression between groups were found in terms of total duration of attacks (A), number of attacks (B), and latency to first attack (C) on any of the three consecutive test days. Bars represent means + SE. White bars indicate WT mice and black bars indicate KO mice.