Fighting in the home cage: Agonistic encounters and effects on neurobiological markers within the social decision-making network of house mice (*Mus musculus*)

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HIGHLIGHTS

- We determined competitive abilities within the homecages of group-housed C57Bl/6 mice.
- Dominance status influenced neurobiological markers associated with aggression.
- We present a "tube test" as a tool for assessing dominance status.

ARTICLE INFO

ABSTRACT

Inbred strains of mice, such as C57Bl/6, have become preferred animal models for neurobehavioral studies. A main goal in creating inbred lines is to reduce the effects of individual genetic variation on observed phenotypes. Most studies use only males, and there is increasing evidence that agonistic interactions within the home cage may produce systematic variability in behavior and brain function. Previous studies have demonstrated that the outcomes of aggressive interactions have powerful effects on the brain and behavior, but less is known about whether aggressive interactions within the home cage have similar effects. We assessed group-housed laboratory mice C57Bl/6 for competitive ability and then tested the extent high competitive ability (CA) or low CA was related to gene and protein expression within related pathways. We focused on a broad social behavior network, including the nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST). High CA mice had significantly more corticotropin releasing hormone receptor 2 (*CRHR2*) and estrogen receptor alpha (*ESR1*) mRNA in the BNST. Our data suggest a simple test of CA could yield valuable information that could be used to reduce error variance and increase power in neurobiological studies using mice.

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1. Introduction

The laboratory mouse (*Mus musculus*) is used as a genetic model for a wide range of human diseases, particularly within clinical neuroscience [1,7]. Interestingly, even though most studies use identical strains of inbred mice, variability in experimental outcomes is often a problem [7,38]. It is well known, although perhaps underappreciated, that many mundane aspects of the lab environment such as diet [9], cage bedding [37], or noise levels [28] can have a major impact on study outcomes. The sex of the animal is also critical, and most neuroscience studies focus exclusively on male rodents [2]. Although typically not acknowledged, a common rationale for focusing on males is to avoid potential confounds related to hormone fluctuations of the female ovarian

cycle. However, in some contexts males can be more variable than females [24]. Dominance hierarchies are an important part of *Mus* life history [23,25,27], and the outcomes of aggressive encounters have important effects on the brain and behavior [4]. Aggressive interactions between males in the home cage are common in many strains of *Mus* [10,17]. Indeed, anxiety-like behavior in male C57Bl/6 mice is reduced in single-housed males compared to group-housed males [34], suggesting that ongoing aggressive interactions have important implications for behavioral testing [35].

Previous studies have demonstrated differences between dominant and subordinate *M. musculus* in behavior and brain function [35,36], but these studies typically used outbred strains, such as CD-1, and observed dominance relationships in dyadic pairs. In contrast, little is known of the potential impact of competitive ability in group-housed C57Bl/6, the predominant background for genetic studies. Here, we investigated the impact of competitive ability on signaling pathways in nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST). The nuclei are components of a social decision-making network known to regulate both social behavior and behavioral responses to stress [26]. We show that dominance hierarchies within the home cage have very strong effects on brain function that could systematically affect the outcomes of neuroscience experiments.

2. Methods

2.1. Animals

Male C57BL/6J mice (~100 days old) were obtained from Jackson Laboratory-West and were housed in groups of 4. For the duration of this study, mice were housed in either a polycarbonate home cage (26 cm × 15.2 cm) or in a behavioral testing apparatus. Housing was lined with a mixture of Carefresh (International Absorbents, Ferndale, WA, USA) and Paperchip beddings (Shepherd Specialty Papers, Kalamazoo, MI, USA) in a 2:1 ratio. Mice were kept on a 14 h light/10 h dark cycle throughout the study, with free access to food (Purina Mills Mouse Chow 5010, St. Louis, MO, USA) and water. Mice were acclimated to the testing apparatus for 72 h before being recorded for 24 h for behavioral coding. The testing apparatus was outfitted with radio frequency identification and digital video recording for automated measurement of animal behavior (see [16]). Following a brief return to the home cage for cleaning, this process was repeated twice for a total of 3 observation periods. Following the 3rd observation period, mice were returned to the home cage for at least one week, anesthetized with isoflurane and euthanized by decapitation. Brains were removed and dissections were made from 2 mm-thick coronal sections taken from a brain matrix. The nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST) were dissected using 1 mm-diameter bilateral punches and then frozen on dry ice [12].

2.2. Competitive ability assessment

Fighting between familiar male C57/Bl6 cagemates begins to occur at approximately 55 days of age [30]. Following a series of agonistic interactions, there is typically a single dominant mouse that receives the least amount of aggression within the cage, while hierarchies form among all remaining subordinates [3,30]. In this study, we identified mice based on their competitive ability. All occurrences of aggression were recorded, and the winner and loser of each interaction were determined by characterizing pursuit and submissive behaviors [15].

While a wide array of assays exist for measuring agonistic behavior in rodents [18], many classic dominance tests promote fighting between subjects [30]. On the other hand, competitive methods commonly consist of two animals competing for a resource from opposite sides of a tube [22], and they can be used to assess dominance status without the experience of wounds or other physical trauma. We confirmed competitive ability using a "tube test" from previously described methods [15]. Briefly, two mice from the same cage were placed simultaneously on opposite sides of a tube for a competitive exclusion test. The mouse that first departed from the tube was recorded as a loser of the trial. For each mouse we calculated the proportion of tube tests trials won out of the total that the mouse participated in. Thirteen mice won more than half of the tube test trials and were classified as high competitive ability (CA) whereas twelve mice won fewer than half of the tube trials and were considered low CA.

2.3. Western blotting

Punch samples from NAc were homogenized in buffer (7.4 pH, 20% glycerol, 0.4 M NaCl, 20 mM HEPES, 5 mM MgCl$_2$, 0.5 mM EDTA in H$_2$O) with protease inhibitor (1% PMSF in EtOH). Laemmli buffer (Sigma, St. Louis, MO) was added to the homogenate at a 1:1 dilution. Proteins were denatured and separated with gel electrophoresis (15% bis-acrylamide resolving gel). Protein was transferred to polyvinylidine fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) which were then rinsed and blocked with 5% skim milk in 0.1%Triton-X with tris-buffered saline (TBS-T). We probed membranes with rabbit anti-AR (Millipore, 1:500) and rabbit anti-β-actin (Cell Signaling, 1:2000), followed by incubation in peroxidase-conjugated anti-rabbit secondary antibody (Vector, 1:2000) in TBS-T. Blots were imaged on a Bio-Rad ChemiDoc. Bands for AR protein were normalized to their respective actin controls.

2.4. qPCR

For the BNST, RNA was extracted with RNaqueous kits (Life Tech) and then reverse transcribed. We then used an ABI 7500 Sequencing Detection system (Applied Biosystems, Foster City, CA) and Taqman chemistry to detect estrogen receptor alpha (*ESR1*) and corticotropin releasing hormone receptor 2 (*CRHR2)* mRNA. Relative gene expression was calculated by comparison to standard curves consisting of serial dilutions of pooled brain cDNA followed by normalization to 18s gene expression. Gene expression was normalized to a cDNA pool run on each plate.

2.5. Statistical methods

We used *Q–Q* plots and histograms to check for normal distributions of datasets. Protein and gene expression data were not
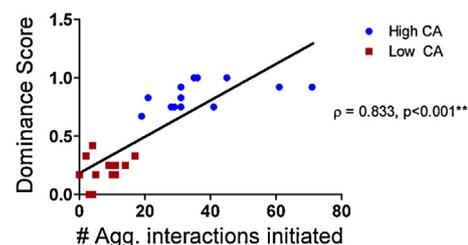
Fig. 1. The "tube test" can be used as a tool to predict competitive ability (CA). Dominance scores (number of trials won/total trials participated in) from the tube test were significantly, positively correlated with the number of aggressive bouts initiated while mice were in the behavioral testing apparatus (*n* = 13 high CA, *n* = 12 low CA, ** *p* < 0.01).
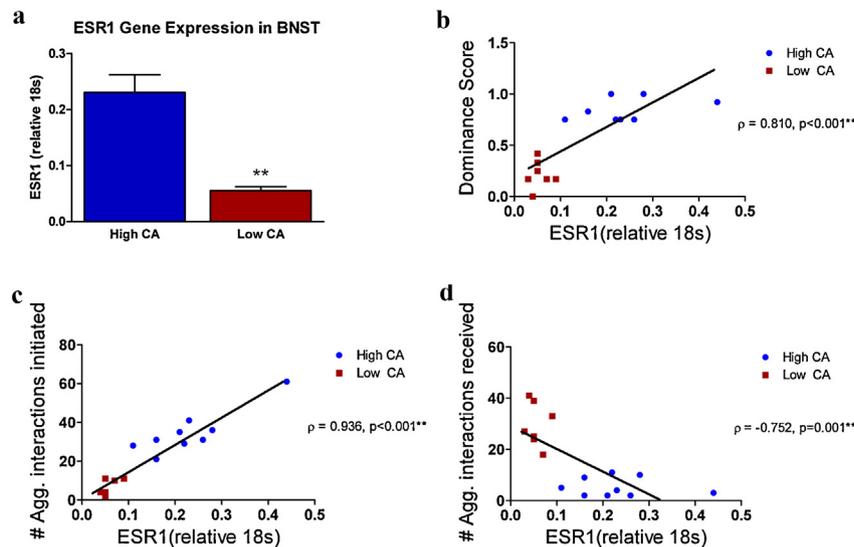
Fig. 2. Competitive ability (CA) affects estrogen receptor alpha (*ESR1*) mRNA in the bed nucleus of the stria terminalis (BNST). (a) High (CA) mice have more *ESR1* mRNA in BNST compared to low CA mice. Data are shown as mean ± SE ($n = 9$ high CA, $n = 7$ low CA, [**] $p < 0.01$). (b) *ESR1* in the BNST is positively correlated with dominance scores from the "tube test." Dominance score was calculated from the ratio of number of trials won:total trials participated in ([**] $p < 0.01$). (c) *ESR1* mRNA in the BNST is positively correlated with the number of aggressive interactions initiated and (d) negatively correlated with the number of aggressive interactions received. ([**] $p < 0.01$).

normally distributed, so we analyzed this data with nonparametric statistics. We used Spearman correlations to examine relationships between protein/gene expression and behavior.

3. Results

3.1. Performance in tube test is associated with experience of aggressive interactions

The proportion of wins from the tube test significantly predicted aggressive bouts initiated while mice were in the behavioral testing apparatus (Fig. 1, $\rho = 0.680$, $p < 0.001$). In addition, the proportion of wins from the tube test was also significantly, negatively correlated with aggressive bouts received while mice were in the behavioral testing apparatus ($\rho = -0.545$, $p < 0.01$).

3.2. Competitive ability is associated with CRHR2 and ESR1 mRNA expression in the BNST

High CA mice had significantly more *ESR1* mRNA in the BNST than low CA mice (Fig. 2a, Mann–Whitney $U < 0.001$, $p < 0.001$). *ESR1* in the BNST was positively correlated with tube test dominance scores (Fig. 2b, $\rho = 0.810$, $p < 0.001$). There was a positive correlation between *ESR1* expression and the number of aggressive bouts initiated (Fig. 2c, $\rho = 0.936$, $p < 0.001$) and negative correlation with the number of aggressive bouts received (Fig. 2d, $\rho = -0.752$, $p < 0.01$). High CA mice had significantly more *CRHR2* expression in the BNST (Fig. 3a, Mann–Whitney $U = 5.00$, $p < 0.05$). *CRHR2* in the BNST was positively correlated with tube test dominance scores (Fig. 3b, $\rho = 0.574$, $p < 0.05$). *CRHR2* was positively correlated with the number of aggressive bouts initiated (Fig. 3c, $\rho = 0.504$, $p < 0.05$)
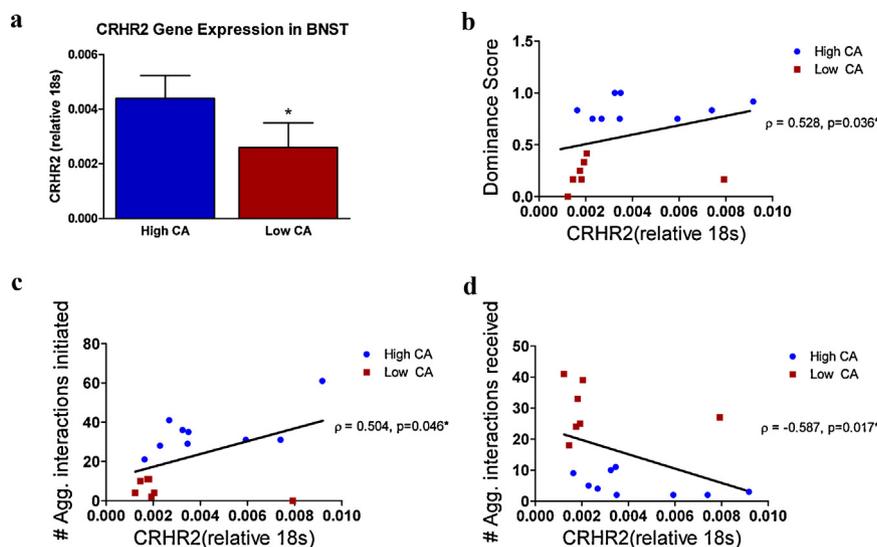
Fig. 3. Competitive ability (CA) affects corticotropin-releasing hormone receptor 2 (*CRHR2*) mRNA in the bed nucleus of the stria terminalis (BNST). (a) High competitive ability (CA) mice have more *CRHR2* mRNA in BNST compared to LCA mice. Data are shown as mean ± SE ($n = 9$ high CA, $n = 7$ low CA, [*] $p < 0.05$). (b) *CRHR2* in the BNST is positively correlated with dominance scores from the "tube test." Dominance score was calculated from the ratio of number of trials won:total trials participated in ([*] $p < 0.05$). (c) *CRHR2* mRNA in BNST is positively correlated with the number of aggressive interactions initiated and (d) negatively correlated with the number of aggressive interactions received. ([*] $p < 0.05$).
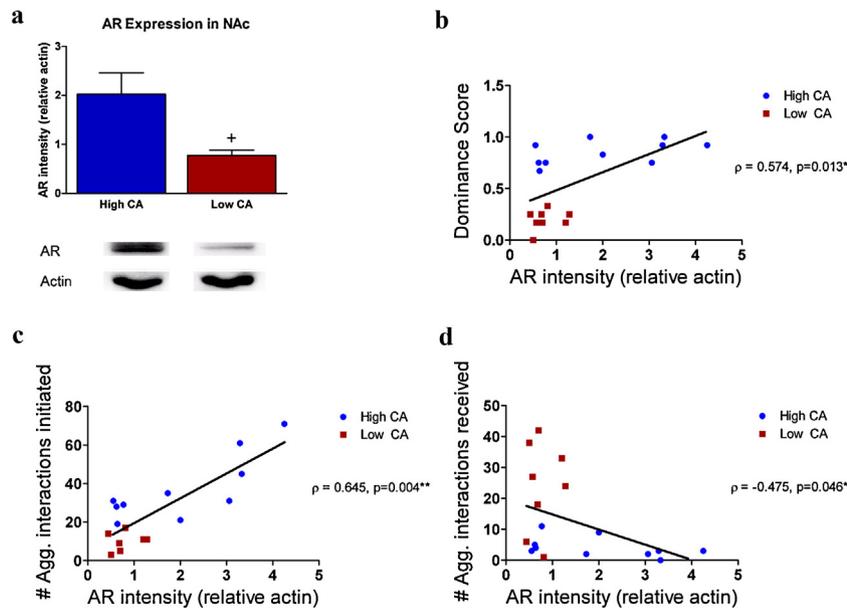
Fig. 4. Competitive ability (CA) affects androgen receptor (AR) protein in the nucleus accumbens (NAc). (a) High CA mice have a near-significant trend for more NAc AR protein than low CA mice. Data are shown as mean ± SE ($n = 10$ HCA, $n = 8$ LCA, + $p = 0.06$). (b) AR protein in NAc is positively correlated with dominance scores from the "tube test." Dominance score was calculated from the ratio of number of trials won:total trials participated in (* $p < 0.05$). (c) AR protein in NAc is positively correlated with the number of aggressive interactions initiated and (d) negatively correlated with the number of aggressive interactions received. AR was calculated from the ratio of AR:Actin. (** $p < 0.01$, * $p < 0.05$).

and negatively correlated with the number of aggressive bouts received (Fig. 3d, $\rho = -0.587$, $p < 0.05$).

3.3. Competitive ability is associated with AR protein expression in the NAc

High CA mice had near-significantly more AR in the NAc than low CA mice (Fig. 4a, Mann–Whitney $U = 19.00$, $p = 0.06$). AR in the NAc was significantly, positively correlated with the dominance score from the tube test (Fig. 4b, $\rho = 0.574$, $p < 0.05$). Additionally, AR in the NAc was significantly, positively correlated with the number of aggressive bouts initiated (Fig. 4c, $\rho = 0.645$, $p < 0.01$) and significantly, negatively correlated with the number of aggressive bouts received in the behavioral testing apparatus (Fig. 4d, $\rho = -0.475$, $p < 0.05$).

4. Discussion

Investigators usually assume there are no systematic differences between individual mice before they are assigned to treatment groups. However, our data suggest aggressive interactions between male *Mus* in the home cage exert a systematic effect on signaling systems that are commonly studied by neuroscientists. High CA mice had increased *ESR1* and *CRHR2* gene expression in the BNST and a marginally-significant increase in AR in the NAc. Differences between high CA and low CA mice were substantial, often more than two fold. The changes occurred in the absence of any outside manipulations and suggest that aggressive interactions are an important source of error variance in neuroscience studies of group-housed *M. musculus.* We also demonstrated that the relatively simple tube test can be used to assess and potentially control for this variability. These findings indicate aggressive interactions in the home cage could contribute to variability in behavior and neurobiology, and they provide further considerations for single- vs. group-housing of laboratory rodents.

4.1. High CA mice had increased ESR1 and CRHR2 gene expression in the BNST

Both *ESR1* and *CRHR2* mRNAs were upregulated in the BNST of high CA mice when compared to low CA mice. The BNST is a brain region known to modulate aggressive behavior [33]. A previous study reported a positive correlation between *ESR1's* translated protein (ERα) and aggression in outbred CD-1 mice [6]. Our results suggest that this individual variation in ERα expression generalizes to an inbred strain. Interestingly, estrogens can be locally synthesized, and local steroid signals are more likely than systemic signals to act via rapid mechanisms [13,29,39]. Increased ERα in the BNST has been linked to increased aggression in male Siberian hamsters during short-day photoperiods [20]. Earlier studies from male songbirds provided experimental support for the regulation of aggressive behavior by estrogens during nonbreeding seasons [31,32].

The BNST serves as a relay point for the "social decision-making network's" superhighway, connecting the mesolimbic dopamine system to the social behavior network [26]. This makes the region well-positioned to integrate intrinsic cues with environmental signals to produce adaptive behavioral responses (e.g. aggression) to social stimuli. Previous studies have linked nodes throughout the "social behavior network" to responses to social stimulation, particularly agonistic encounters. Neuronal activity in the mesolimbic pathway [21], social behavior network [19] and the BNST [19] is modulated following social stress in rodents. Additionally, dominant naked mole-rats have a larger BNST than that of subordinates [14]. Changes in the BNST could modulate aggressive-like behaviors in mice.

4.2. AR protein in NAc is associated with competitive ability

There is considerable evidence that androgens facilitate aggressive behavior in rodents, including *Mus.* Increased AR expression has also been observed in dominant naked mole-rats [14], suggesting that increased AR expression is an intrinsic factor that

confers dominance. However, it is also possible that winning aggressive encounters increases AR expression. The experience of winning aggressive encounters (independent of intrinsic fighting ability) increases AR in the NAc of male California mice [11]. This mechanism may be more important in genetically inbred lines such as C57Bl/6, and suggest that altering housing conditions to reduce aggressive interactions might reduce dominance hierarchy-induced variance in behavior and brain function.

5. Conclusions

One justification for the use of inbred *Mus* strains is to reduce individual variation and maximize power [8]. Although researchers create controlled laboratory environments, our results show the standard procedure of group housing male C57Bl/6 produces aggressive interactions that have important effects on neural signaling systems. However, we also show that the "tube test" can be used as a tool to easily assess exposure to aggressive interactions, which could be used as a covariate in statistical analyses. Importantly, researchers commonly exclude injured mice from their studies, creating a bias in sampling towards dominants which typically do not incur physical injury in the home cage [30]. Alternatively, adjusting husbandry conditions to reduce intermale aggression might reduce variation. For example, transferring nesting material from dirty cages to clean cages has been found to reduce aggression in the home cage [35]. Considerations for housing and social structure in *Mus* studies of aggression have been proposed for some time [5], but few studies incorporate this variable into analyses. Overall, controlling for dominance hierarchies in *Mus* should reduce variability and increase power for neurobiological studies of social behavior.

Acknowledgments

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