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## High Novelty-Seeking Predicts Aggression and Gene Expression Differences within Defined Serotonergic Cell Groups

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### Abstract

Aggression frequently coincides with specific dimensions of emotionality, such as impulsivity, risk-taking, and drug abuse. Serotonergic (5-HTergic) neurotransmission contributes to the regulation of numerous neurobiological functions, and is thought to play a key role in modulating aggressive responses. The current study uses selectively-bred High (bHR) and Low (bLR) Responder rats that exhibit differences in emotionality and behavioral control, with bHRs exhibiting heightened novelty-induced exploration, impulsivity, and increased sensitivity to drugs of abuse, and with bLRs characterized by exaggerated depressive- and anxiety-like behaviors. Based on this behavioral profile we hypothesized that bHR rats exhibit increased aggression along with changes in testosterone and corticosterone secretion characteristic of aggression, and that these changes are accompanied by alterations in the expression of key genes that regulate 5-HTergic neurotransmission (*Tph2* and *Sert*) as well as in the activation of 5-HTergic cell groups following aggressive encounter. Our data demonstrate that when compared to bLR rats, bHRs express increased baseline *Tph2* and *Sert* in select brainstem nuclei, and when tested on the resident-intruder test they exhibited: 1) increased aggressive behavior; 2) potentiated corticosterone and testosterone secretion; and 3) diminished intrusion-induced *c-fos* expression in select 5-HTergic brainstem cell groups. The most prominent gene expression differences occurred in the B9 cell group, pontomesencephalic reticular formation, median raphe, and the gigantocellular nucleus pars  $\alpha$ . These data are consistent with the notion that altered 5-HT neurotransmission contributes to bHRs' heightened aggression. Furthermore, they indicate that a specific subset of brainstem 5-HTergic cell groups contributes to the regulation of intrusion-elicited behavioral responses.

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## Keywords

emotionality; brainstem; TPH2; SERT; c-fos; resident-intruder test

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## 1. Introduction

Serotonergic (5-HTergic) neurotransmission contributes to the regulation of virtually every neurobiological function, including sensory, motor, homeostatic, cognitive, and higher order executive functions. Furthermore, dysregulated 5-HTergic neurotransmission contributes to diverse neuropsychiatric illnesses, including major depression, bipolar disorder, suicide, sleep disorders, conduct disorders, Tourette's syndrome, and psychosis (Arango et al., 2002; Asberg et al., 1976; de Boer and Koolhaas, 2005; Lopez-Figueroa et al., 2004; Parsey et al., 2006; Rodrigo-Angulo et al., 2000; Thase, 1999). Despite such widespread functional roles, 5-HTergic neurons number only in the tens of thousands in the mammalian brain, yet these cells are highly complex, sending collateralized projections to multiple targets (Jacobs and Azmitia, 1992; Steinbusch, 1984). Emerging evidence points to functional and structural heterogeneity in the organization of these cell groups. (Abrams et al., 2004; Clark et al., 2006; Jones and Light, 1992; Lowry, 2002; Lowry et al., 2008; Waselus et al., 2006). For example, 5-HTergic neurons within the caudal dorsal raphe nucleus (DRC) project to limbic targets, including septum and the bed nucleus of the stria terminalis (Waselus et al., 2006), are thought to regulate mood and affect, and are potentially involved in anxiety, depression, and suicide (Lowry et al., 2008). On the other hand, 5-HTergic neurons located more rostrally and ventrally project to the basal ganglia (Waselus et al., 2006), have been implicated in motor control and cognitive regulation, and may play a role in Tourette's syndrome and obsessive compulsive disorder (Lowry et al., 2008). Together these observations suggest the existence of parallel 5-HTergic circuits that mediate specific behaviors and physiological states.

One such behavior is aggression, which has been linked with altered 5-HTergic tone in numerous clinical studies and rodent models (Davidson et al., 2000; Kruk et al., 1998; Miczek et al., 2007; Nelson and Trainor, 2007). Interestingly, while there is a well-established link between aggression and 5-HT, the exact nature of this relationship is complex. Distinct types of aggressive displays (e.g. pathological aggression versus territorial aggression) have been associated with distinct neurochemical changes (either diminished or potentiated 5-HTergic transmission (Nelson and Trainor, 2007)). Moreover, individual susceptibility to aggression is influenced by a variety of biological factors as well as behavioral traits.

We recently developed two contrasting rodent lines -- High Responder (bHR) and Low Responder (bLR) rats that were selectively-bred for their propensity to explore a novel environment. Compared to their bLR counterparts, bHR rats exhibit heightened novelty exploration, enhanced impulsivity and reward drive, as well as decreased depressive- and anxiety-like behaviors (Clinton et al., 2007; Flagel et al., 2010; Perez et al., 2009; Stead et al., 2006). These behavioral responses are reminiscent of human externalizing neuropsychiatric disorders, which also include pathological aggression (Flagel et al., 2010). Thus, in the current study we hypothesized that bHR rats exhibit heightened aggressive behavior that is correlated with altered endocrine and gene expression changes within the brainstem 5-HTergic cell groups.

## 2. Results

### 2.1 Behavior on the Resident-Intruder Test

When exposed to the intruder, bHR residents spent markedly more time engaged in aggressive behaviors compared to bLR residents ( $F_{1,33} = 34.83$ ,  $p < 0.0001$ ; Fig. 1A). In contrast, resident bLRs spent significantly more time in defensive freezing behavior ( $F_{1,33} = 4.18$ ,  $p < 0.05$ ; Fig. 1C). No difference in aggressive behavior was detected between intruder animals paired either with bHR or bLR rats (Fig. 1B); however, time spent in defensive freezing was significantly higher in intruder rats paired with bHRs ( $F_{1,33} = 4.69$ ,  $p < 0.05$ ; Fig. 1D). Overall, bLR residents spent the vast majority of their time engaged in other (non-aggressive) behaviors, which was significantly greater than their bHR counterparts ( $F_{1,33} = 7.67$ ,  $p < 0.01$ ). Details about specific aggressive and non-aggressive behaviors are shown in Table 1.

### 2.2 Testosterone and Corticosterone Levels

Increased aggressive behavior in bHR resident rats was accompanied by a significant increase in circulating testosterone levels (Fig. 2A). In contrast, bLR residents did not increase their secretion of testosterone; consequently, post-intrusion testosterone levels were more than two-fold greater in bHR vs. bLR rats ( $F_{1,38} = 9.03$ ,  $p < 0.01$ ; Fig. 2A).

Exposure to the intruder elicited increased secretion of corticosterone in both bLR and bHR rats (main effect of intrusion compared to baseline,  $F_{1,44} = 181.21$ ,  $p < 0.0001$ ). However, this response was significantly greater in bHR animals (main effect of phenotype  $F_{1,44} = 4.52$ ,  $p < 0.05$  and significant phenotype  $\times$  intrusion interaction  $F_{1,44} = 4.91$ ,  $p < 0.05$ ; Fig. 2B).

### 2.3 Characterization of Raphe Cell Groups

Examination of *Sert* autoradiograms revealed distinct clusters of 5-HTergic cell groups throughout the brainstem. Such neurochemical parcellation agreed with cytoarchitectonic differences, as was apparent when *Sert* signal was digitally projected onto adjacent cresyl violet-stained tissue sections (Supplementary Fig. 1).

Using a combination of neurochemical and cytoarchitectonic criteria, we examined tissue sections at 240  $\mu\text{m}$  intervals and identified distinct 5-HTergic cell groups throughout the brainstem (Fig. 3). We observed differences in density, intensity, and distribution of *Sert* signal associated with different cell groups. These differences also corresponded with alterations in cell size, cell shape, and packing density that were apparent in cresyl violet-stained material. Tissue processed for *Tph2* immunocytochemistry revealed the same parcellation of 5-HT-ergic cell groups as revealed by *Sert* ISH, and similar differences in cell size, shape and density as suggested by cresyl violet stain (Supplementary Fig. 2).

Caudal 5-HTergic cell groups were located within the rostral medulla and caudal pons, while rostral groups were located within rostral pons and the midbrain (Fig. 3). Intensity and area occupied by the *Sert* signal was clearly greater within the more rostral cell groups (compare panels K-R to panels A-H in Figure 3 and Supplemental Figure 2). Among these rostral cell groups, dorsal raphe nucleus had the most complex organization and consisted of caudal (DRC), dorsal (DRD) and ventral divisions. Its ventral division was further parcellated into caudal (DRVc), rostral (DRVr), ventral (DRVv), dorsal (DRVd), and lateral (DRVl) subdivisions. Intrafascicular raphe (IF) was located along the midline extending from the dorsal to ventral extent of the medial longitudinal fasciculus. Median raphe (MR) contained dense signal and was likewise located along the midline, but ventral to IF. In addition, laterally-displaced *Sert* signal was detected within the pontomesencephalic

reticular formation (PMRF), as well as more ventrally in and around the medial lemniscus, corresponding to the B9 cell group.

More caudally, much of the *Sert* signal was confined to the ventromedial medulla and the gigantocellular nucleus pars  $\alpha$  (GiA), as well as the caudal raphe nuclei, including: obscurus (ROb), magnus (RMg), and pallidus (RPa). More laterally, *Sert* was expressed ventrally and laterally to the pyramidal tract in the medulla-pons within the parapyramidal cell group (PPy).

## 2.4 Tph2 and Sert Expression

Our initial observations revealed greater signal for *Tph2* and *Sert* in bHR rats as compared to bLRs (Fig. 4). Subsequent analysis of *Tph2* gene expression revealed a significant main effect of bHR/bLR phenotype within: the B9 cell group ( $F_{1,51} = 8.47$ ,  $p < 0.01$ ; Fig. 5A) and the PMRF ( $F_{1,78} = 8.47$ ,  $p < 0.05$ ; Fig. 5B), GiA ( $F_{1,36} = 4.67$ ,  $p < 0.05$ ; Fig. 5E). An interaction between phenotype and the rostro-caudal anatomical position was observed within MR ( $F_{8,94} = 8.47$ ,  $p < 0.01$ ; Fig. 5C); in DRC a main effect of phenotype (bHR/bLR) along with an interaction between phenotype and rostrocaudal position was detected: ( $F_{1,41} = 6.64$ ,  $p < 0.05$  and  $F_{3,41} = 5.00$ ,  $p < 0.01$ , respectively; Fig. 5D). These differences appeared to be anatomically segregated, so that greater *Tph2* expression levels were detected in bHR rats within the middle and middle-caudal portions of B9, PMRF, MR, and GiA (Fig. 4; Fig. 5A-C, E). Within DRC, bHR rats similarly exhibited elevated *Tph2* expression, but at the rostral pole of this cell group (Fig. 4; Fig. 5D).

Analysis of *Sert* expression revealed bHR/bLR group differences within B9, PMRF, DRV1, and GiA. These differences were dependent on the rostro-caudal position within each nucleus as evidenced by a significant interaction between phenotype and rostrocaudal position within all regions illustrated in Fig. 5, including the B9 cell group ( $F_{4,75} = 5.95$ ,  $p < 0.001$ ; Fig. 6A); PMRF ( $F_{1,40} = 8.53$ ,  $p < 0.01$ ;  $-9.12$  mm to  $-8.64$  mm from Bregma), but not more rostrally ( $F_{1,47} = 1.70$ ,  $p = 0.2$ ;  $-8.4$  mm to  $-7.92$  mm from Bregma; Fig. 6B); DRV1 ( $F_{3,61} = 6.38$ ,  $p < 0.001$ ; Fig. 6C); and GiA where *Sert* levels significantly greater in bHR rats rostrally ( $F_{3,23} = 5.34$ ,  $p < 0.05$ ;  $-10.32$  mm to  $-10.08$  mm from Bregma), but not caudally ( $F_{1,19} = 5.34$ ,  $p > 0.5$ ;  $-0.56$  mm to  $-10.80$  mm from Bregma; Fig. 6D). These differences were similar to those observed for *Tph2*, which was also increased in bHRs within some of the same cell groups, including: B9, PMRF, and GiA.

## 2.5 C-fos Expression

We used *c-fos* expression as an indicator of the activation of brainstem cell groups by the intruder exposure. Within bLR brains we observed distinct *c-fos* expression within IF, PMRF, and B9 that was absent in the bHRs (Fig. 7 A-C versus D-F). Statistical analyses confirmed this observation, regarding increased post-intrusion *c-fos* mRNA levels in bLR rats as compared to bHRs in multiple cell groups (Fig. 8). Prominent differences were detected within B9 ( $F_{1,22} = 6.45$ ,  $p < 0.05$ ; levels  $-8.64$  mm,  $-8.4$  mm, and  $-8.16$  mm from Bregma) and PMRF ( $F_{1,47} = 8.94$ ,  $p < 0.01$ ; Fig. 8A, B), two areas in which we also detected bHR/bLR differences in *Tph2* and *Sert* expression (Figs. 5A, B and 6A, B). Similar differences were also observed in MR ( $F_{1,44} = 7.47$ ,  $p < 0.05$ ; Fig. 8C), DRVv ( $F_{1,27} = 6.41$ ,  $p < 0.05$ ; Fig. 8D), DRD ( $F_{1,29} = 5.23$ ,  $p < 0.05$ ; Fig. 8E), and IF ( $F_{1,32} = 13.22$ ,  $p < 0.01$ ; Fig. 8F).

## 3. Discussion

Our data demonstrate that compared to bLRs, bHRs exhibit: 1) enhanced aggression when facing an intruder; 2) potentiated intruder-induced corticosterone and testosterone secretion;

3) increased baseline *Tph2* and *Sert* gene expression; and 4) decreased intrusion-evoked *c-fos* mRNA in a subset of 5-HTergic cell groups. While a number of brainstem 5-HTergic cell populations were examined, gene expression differences were restricted to a subset of groups (B9, PMRF, MR, GiA) suggesting that these areas may play a specific role in shaping intrusion-evoked behavioral and endocrine responses. It also seems feasible that a different collection of 5-HT cell groups mediate other actions of 5-HT (e.g. homeostasis, sensory processing, executive function, etc.)

### 3.1 Behavioral and Endocrine Differences

The bHR/bLR aggression differences were not associated with divergent behavior of the intruders themselves, since bHR and bLR opponents exhibited similarly low levels of aggression. Rather, our findings point to inherent bHR/bLR differences in predisposition toward aggression, and complement earlier work showing increased impulsivity, stronger reward drive, and decreased depression and anxiety behaviors (all of which co-occur with increased aggression) in bHR versus bLR rats (Flagel et al., 2010; Stead et al., 2006). bHRs' collection of behavioral traits closely resembles human externalizing disorders, including substance abuse, conduct disorders, and pathological aggression (Dawe et al., 2004). Intruder exposure also elicited a testosterone surge in bHRs but not bLRs, which is consistent with human studies linking elevated plasma testosterone levels and enhanced violence (Ehrenkranz et al., 1974; Kruk et al., 1998). Interestingly, testosterone and its metabolites interact with 5-HT within forebrain areas, and may inhibit or facilitate aggression depending on the endocrine milieu and anatomical location (Simon et al., 1998).

Intruder exposure induced corticosterone secretion in both groups with a significantly greater response in bHRs. These findings are consistent with other reports of increased corticosterone secretion following social encounters (File and Seth, 2003). While elevated corticosterone levels often correlate with aggression in rodents (Hayden-Hixson and Ferris, 1991) and humans (van Bokhoven et al., 2005), this relationship is complicated and the literature is not entirely in agreement (Summers and Winberg, 2006). This heterogeneity may represent inherent individual differences in emotionality, risk-taking, and impulsivity. For example, stress exposure elicits greater cortisol secretion in risk-seeking individuals, suggesting a rewarding effect of stress (Filaire et al., 2007). Likewise, mild stress in bHRs elicits a greater corticosterone response compared to bLRs (Kabbaj et al., 2000). Since corticosterone has rewarding properties (Piazza et al., 1993), and bHR rats have an increased reward drive, it is feasible that aggressive behavior has rewarding properties for the bHR residents.

### 3.2 Gene Expression Differences

A large literature in humans and animals has linked altered 5-HT transmission and aggression (de Boer and Koolhaas, 2005). Since bHRs exhibited increased intrusion-induced aggression, testosterone and corticosterone secretion, we hypothesized that these differences are accompanied by baseline differences in the expression of key 5-HTergic genes, and in turn, basal differences in 5-HTergic tone. Because emerging evidence points to anatomical and functional heterogeneity within brainstem 5-HTergic cell groups (Clark et al., 2006; Lowry et al., 2008), we initially characterized the location and borders of 5-HTergic cell groups in our material using a combination of neurochemical and cytoarchitectonic criteria. The fact that bLR/bHR differences were not only confined to a subset of cell groups but were detected at precise rostro-caudal positions within these groups (see below) lends further support to the notion of functional and anatomical heterogeneity as well as specificity within these regions.

Although 5-HT levels at the synapse are determined by several factors, activities of *Tph2* and *Sert* (which regulate synthesis and reuptake, respectively) are critical for the setting of overall 5-HTergic tone (Cooper et al., 2003). At baseline, bHRs exhibited higher *Tph2* and *Sert* mRNA within three areas: B9, PMRF, and GiA. bHRs also showed increased *Tph2* in MR and DRC, and increased *Sert* in DRVl. Although *Tph2* and *Sert* expression were assayed immediately following homecage intrusion, it is unlikely that observed bHR/bLR differences represent behavior-induced effects. Stimulus-evoked mRNA alterations for non-immediate early genes (such as *Tph2* and *Sert*) usually require several hours to appear (Curran and Morgan, 1995). Other reports demonstrate behaviorally- or pharmacologically-induced *Tph2* and/or *Sert* alterations after a delay of several hours or days, rather than five minutes as in our study (Oliva et al., 2005; Semple-Rowland et al., 1996). Therefore, bHRs' observed *Tph2* and *Sert* increases likely represent greater 5-HT synthesis and turnover, at least within a subset of 5-HT innervated areas (Kim et al., 2005).

Our study also measured expression of *c-fos*, a reliable and robust marker of neuronal activation (Curran and Morgan, 1995), following intruder exposure. We found diminished *c-fos* mRNA expression in bHR versus bLR rats within a specific subset of 5-HTergic cell groups. There were prominent *c-fos* differences within cell groups with significant *Tph2* and/or *Sert* alterations (B9, PMRF, and MR), as well as within three additional groups: DMVv, DRD, and IF.

Together these mRNA differences suggest that four cell groups – B9, PMRF, MR, and GiA are key regulators of 5-HT transmission in shaping individual differences in aggressive behavior. It is feasible that baseline bHR/bLR differences in *Tph2* and *Sert* impact their inborn predisposition to aggression and contribute more broadly to their distinct behavioral profiles. Furthermore, *c-fos* differences within the same cell groups suggest altered recruitment of these regions in response to homecage intrusion. Since these *c-fos* changes occurred within the same brainstem cell groups showing *Tph2* and *Sert* differences, they suggest that the baseline and intrusion-evoked expression alterations are functionally related. However, since our autoradiogram-based ISH method lacks cellular resolution, we cannot be sure whether or not the *c-fos* differences are confined to 5-HTergic neurons. Numerous studies have characterized non-5-HTergic neurons within the raphe that powerfully modulate 5-HTergic neurons, such as GABA-mediated inhibition of cell firing and transmitter release (Hurley et al., 2003; Melander et al., 1986). We are currently refining a dual-label ISH methodology for quantifying *c-fos* within neurochemically defined raphe neurons, as well as with laser-capture microdissection coupled with high-throughput gene expression assays (Kerman et al., 2006a). With these tools in-hand, future studies will focus on identifying the neurochemical content of neurons that express intrusion-evoked *c-fos* in bHRs versus bLRs. Furthermore, these gene expression differences may depend on aggressive experience of individual animals, because Caramaschi and colleagues have demonstrated that in mice selected for short attack latencies serotonin levels decrease in the prefrontal cortex with repeated resident-intruder fighting experience (Caramaschi et al., 2008).

While the resident-intruder paradigm is typically used to assess aggression, it is notable that this stimulus potentially evokes a wide range of behavioral responses, including fear and anxiety. Therefore, our observed *c-fos* differences may reflect increased behavioral inhibition (e.g. fear, anxiety) in bLRs facing intrusion. Our corticosterone data demonstrate that the intruder encounter is stressful, and previous reports show that bLRs exhibit high anxiety in stressful situations (Stead et al., 2006). Thus, these *c-fos* differences may reflect bLRs' anxiety during the novel social encounter, as evidenced by their increased defensive freezing. Future work will be required to fully explore these possibilities.

Previous studies have implicated these 5-HTergic brainstem cell groups in the regulation of behavioral responses that may be elicited by intrusion and other types of stressors. For example, anatomical studies show that 5-HTergic neurons within B9, DRC, and MR project to septum and hippocampus, which both play prominent roles in regulating affect and stress responsivity (Lowry et al., 2008; Waselus et al., 2006). 5-HTergic neurons within GiA project to the spinal cord to innervate motoneurons, sympathetic preganglionic neurons, and the dorsal horn (Kerman et al., 2003; Kerman et al., 2006b; Kerman, 2008; Mason, 2001). This region also contains high levels of  $\mu$  and  $\kappa$  opioid receptors, and is thought to mediate stress-induced motor, autonomic, and analgesic responses characteristic of 'fight-or-flight' (Mansour et al., 1988; Mason, 2001). Although B9 and PMRF contain over 20% of the total number of serotonergic neurons in the brain, second only to the dorsal raphe (Lowry et al., 2008), their function is unknown. Anatomical studies in several species demonstrated that B9 neurons project to the hypothalamus, cortex, hippocampus, septum, MR, and basal forebrain (Vertes and Martin, 1988). Less is known about connectivity of PMRF neurons, with a single report of its projection to the basal forebrain (Vertes and Martin, 1988). Together these observations suggest that B9 and PMRF participate in regulating vigilance, affect, hormonal stress responses, and homeostasis. Since homecage intrusion engages all of these neurobiological systems, it is feasible that B9 and PMRF play a prominent role in shaping aggression. In addition, most of the bHR/bLR gene expression differences in these regions were detected within their caudal portions, suggesting rostro-caudal functional topography of these groups.

### 3.3 Conclusions

Together our data indicate that bHR animals represent a novel rodent model of aggressive behavior that co-occurs with increased novelty-seeking, impulsivity, and propensity for drug self-administration. bHRs exhibit basally altered expression of genes that regulate synaptic 5-HT levels, and diminished intrusion-evoked *c-fos* in brainstem nuclei that are enriched in their content of 5-HT cells. These gene expression differences were consistently detected in a subset of 5-HTergic cell groups, suggesting existence of a dedicated 5-HTergic circuit that regulates aggressive behavior. Various nodes of this circuit likely modulate distinct facets of aggression, including affective, endocrine, and physiological responses, although future work is required to investigate these possibilities.

## 4. Experimental Procedure

### 4.1 Animals

Adult male bHR (total n = 40) and bLR (total n = 40) rats were acquired from our in-house breeding colony where we have maintained these lines for several generations (Stead et al., 2006). bHR and bLR rats used in these studies were from the 21<sup>st</sup> and 22<sup>nd</sup> generations of our colony. In addition, a group of adult Sprague-Dawley male rats (n=60) was purchased from Charles River (Wilmington, MA) and allowed to acclimate to our housing facilities for at least 1 week prior to any behavioral testing. All rats were housed in a dedicated animal facility with 12:12 light-dark cycle (lights on at 6 a.m.). Rat chow and water were available *ad libitum*. All experiments were approved by the University of Michigan University Committee on Use and Care of Animals, and were conducted in accordance with the National Institute of Health (NIH) guidelines on laboratory animal use and care dictated by the National Research Council in 1996. All work was carried out in accordance with European Commission (EC) Directive 86/609/EEC for animal experiments and the Horizontal Legislation on the Protection of Animals Used for Scientific Purposes ([http://ec.europa.eu/environment/chemicals/lab\\_animals/legislation\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm)).

## 4.2 Experimental Design

Four different experimental groups were employed, with each group consisting of 10 bHR and 10 bLR male rats. Animals in Group 1 were tested on the resident-intruder test (described below) only; animals in Group 2 were used for replication of behavioral testing and collection of trunk blood for endocrine assays (total testosterone and corticosterone); Group 3 animals were exposed to female rats for 14 days, but were not presented with intruders. These animals were euthanized on day 15, and their trunk blood was used for the determination of baseline testosterone and corticosterone levels. Group 4 animals were tested in the resident-intruder paradigm, and their brains were used for *in situ* hybridization (ISH) studies.

## 4.3 Behavioral Testing

Male bHR/bLR rats were housed with a female mating partner of the same selectively-bred line (i.e. male bHR with female bHR, and male bLR with female bLR) for 14 days. On the day of testing (day 15), the female partner was removed and each resident was presented for 10 min. with a size-matched commercially-purchased male Sprague-Dawley rat classified as the intruder. Each intruder rat was only used once in the resident-intruder paradigm. This social encounter was videotaped, and was subsequently scored (Observer 5.0.20, Noldus Information Technologies, The Netherlands) by a blinded observer for aggressive behaviors: sniffing, boxing, and biting; defensive behavior: freezing; and other behaviors: rearing, running, and eating/drinking in resident and intruder rats. This resident-intruder paradigm has been validated by numerous laboratories as a reliable tool for the characterization of aggression in rodents (Bannai et al., 2007; de Almeida et al., 2008; de Boer and Koolhaas, 2005; van Erp and Miczek, 2007).

## 4.4 Hormone Assays

Five minutes following resident-intruder testing (15 minutes after beginning of testing) bHR/bLR resident animals were sacrificed by rapid decapitation. Brains were removed, snap frozen and stored at  $-80^{\circ}\text{C}$  for later *in situ* hybridization (ISH) studies (see next section). Trunk blood samples were collected in EDTA-coated tubes to assess testosterone and corticosterone levels. Blood samples were separated by centrifugation ( $1,000\text{g} \times 10\text{ min}$  at  $4^{\circ}\text{C}$ ), and plasma was removed, frozen and stored at  $-80^{\circ}\text{C}$ . Testosterone and corticosterone levels were measured using commercially available radioimmunoassay kits (MP Biomedicals, Solon, OH) according to manufacturer instructions. The sensitivity of the assays was  $12.5\text{ ng/ml}$ , and intra- and inter-assay coefficients of variation were less than 5%.

## 4.5 Tissue Processing

Brains were cryostat sectioned at  $12\text{ }\mu\text{m}$ , immediately thaw-mounted onto Fisherbrand Superfrost Plus Microscope Slides (Fisher Scientific, <http://www.fishersci.com/>), and subsequently prepared for ISH. The expression of *Sert*, *Tph2*, and *c-fos* mRNAs was assessed at  $240\text{ }\mu\text{m}$  intervals throughout rostrocaudal extent of the brainstem. *Tph2* and *Sert* were selected for analysis because of their critical roles in regulating 5-HT synthesis and synaptic reuptake, respectively. We examined expression of the immediate early gene *c-fos* as an indicator of regional activation following exposure to the resident-intruder paradigm. At each anatomical level, adjacent slides were processed for each of the 3 transcripts, with an additional slide stained with cresyl violet.

Our ISH protocol has been previously described (Kabbaj et al., 2000). Briefly, sections were fixed in 4% paraformaldehyde at room temperature for 1 hour. The slides were then washed  $5\text{ min.} \times 3$  in 2X SSC (300mM NaCl/30mM sodium citrate, pH 7.2). Next, the slides were placed in a solution containing acetic anhydride (0.25%) in triethanolamine (0.1 M), pH 8.0,



for 10 min at room temperature, rinsed in distilled water, and dehydrated through graded ethanol washes (50%, 75%, 85%, 95%, and 100%). After air drying, adjacent tissue sections were hybridized overnight at 55°C with a <sup>35</sup>S-labeled cRNA probes (see below). Following hybridization, coverslips were removed and the slides were washed with 2X SSC and incubated for 1 hour in RNaseA at 37°C, followed by multiple washes in increasingly stringent SSC solutions. Slides were then rinsed in distilled H<sub>2</sub>O, dehydrated through graded ethanol washes, air-dried, and apposed to Kodak XAR film (Eastman Kodak, Rochester, NY). Slides processed for *Tph2* and *Sert* were exposed to film for 48 hours, while slides processed for *c-fos* were exposed to film for 7 days; these development times were chosen since they were found to be within the linear portion of the development curve for each probe for the brain regions of interest.

The following cRNA riboprobes were used: *Sert* (alias *Slc6a4*; NCBI reference sequence: X63253, pos. – 655-1,234), *Tph2* (alias *Ntph*; NCBI reference sequence – AY098915, pos. – 1,550-2,580), and *c-fos* (alias *Fos*; NCBI reference sequence – X06769, pos. – 585-1,368). Specificity of labeling was confirmed by the absence of signal following hybridization with sense riboprobes generated for the same positions of the target mRNAs. Autoradiograms were developed and digitized using a ScanMaker 1000XL Pro flatbed scanner (Microtek, Carson, CA) with LaserSoft Imaging software (AG, Kiel, Germany) at 1,600 dpi. Digitized images were analyzed using ImageJ Analysis Software for PC from NIH. Optical density measurements were corrected for background, and then multiplied by the area sampled in μm<sup>2</sup> to produce an integrated optical density (IOD) measurement.

#### 4.6 Quantification of in Situ Hybridization Data

Adjacent cresyl violet-stained sections were digitized at 1,600 dpi using Super CoolScan 4000 ED slide scanner (Nikon, <http://www.nikon.com/>). Parcellation of the brainstem 5-HT cell groups was carried out by using *Sert* autoradiograms and adjacent cresyl violet stained sections, which were digitally overlaid using Adobe Photoshop CS2 (<http://www.adobe.com/>). This method afforded the use of neurochemical and cytoarchitectonic criteria for the delineation of separate 5-HTergic cell groups. We generated a detailed map of the brainstem 5-HTergic cell groups, and then used it to guide quantification of *Tph2* and *Sert* expression.

For quantification of *c-fos* expression, *Sert* and *c-fos* autoradiograms from adjacent tissue sections were digitally aligned in Photoshop and exported to ImageJ 1.41o (<http://rsb.info.nih.gov/ij/>). *Sert* ISH signal was then used to delineate the boundaries of 5-HTergic cell groups, which were projected onto *c-fos* autoradiograms from adjacent tissue sections. Background adjusted signal and integrated optical density (IOD) were quantified for each probe within each cell group.

#### 4.7 Statistical Analyses

Data were analyzed using either a one-way ANOVA with (bLR/bHR) phenotype as the independent variable (for analysis of behavioral data), or a two-way ANOVA with phenotype and either rostro-caudal position (for analysis of ISH data) or intruder exposure status (for endocrine assays) as independent variables. Significant main effects or interactions were followed up by post-hoc comparisons using Fisher's Exact Test. Data were analyzed using SPSS 17.0 for Windows (SPSS Institute, <http://www.spss.com/>) and are presented as mean ± SEM. For all tests α=0.05.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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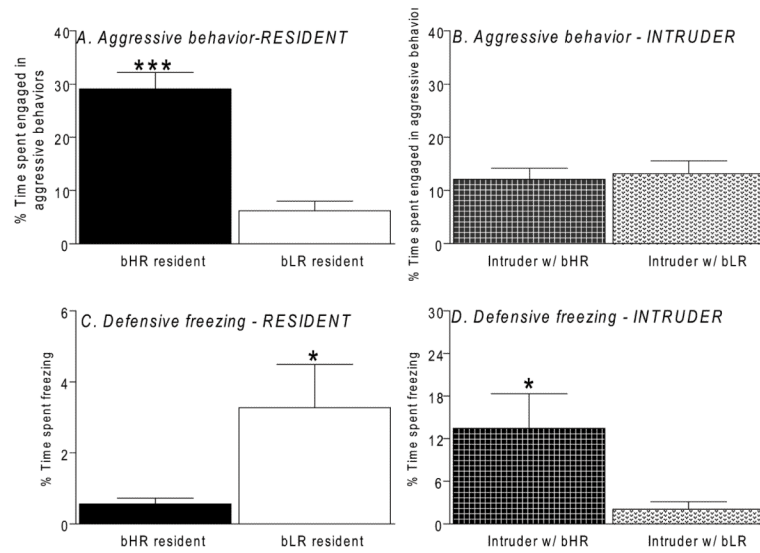
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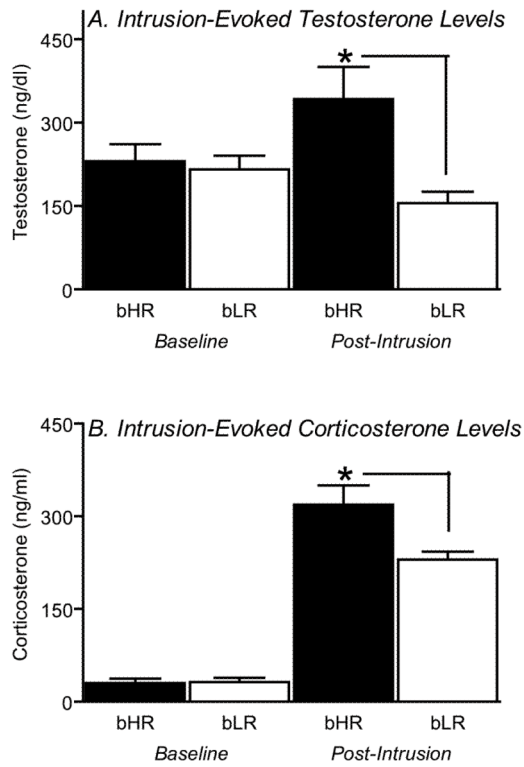
**\*Highlights**

Highlights for Kerman, Clinton et al “High Novelty-Seeking Predicts Aggression and Gene Expression Differences within Defined Serotonergic Cell Groups”:

- We examined aggression in two rat lines bred for high versus low novelty-seeking.
- As predicted, high novelty-seeking rats exhibited exaggerated aggressive behavior.
- Gene expression studies point to altered serotonin levels leading to aggression.
- Aggression-related serotonin differences occur in specific brainstem nuclei.

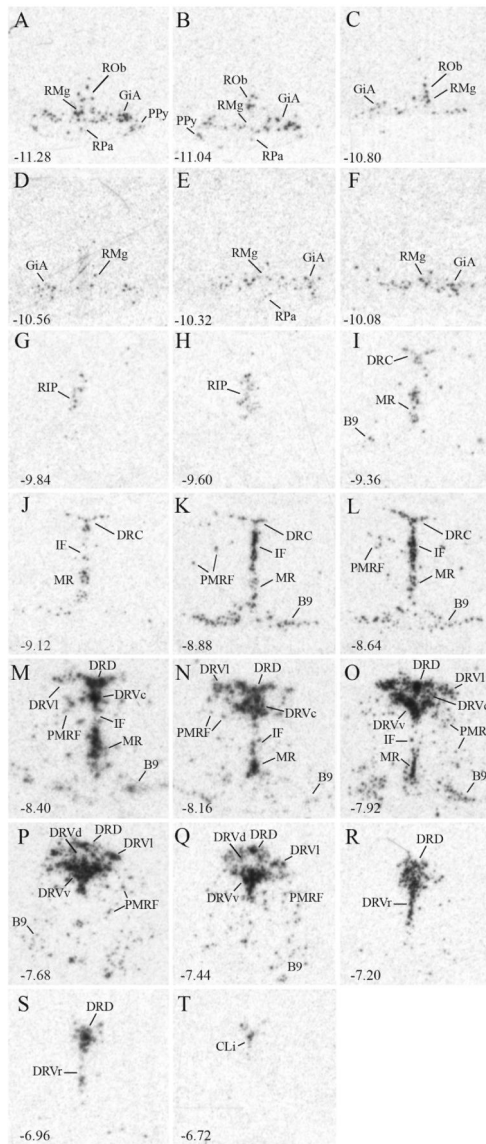


**Figure 1.** Behavioral differences in bHR/bLR rats on the resident-intruder test. Selectively-bred HR resident male rats exhibited increased aggressive behavior compared to their bLR counterparts (A;  $p < 0.001$ ); intruder male rats showed similarly low levels of aggressive behavior when exposed to either bHR or bLR residents (B;  $p > 0.05$ ). bLR residents exhibited increased defensive behaviors (C;  $p < 0.05$ ). Intruder rats exposed to bHR residents exhibited markedly increased defensive freezing compared to those interacting with bLR residents (D;  $p < 0.05$ ). \* –  $p < 0.05$ , \*\*\* –  $p < 0.001$  compared to bLR rats.



**Figure 2.**

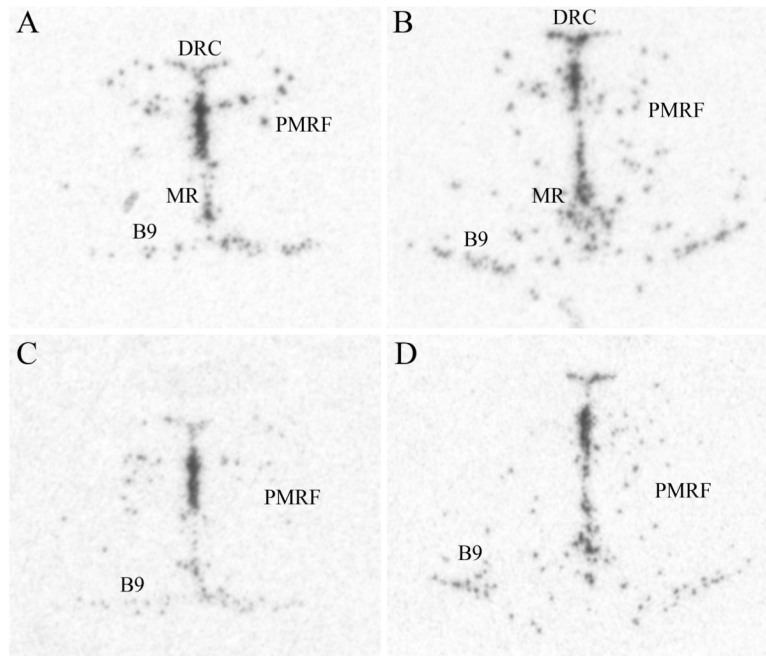
Total testosterone and corticosterone levels in bHR and bLR rats. Selectively-bred bHR, but not bLR, resident rats increased their total testosterone levels in response to intruder exposure (A;  $p < 0.01$ ). Intruder exposure elicited an increase in circulating corticosterone levels in both types of rats, but this response was significantly greater in bHRs (B;  $p < 0.05$ ). \* –  $p < 0.05$  vs. bLR.



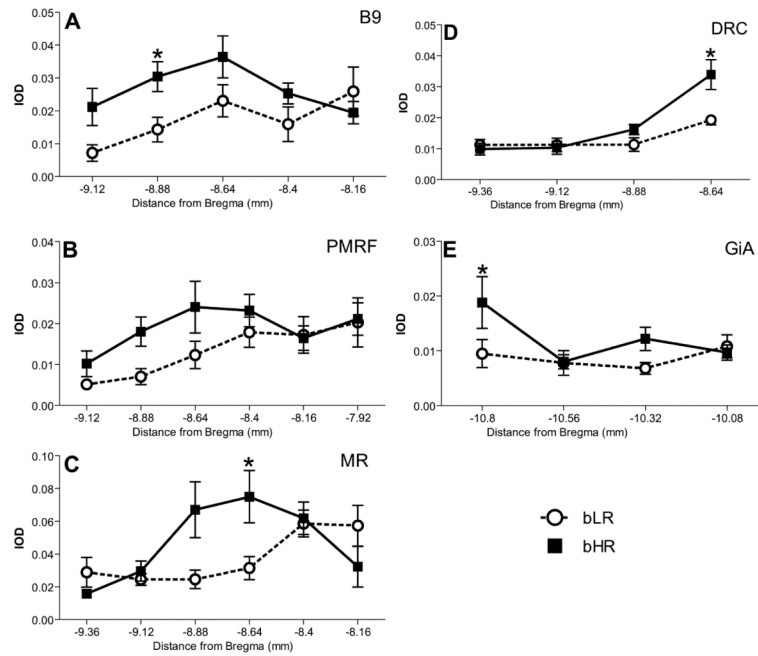
**Figure 3.** Serotonergic cell groups in the rat brainstem. Cell groups were parceled based on a combination of neurochemical and cytoarchitectonic criteria (see text and Supplementary Fig. 1 for additional details). *Sert* expression across the rostro-caudal extent of the brainstem was used to delineate cell group boundaries. Data are presented caudal (A) to rostral (T) from tissue sections 240  $\mu$ m apart, and numbers in the lower left of each panel correspond to distances from bregma in mm. Note differences in shape, packing density, and signal intensity among different cell groups and along the rostro-caudal extent. Abbreviations: B9 – B9 cell group; CLi – caudal linear raphe; DRC – caudal dorsal raphe; DRD – dorsal division of the dorsal raphe; DRVc – ventral division of the dorsal raphe, caudal portion; DRVd – ventral division of the dorsal raphe, dorsal portion; DRVl – ventral division of the dorsal raphe, lateral portion; DRVr – ventral division of the dorsal raphe, rostral portion; DRVv – ventral division of the dorsal raphe, ventral portion; GiA – gigantocellular nucleus pars  $\alpha$ ; IF – interfascicular raphe; MR – median raphe; PMRF – pontomesencephalic



reticular formation; PPy – parapyramidal cell group; RIP – raphe interpositus; RMg – raphe magnus; ROb – raphe obscurus; RPa – raphe pallidus.

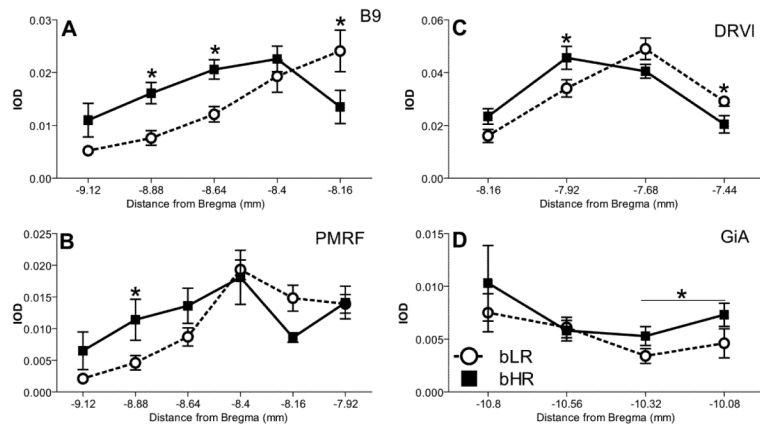


**Figure 4.** Examples of *Tph2* (A, B) and *Sert* (C, D) expression differences between bLR (A, C) and bHR (B, D) rats. Images were taken from the adjacent sections of the same bLR and bHR rats at  $-8.64$  mm from Bregma. Note an increase in signal in the bHR images as compared to bLR. These differences reached significance in the labeled cell groups at this anatomical level (see subsequent figures and text). Abbreviations same as in Fig. 3

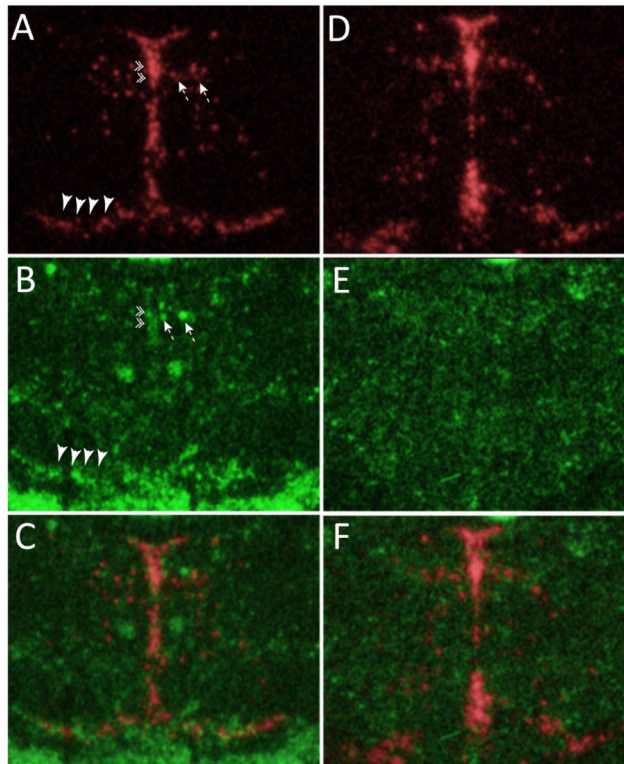


**Figure 5.**

Greater *Tph2* expression in bHR versus bLR rats. Significant bHR/bLR differences in *Tph2* expression were detected in the: B9 cell group (A), pontomesencephalic reticular formation (PMRF; B), median raphe (MR; C), caudal dorsal raphe (DRC; D), and gigantocellular nucleus pars  $\alpha$  (GiA; E). These expression differences were influenced by the rostro-caudal position, so that within B9, PMRF, and MR expression differences were observed caudally. In contrast, within DRC expression differences were detected near its rostral pole. \* – p < 0.05 vs. bLR post- hoc comparison at the indicated level.

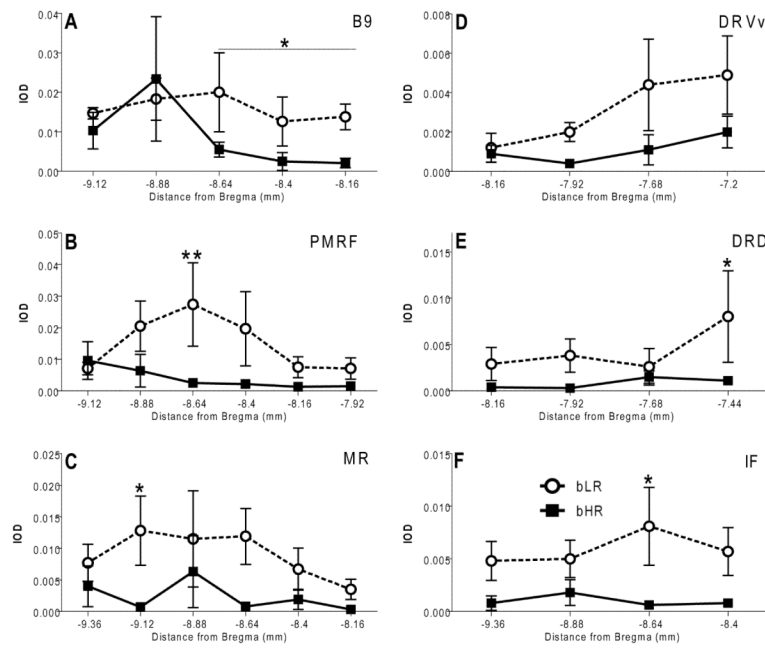


**Figure 6.** Greater *Sert* expression in bHR versus bLR rats. Significant differences in *Sert* expression were detected within: the B9 cell group (A); pontomesencephalic reticular formation (PMRF; B); ventral division of the dorsal raphe, lateral portion (DRVI; C); and gigantocellular nucleus pars  $\alpha$  (GiA; D). As with *Tph2* these differences were more pronounced caudally within B9 and PMRF. \* –  $p < 0.05$  vs. bLR post-hoc comparison at the indicated level.



**Figure 7.**

*Sert* and *c-fos* expression. *Sert* (A, D) and *c-fos* (B, E) autoradiograms were digitally inverted and pseudocolored (*Sert* – red, *c-fos* – green). *Sert* and *c-fos* images from adjacent sections were digitally overlaid and linked (C, F). *Sert* signal was then used to identify different serotonergic cell groups, and the boundaries of these cell groups were then projected onto the adjacent *c-fos* image for quantification. Data in panels A-C are from a bLR animal, while those in D-F are from a bHR rat. Note increased *c-fos* expression in the bLR brain within: intrafascicular raphe (double arrowheads), pontine mesencephalic reticular formation (dashed arrows), and the B9 cell group (large arrowheads).



**Figure 8.**

Increased intruder-induced activation of serotonergic cell groups in bLR rats. Following the intruder exposure, *c-fos* expression was significantly increased within several serotonergic cell groups in bLR residents relatively to bHRs. Similar to *Tph2* and *Sert*, in three cell groups these expression differences were influenced by the rostro-caudal position and were most prominent rostrally within: the B9 cell group (A), ventral portion of the ventral dorsal raphe (D), and the dorsal subdivision of the dorsal raphe (E). In contrast, significant bHR/ bLR differences in *c-fos* expression were detected within the middle-caudal portion of the pontomesencephalic reticular formation (B). Similarly increased expression of *c-fos* in bLR residents was also observed in the median (MR; C) and intrafascicular (IF; F) raphe. \* –  $p < 0.05$  vs. bHR.

**Table 1**

Behavioral profiles of bHR/bLR rats on the resident intruder test.

	HR Resident		LR Resident		p-value
	mean	SEM	mean	SEM	
<b>DEFENSIVE BEHAVIOR TOTAL</b>	0.56	0.18	3.37	1.58	<0.05
Aggressive sniff	12.05	1.47	5.15	1.72	<0.0005
Aggressive groom/bite	5.02	1.76	0.32	0.19	<0.05
Boxing	12.06	1.80	0.71	0.23	<0.0001
<b>AGGRESSIVE BEHAVIOR TOTAL</b>	29.12	3.07	6.18	1.82	<0.0001
Eat/Groom/Drink	2.95	0.84	10.76	2.81	<0.01
Rear	31.31	3.21	30.38	3.26	0.84
Run	35.99	2.93	44.70	3.56	0.07
Rest	0.03	0.01	0.02	0.02	0.86
<b>OTHER BEHAVIOR TOTAL</b>	70.27	3.08	85.86	5.05	<0.01

Values represent percentage of time spent performing each behavior during the 10-minute intruder encounter. Data are presented as mean  $\pm$  SEM.