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Aggressive Encounters Alter the Activation of Serotonergic Neurons and the Expression of 5-HT1A mRNA in the Hamster Dorsal Raphe Nucleus

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Abstract

Serotonergic (5-HT) neurons in the dorsal raphe nucleus (DRN) have been implicated in stress-induced changes in behavior. Previous research indicates that stressful stimuli activate 5-HT neurons in select subregions of the DRN. Uncontrollable stress is thought to sensitize 5-HT neurons in the DRN and allow for an exaggerated 5-HT response to future stimuli. In the current study, we tested the hypothesis that following aggressive encounters, losing male Syrian hamsters would exhibit increased c-Fos immunoreactivity in 5-HT DRN neurons compared to winners or controls. In addition, we tested the hypothesis that losers would have decreased 5-HT1A mRNA levels in the DRN compared to winners or controls. We found that a single 15-min aggressive encounter increased c-Fos expression in 5-HT and non-5-HT neurons in losers compared to winners and controls. The increased c-Fos expression in losers was restricted to ventral regions of the rostral DRN. We also found that four 5-min aggressive encounters reduced total 5-HT1A mRNA levels in the DRN in losers compared to winners and controls, and that differences in mRNA levels were not restricted to specific DRN subregions. These results suggest that social defeat activates neurons in select subregions of the DRN and reduces message for DRN 5-HT1A autoreceptors. Our results support the hypothesis that social stress can activate 5-HT neurons in the DRN, reduce 5-HT1A autoreceptor-mediated inhibition, and lead to hyperactivity of 5-HT neurons.

Keywords

social defeat; aggression; stress; serotonin; c-Fos; 5-HT1A; autoreceptor

Psychosocial stress, be it acute social defeat or chronic subordination in a social group, is a potent stressor that activates the hypothalamic-pituitary-adrenal (HPA) axis (Blanchard et al.,

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1995, Koolhaas et al., 1997). Both acute social defeat and chronic subordination have been shown to produce alterations in serotonin (5-HT) systems (McKittrick et al., 1995, Berton et al., 1998), as well as marked behavioral changes such as reduced locomotor activity (Meerlo et al., 1996, Berton et al., 1998), changes in feeding (Bartolomucci et al., 2004, Foster et al., 2006), disruption of circadian and sleep rhythms (Harper et al., 1996, Meerlo et al., 2002), and depressive-like and anxiety-like behavior (Rodgers and Cole, 1993, Berton et al., 1998, Keeney et al., 2006). Some of these behavioral effects can be reversed with administration of selective serotonin reuptake inhibitors (SSRIs) (Fuchs et al., 1996, Berton et al., 1999). In Syrian hamsters, social defeat results in a complete loss of species-typical territorial aggression and a substantial increase in submissive and defensive behavior when individuals are later tested with a non-aggressive opponent, and we have called this behavioral change conditioned defeat (Huhman et al., 2003).

The dorsal raphe nucleus (DRN), as well as the median raphe nucleus (MRN), gives rise to the vast majority of 5-HT neurons innervating forebrain structures. Neuroanatomical studies have shown that specific subregions of the DRN and MRN project to different forebrain targets (Imai et al., 1986, Kazakov et al., 1993, Hensler et al., 1994). Exposure to diverse stressors such as forced swimming (Kirby et al., 1995) and footshock (Yoshioka et al., 1995) increase 5-HT concentrations in DRN projection regions. However, diverse stressors, as well as intra-DRN administration of corticotropin-releasing factor (CRF), produce distinct patterns of 5-HT release in forebrain target areas (Lee et al., 1987, Adell et al., 1997, Forster et al., 2006). Also, individual stressors can increase 5-HT release in some DRN projection regions, while at the same time decrease or produce no change in 5-HT release in other regions (Kirby et al., 1995). The topographical organization of the DRN no doubt contributes to the diversity of 5-HT responses to stress and is another aspect of DRN function that needs to be explored. Urocortin administration, anxiogenic drugs, and social defeat have been shown to selectively activate 5-HT neurons in the dorsal part of the mid-rostrocaudal and caudal DRN (Abrams et al., 2005, Gardner et al., 2005, Staub et al., 2005). Also, the prevention of learned helplessness is associated with the reduced activation of 5-HT neurons within the middle and caudal DRN (Amat et al., 2006).

5-HT_{1A} receptors are located on the soma and dendrites of 5-HT DRN neurons where they function as inhibitory autoreceptors (Miquel et al., 1992). 5-HT_{1A} receptor agonists administered into the DRN have been shown to inhibit DRN electrical activity (Sprouse and Aghajanian, 1987), 5-HT synthesis (Hamon et al., 1988), and 5-HT release in DRN projection regions (Sharp et al., 1989). Injection of a 5-HT_{1A} agonist into the DRN diminishes anxiety-like behavior on the elevated plus maze (File and Gonzalez, 1996), and blocks the development of learned helplessness (Maier et al., 1995). Similarly, we have shown that pharmacological activation of 5-HT_{1A} autoreceptors in the DRN reduces conditioned defeat (Cooper et al., 2008). The desensitization of 5-HT_{1A} autoreceptors has been reported following chronic mild stress (Lanfumey et al., 1999), as well as following chronic social defeat (Flugge, 1995). Acute stress may also alter the sensitivity or expression of 5-HT_{1A} autoreceptors. It has been proposed that an acute bout of inescapable shock produces a desensitization of 5-HT_{1A} autoreceptors, thus removing an important source of inhibition on DRN 5-HT neurons and allowing for the exaggerated activity of DRN 5-HT neurons that underlies learned helplessness (Maier and Watkins, 2005). Likewise, increased 5-HT_{1A} autoreceptor levels may protect against learned helplessness as shown by the finding that wheel running up-regulates 5-HT_{1A} autoreceptor mRNA and prevents the development of learned helplessness (Greenwood et al., 2003).

The purpose of this study was to examine the activity of 5-HT cells and the expression of 5-HT_{1A} autoreceptor mRNA in the DRN following aggressive encounters in Syrian hamsters. We hypothesized that losers would show more DRN cells double-labeled for 5-HT and c-Fos

immunoreactivity than would winners or controls. Also, we hypothesized that losers would show decreased 5-HT1A mRNA expression in the DRN compared to winners or controls. These experiments address how aggressive experience produces functional changes in 5-HT signaling in the DRN that can, in turn, modify future social behavior.

EXPERIMENTAL PROCEDURES

Animals

We used male Syrian hamsters (*Mesocricetus auratus*) that weighed 120–140 g (3–4 months) at the start of the study. Animals were individually housed in polycarbonate cages (20 × 40 × 20 cm) with corncob bedding, cotton nesting materials, and wire mesh tops. Animals were allowed to scent mark their cage for two weeks prior to behavioral testing. Animals were housed in a temperature-controlled colony room (20 ± 2 °C) and maintained on a 14:10 hr light-dark cycle with food and water available *ad libitum*. We performed behavioral testing during the first three hours of the dark phase of the daily light-dark cycle to control for circadian rhythmicity of physiology and behavior. All procedures were approved by the Georgia State University Animal Care and Use Committee and are in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

We used a resident-intruder paradigm to generate aggressive encounters, and hamsters were weight-matched and randomly assigned as resident, intruder, or control. In experiment 1, an intruder was placed into the home-cage of a resident for a single 15-min aggressive encounter. Animals were later identified as winners (N = 10) and losers (N = 10) based on the outcome of the encounter, and residency did not necessarily confer dominance. In no case did the aggressive encounters result in tissue damage. Control animals (N = 10) were placed in a novel, empty cage for 15 minutes. We perfused animals with 0.01% phosphate-buffered saline (PBS) and 4% paraformaldehyde 75 minutes following aggressive encounters, a latency which is consistent with previous research (Grahn et al., 1999, Delville et al., 2000). Brains were post-fixed in paraformaldehyde for 24 hrs and subsequently transferred to PBS sucrose.

In experiment 2, we modified our resident-intruder paradigm so that intruders were placed in the home-cage of the same resident animal for four, 5-min aggressive encounters at 1hr intervals. We used four aggressive encounters spread over a three hour period because we did not know beforehand when to expect optimal changes in 5-HT1A mRNA expression, and we reasoned that extending the period of aggressive encounters would maximize our chance of finding a difference in 5-HT1A mRNA. We have previously shown that both single, 15-min aggressive encounters and four, 5-min aggressive encounters produce robust changes in the future agonistic behavior of defeated animals and are both effective at creating winners and losers (Huhman et al., 2003, Cooper et al., 2008). We identified winners (N = 10) and losers (N = 10) based on the outcome of the encounter. Winners and losers did not reverse dominance status after the first encounter. Control animals (N = 9) were placed in a novel, empty cage for four, 5-min sessions. Immediately following the last aggressive encounter, we rapidly decapitated the animals, collected the brains, and stored them at –80°C.

Behavior scoring

Winners and losers were identified by live observation of the aggressive encounters. Encounters also were recorded and later scored by an observer using behavioral definitions adapted from Albers et al. (2002). We recorded the total duration of four classes of behavior during aggressive encounters: (a) social (attend, approach, investigate, sniff, nose touch, and flank mark); (b) nonsocial (locomotion, exploration, self-groom, nest build, feed, and sleep); (c) submissive and defensive (flight, avoid, tail up, upright and side defense, full submissive

posture, stretch-attend, head flag, attempt to escape from cage); and (d) aggressive (upright and side offense, chase, and attack including bite). We also recorded the frequency of flight and attack during each encounter. The behavioral data were used to confirm the identity of winners and losers. We defined winners as those individuals that never displayed submissive behavior and losers as those individuals that never displayed aggression following their initial submission. In four winner/loser pairs (two pairs from experiment 1 and two pairs from experiment 2) the outcome of the encounters was not clear, and the behavioral data did not indicate an obvious distinction in agonistic behavior. Consequently, these four winner/loser pairs were excluded from the study.

Immunohistochemistry and cell counting

We sliced brains into 40 μ m coronal sections on a vibratome and placed them directly into glass scintillation vials containing cryoprotectant. Labeling for c-Fos and 5-HT occurred sequentially on one set of free-floating sections which contained the mid-brain raphe complex. For immunostaining we used primary antisera directed against the protein product of the immediate early gene c-fos (rabbit anti-c-Fos polyclonal antibody, 1:5000; Santa Cruz Biotechnology) and 5-HT (goat anti-5-HT polyclonal antibody, 1:10,000; ImmunoStar, Inc.).

All washes, rinses, and incubations were performed in glass vials which were gently shaken on an orbital shaker throughout the double immunostaining. Briefly, we rinsed sections in 0.1 M PBS containing 0.2% Triton X-100 (PBS-Triton), incubated them for 20 min with 0.3% hydrogen peroxide, and rinsed them again with PBS-Triton. Then sections were incubated overnight at room temperature in a PBS-Triton solution containing 1% normal donkey serum and the rabbit anti-c-Fos antibody. The next day sections were rinsed in 0.1 M PBS-Triton, followed by incubation for 90 min in a PBS-Triton solution containing 1% normal donkey serum and a biotinylated donkey anti-rabbit IgG polyclonal antibody (1:500, Vector Laboratories). The sections were then rinsed in PBS-Triton, followed by incubation for 90 min with an avidin-biotin complex reagent (Vectastain Elite ABC kit, Vector Laboratories). After rinsing with PBS-Triton, sections were placed in a solution containing 3,3'-diaminobenzidine (DAB), hydrogen peroxide, and nickel ammonium sulfate for 10 min. The peroxidase reaction was stopped with a series of PBS rinses.

The next day, we processed sections for 5-HT immunostaining. After PBS-Triton rinses, sections were incubated at 4°C for 72 hrs in a PBS-Triton solution containing 2% normal donkey serum and the goat anti-5-HT antibody. The subsequent steps were the same as described for c-Fos immunostaining except that sections were incubated with a non-biotinylated donkey anti-goat IgG (1:200, Jackson ImmunoResearch), then with a peroxidase anti-peroxidase reagent (1:500, Sigma-Aldrich), and then exposed to a DAB reaction without nickel for 10 min. The peroxidase reaction was stopped with PBS rinses. Then sections were rinsed in distilled water, mounted onto microscope slides, air-dried, dehydrated with an ethanol series, cleared with xylene, and coverslipped. The color reaction of the c-Fos immunostaining was blue-black and localized to the nucleus while the 5-HT immunostaining was light brown and localized to the cytoplasm.

An observer blind to the treatment conditions performed all cell counts using brightfield microscopy at 40X magnification. The number of c-Fos-immunopositive/5-HT-immunopositive cells (i.e., double-labeled cells), the number of c-Fos-immunopositive/5-HT-immunonegative cells (i.e., c-Fos signal-labeled cells), and the total number of 5-HT-immunopositive cells (i.e., 5-HT signal-labeled and double-labeled cells) were counted in different subdivisions of the DRN (Figure 1). Because the DRN is known to consist of a heterogeneous population of neurons (Lowry, 2002), we divided the DRN into 10 subdivisions based on the hamster atlas of Morin and Wood (2001). Our DRN subdivisions were taken from four rostrocaudal levels including rostral (−5.2 mm bregma), mid-rostral (−5.4mm bregma),

mid-caudal (−5.7mm bregma), and caudal (−6.0 bregma). At each rostrocaudal level the DRN was divided into dorsal and ventral regions, and at the mid-rostral and mid-caudal levels lateral subdivisions were delineated. It should be noted that lateral subdivisions do not exist at rostral and caudal levels. Immunostaining was recorded once in each DRN subdivision per individual. Lateral subdivisions were quantified bilaterally, and dorsal and ventral subdivisions were quantified unilaterally because they are midline structures. Small blue-black particles were counted as single c-Fos-stained nuclei. Larger, light brown particles without a darker nucleus were counted as single 5-HT-stained cells. Larger, light brown particles that contained a darker nucleus were counted as double-labeled c-Fos/5-HT cells.

In situ hybridization and image analysis

We sliced brains into 20 μ m coronal sections on a cryostat and then mounted sections directly onto microscope slides and stored the slides at -80°C until processing for isotopic *in situ* hybridization. Briefly, we fixed the sections using 4% paraformaldehyde for 5 min immediately upon thawing the slides. Slides were rinsed in 0.1M PBS, acetylated in 0.1 M triethanolamine buffer containing 0.25% acetic anhydride, dehydrated in a graded series of ethanol, delipidated with chloroform, and returned to ethanol. Dried sections were then exposed to pre-hybridization buffer containing diethylpyrocarbonate (DEPC) treated water, 25% formamide, 10% dextran sulfate, 4X saline sodium citrate (SCC), 2.5X Denhardt's solution, 4 mM ethylenediamine tetraacetic acid (EDTA), 500 $\mu\text{g/ml}$ salmon testes DNA, and 750 $\mu\text{g/ml}$ yeast tRNA. We used an oligonucleotide probe complimentary to a published sequence of Syrian hamster 5-HT1A mRNA (GenBank #DQ217601). The probe was end-labeled with $\alpha\text{-}^{32}\text{P}$ dATP using terminal deoxytransferase (US Biochemicals). The labeled probe was added to the hybridization buffer and applied to slides at a concentration of approximately 2×10^6 dpm. Sections were incubated in hybridization buffer overnight at 37°C . The next day, sections were washed to a final stringency of 1X SSC at 65°C for 1 hr. Then sections were dehydrated in ethanol, air-dried, and together with ^{14}C microscale calibration strips, exposed to Fuji MS digital imaging plates (FujiFilm Corporation) for 48 hrs. Slides were processed in two separate *in situ* hybridization runs. All slides of a given DRN subdivision were processed in the same run, although treatment groups were counterbalanced across two imaging plates. Control experiments with sense probes indicated that the labeling observed with the antisense probes was anatomically specific (data not shown).

The imaging plates were scanned by a BAS 5000 phosphorimager (FujiFilm Corporation) and the associated computer software calculated relative 5-HT1A mRNA levels using photo stimulus luminescence (PSL). The PSL values were calibrated for each imaging plate using a standard curve generated from the ^{14}C microscales. To make our analysis comparable to experiment 1, we used the same 10 DRN subdivisions shown in Figure 1. We calculated PSL levels for each subdivision of the DRN by quantifying two or three sections per individual and then averaging the PSL values at each subdivision for each individual. In the dorsal and ventral regions of the rostral DRN, two sections per subject were available for quantification while three sections per subject were available in other DRN subdivisions. Lateral subdivisions were quantified bilaterally whereas dorsal and ventral subdivisions were quantified unilaterally. For each section a background PSL value was obtained from an area adjacent to, but outside, the DRN. Background values were subtracted from individual PSL values for each DRN subdivision. We took care to ensure that equivalent areas were analyzed for each subject. Similarly, we quantified PSL levels in the lateral septum (LS), ventromedial hypothalamus (VMH), and CA1 layer of the hippocampus because these brain regions showed high hybridization signal and are important substrates for agonistic behavior and emotional memories (Delville et al., 1996, Price et al., 2002, Li et al., 2006). In these forebrain regions we quantified bilaterally three consecutive sections per individual and subtracted background for each tissue section.

Statistical analysis

Behavioral data were analyzed using dependent t-tests, as the behavior of one opponent depends on the other. We used Pearson correlations to test for correlations between behavioral responses and brain changes. The immunohistochemistry and *in situ* hybridization data were analyzed using two-way repeated measures ANOVAs, with treatment condition as the between-subjects factor and DRN subdivision as the within-subjects factor. ANOVA analyses were followed, when appropriate, by Tukey post-hoc tests. A Greenhouse-Geisser correction epsilon (ϵ) was used for repeated measures analysis to correct for potential violation of the sphericity assumption using SPSS software (version 15.0 for Windows; SPSS, Inc.). The α level was set at 0.05 for each analysis. One control individual in experiment 2 was excluded from statistical analysis because of tissue damage sustained during brain removal and slicing.

RESULTS

Experiment 1: Behavior of winners and losers

Our resident-intruder paradigm reliability established winners and losers such that winners were aggressive and losers were submissive. Winners attacked on average 14.5 (SE = 2.9) times during the aggressive encounter whereas losers attacked 0.3 (SE = 0.2) times ($t_{(9)} = 4.92$, $p = .001$). Also, winners were aggressive for 306.8 sec (SE = 40.4) during the encounter and losers were aggressive for 2.1 sec (SE = 1.0) ($t_{(9)} = 7.55$, $p = .0001$). Likewise, losers were submissive for 452.2 sec (SE = 69.1) during the encounter and winners did not show any submissive behavior ($t_{(9)} = 6.55$, $p = .0001$). Losers fled from winners 14.2 (SE = 3.3) times and winners never fled ($t_{(9)} = 4.29$, $p = .002$).

c-Fos expression in the DRN—A representative photomicrograph illustrating c-Fos/5-HT double-labeling is shown in Figure 2. We did not find significant differences in the total numbers of c-Fos-immunopositive, 5-HT-immunopositive, or double-labeled cells within the DRN as a whole (Table 1). Specifically, the number of c-Fos-immunopositive/5-HT-immunopositive neurons within the total DRN showed no significant main effect for treatment groups (double-immunostained; $F_{(2,27)} = 0.96$, $p = .40$, $\epsilon = 0.35$). Likewise, we did not find a significant main effect for treatment groups for both the total number of c-Fos-immunopositive non-5-HT cells or the total number of 5-HT-immunopositive neurons within the DRN ($F_{(2,27)} = 1.82$, $p = .18$, $\epsilon = 0.24$; $F_{(2,27)} = 0.87$, $p = .43$, $\epsilon = 0.57$; respectively).

Importantly, we did find significant interactions between treatment groups and DRN subdivisions. The repeated measures ANOVA of the number of c-Fos-immunopositive/5-HT-immunopositive neurons within specific subdivisions of the DRN revealed a significant interaction for brain region and treatment ($F_{(18,243)} = 1.92$, $p = .015$, $\epsilon = 0.35$). Post-hoc analysis showed that losers had an increased number of c-Fos-immunopositive 5-HT neurons in the ventral region of the rostral DRN compared to both winners and controls ($p = .013$, $p = .001$; respectively). Treatment effects were not observed in other DRN subdivisions (Figure 3). We tested whether the duration of agonistic behavior correlated with number of double-labeled c-Fos/5-HT positive cells in the ventral region of the rostral DRN. The number of double-labeled cells was not significantly correlated with the duration of submissive behavior in losers or with the duration of aggression in winners ($r = -.32$, $p = 0.37$; $r = -.21$, $p = 0.55$, respectively).

Analysis of the number of c-Fos-immunopositive/5-HT-immunonegative cells within specific subdivisions of the DRN also revealed a significant interaction for brain region and treatment ($F_{(18,243)} = 2.34$, $p = .002$, $\epsilon = 0.24$). Post-hoc analysis showed that losers had an increased number of c-Fos-immunopositive, non-5-HT neurons in the ventral region of the rostral DRN compared to both winners and controls ($p = .001$, $p = .0002$; respectively). Treatment effects were not observed in other DRN subdivisions (Figure 4). Also, we found that the number of

single-labeled c-Fos positive cells was not significantly correlated with the duration of submissive behavior in losers or with the duration of aggression in winners ($r = -.11$, $p = 0.76$; $r = .07$, $p = 0.87$, respectively).

Although the number of 5-HT-immunopositive neurons varied across DRN subdivisions ($F_{(9,243)} = 116.13$, $p = .0001$, $\epsilon = 0.57$), there was no interaction between brain region and treatment ($F_{(18,243)} = 1.21$, $p = .26$, $\epsilon = 0.57$) (data not shown).

Experiment 2: Behavior of winners and losers

The resident-intruder paradigm successfully produced winners and losers as indicated by their combined agonistic behavior during all four encounters. Winners attacked losers 18.8 (SE = 3.3) times during the aggressive encounters whereas losers attacked 0.9 (SE = 0.3) times ($t_{(9)} = 5.43$, $p = .0003$). Also, winners were aggressive for a total of 374.4 sec (SE = 64.1) during the encounters and losers were aggressive for 9.9 sec (SE = 3.9) ($t_{(9)} = 5.72$, $p = .0002$). Likewise, losers were submissive for a total of 546.5 sec (SE = 95.3) during the encounters and winners did not show any submissive behavior ($t_{(9)} = 5.73$, $p = .0002$). Losers fled from winners 22.9 (SE = 6.3) times whereas winners never fled ($t_{(9)} = 3.61$, $p = .006$). Although the number of attacks showed a slight decrease with repeated encounters, there were no significant differences in the above measures of aggressive or submissive behavior over trials.

5-HT1A receptor mRNA—Representative autoradiographs illustrating the relative levels of 5-HT1A mRNA in the DRN for winners and losers are shown in Figure 5. We found that the total level of 5-HT1A mRNA in the DRN varied with treatment groups. A repeated measures ANOVA of 5-HT1A mRNA levels within the DRN showed a significant main effect of treatment ($F_{(2,26)} = 3.73$, $p = .038$, $\epsilon = 0.31$). Post-hoc analysis revealed that winners had elevated 5-HT1A mRNA levels compared to losers ($p = .031$), and control animals were intermediate (Figure 6). Although the repeated measures ANOVA of 5-HT1A mRNA levels showed a significant effect of brain region ($F_{(9,234)} = 21.61$, $p = .0001$, $\epsilon = 0.31$), there was no significant interaction between brain region and treatment group ($F_{(18,234)} = 1.08$, $p = .37$, $\epsilon = 0.31$). Thus, winners had greater relative mRNA levels than did losers in each DRN subdivision, and the difference between winners and losers did not depend on the subdivision. Also, we found that 5-HT1A mRNA levels in the DRN were not significantly correlated with the duration of submissive behavior in losers or with the duration of aggression in winners ($r = .04$, $p = 0.91$; $r = -.17$, $p = 0.63$, respectively).

We also quantified 5-HT1A mRNA levels for winners, losers, and controls in forebrain regions including the LS, VMH, and CA1 layer of the hippocampus. We did not find significant treatment effects in these forebrain regions (Table 2; LS: $F_{(2,26)} = 1.55$, $p = .23$; VMH: $F_{(2,26)} = 0.95$, $p = .40$; CA1: $F_{(2,26)} = 1.15$, $p = .33$).

DISCUSSION

Activation of DRN neurons after aggressive encounters

We found that losers of aggressive encounters showed increased c-Fos immunoreactivity in 5-HT and non-5-HT neurons in discrete subregions of the DRN compared to winners and handled controls. Our results extend previous research on Syrian hamsters which showed that 30-min aggressive encounters produce elevated c-Fos mRNA in several brain regions of subordinate animals, including the DRN (Kollack-Walker et al., 1997). Our results are also consistent with other research showing that 5-HT neurons in the DRN are activated by stressful or aversive events such as inescapable footshock (Grahn et al., 1999), urocortin administration (Staub et al., 2005), and social defeat (Gardner et al., 2005). Further, we have reported that the pharmacological activation of 5-HT1A autoreceptors in the DRN during social defeat impairs

the development and expression conditioned defeat (Cooper et al., 2008). In sum, the current results are consistent with our previous findings and together suggest that the activation of DRN 5-HT neurons is an important component of the neural circuitry underlying behavioral changes that occur after losing an aggressive encounter.

Regional variation in 5-HT activation within the DRN is important because the DRN is a heterogeneous structure in which different subregions contain cells with unique axonal projections, neurochemical phenotypes, and receptor expression (Lowry, 2002, Commons et al., 2003, Day et al., 2004). The heterogeneous nature of the DRN is thought to be responsible for the diverse 5-HT responses observed following the administration of CRF and other stressors. CRF administration has been reported to inhibit 5-HT neurons in the rostral DRN (Price et al., 1998, Kirby et al., 2000), while it excites 5-HT neurons in the caudal DRN (Lowry et al., 2000). It is thought that activation of CRF type-1 receptors might excite GABA neurons and thereby mediate inhibitory effects on rostral 5-HT neurons (Roche et al., 2003), whereas activation of CRF type-2 receptors might inhibit non-5-HT interneurons (presumably GABAergic) and disinhibit caudal 5-HT neurons (Pernar et al., 2004). Distinct projections from DRN subregions allow for a diverse pattern of 5-HT responses to stressful events. Neuroanatomical data indicate that the rostral DRN sends 5-HT projections to forebrain regions including the lateral septum and caudate putamen (Steinbusch et al., 1981, Imai et al., 1986). Stressors that increase motor activity, such as swim stress, have been shown to increase extracellular 5-HT levels in the caudate putamen and decrease it in the lateral septum (Kirby and Lucki, 1998, Price et al., 2002). Projection targets of the caudal DRN include the central amygdala, hippocampus, and paraventricular nucleus of the hypothalamus (Imai et al., 1986, Kazakov et al., 1993), which are thought to underlie autonomic, neuroendocrine, as well as emotional responses to stress (Lowry, 2002).

In the current study, we found that losing aggressive encounters increased c-Fos expression in rostral 5-HT DRN neurons. One possibility is that losing aggressive encounters results in the release of CRF and/or urocortins within the DRN which in turn activates CRF type-2 receptors and ultimately activates rostral 5-HT DRN neurons. In fact, our lab has previously shown that injection of a CRF type-2 receptor antagonist into the DRN reduces the changes in agonistic behavior associated with conditioned defeat (Cooper and Huhman, 2007). We also found that losing aggressive encounters increased c-Fos expression in non-5-HT cells within the rostral DRN. Although we do not know the neurochemical phenotype of the c-Fos-positive non-5-HT cells, one possibility is that they are GABAergic interneurons activated by neurotransmission at CRF type-1 receptors. Thus, we might speculate that losing aggressive encounters activates both 5-HT and non-5-HT neurons within the rostral DRN by increasing neurotransmission at both CRF type-1 and CRF type-2 receptors. Although pharmacological and neuroanatomical data in rats indicate a greater role for CRF type-1 receptors than CRF type-2 receptors in the rostral DRN, it should be noted that the organization of hamster DRN is unknown.

We have shown that the basolateral amygdala, central amygdala, medial amygdala, and bed nucleus of the stria terminalis (BNST) are critical components of the neural circuitry controlling conditioned defeat (Jasnow and Huhman, 2001, Jasnow et al., 2004a, Jasnow et al., 2004b, Cooper and Huhman, 2005, Markham and Huhman, 2008). Neuroanatomical data in rats indicate that the amygdala and BNST have reciprocal connections with the DRN (Imai et al., 1986, Peyron et al., 1998, Dong et al., 2001, Lowry, 2002, Lee et al., 2003). Although the mid and caudal DRN are more well-known for 5-HT projections to limbic structures such as the amygdala, the rostral DRN also sends projections to the amygdala (Steinbusch et al., 1981, Imai et al., 1986). Also, CRF administration into the basolateral amygdala has been shown to enhance c-Fos expression in the rostral DRN, although the connection from the basolateral amygdala to the DRN is likely indirect (Spiga et al., 2006). The exact topography of DRN efferent projections to the BNST have not been defined (Weller and Smith, 1982). Interestingly,

glutamatergic projections from the lateral ventral portion of the BNST have been shown to target ventral portions of the middle DRN, but not the caudal DRN (Lee et al., 2003). It has been hypothesized that a glutamatergic connection between the lateral ventral BNST and the rostral-mid DRN might contribute to the ability of wheel running to attenuate stress-induced c-Fos expression in DRN 5-HT neurons and to prevent learned helplessness (Greenwood et al., 2005). Collectively, the available behavioral and anatomical data indicate that the reciprocal connections between the rostral DRN and the amygdala and BNST are in position to modulate how winners and losers might respond in future social interactions.

5-HT1A gene expression after aggressive encounters

In the present study, we found that losers of aggressive encounters had reduced 5-HT1A mRNA throughout the DRN compared to winners. The reduced 5-HT1A mRNA expression in losers appears to be specific to the DRN because mRNA levels did not significantly differ in several forebrain regions including the LS, VMH, and CA1 layer of the hippocampus. Taking the 5-HT1A mRNA and c-Fos data together, our results suggest that losing aggressive encounters both decreases message for 5-HT1A autoreceptors and increases the activity of 5-HT neurons in the DRN. Further, the brain changes that occurred following aggressive encounters appear related to final status rather than the amounts of agonistic behavior because mRNA and c-Fos levels were not correlated with behavior in either winners or losers.

Our data on changes in 5-HT1A mRNA expression have three possible limitations that should be considered. First, we found that c-Fos expression was selectively increased in specific DRN subregions in losers of aggressive encounters, whereas 5-HT1A mRNA expression was altered throughout the DRN. While a decrease in 5-HT1A autoreceptors might allow for increased future activation of DRN 5-HT neurons, it is unclear how uniform changes in 5-HT1A autoreceptors might mediate subregional changes in c-Fos expression. A link between uniform changes in 5-HT1A mRNA expression and selective increases in neural activity would require regionally specific post-transcriptional processing of 5-HT1A autoreceptors. Second, although hamsters that lose aggressive encounters exhibit robust changes in their future agonistic behavior compared to controls (Huhman et al., 2003), in the current study we found that losers showed decreased 5-HT1A mRNA expression in the DRN compared to winners only. It is problematic to conclude that neurochemical differences between winners and losers could mediate behavioral differences between losers and controls. The optimal time point to observe acute changes in 5-HT1A mRNA expression is unknown and, because we investigated mRNA changes at a single time point, it is possible that losers and controls may differ in their mRNA expression at other time points. We predict that changes in 5-HT1A receptor levels, rather than 5-HT1A mRNA levels, should correspond to the behavioral changes that occur following social defeat and expect that measuring the time course of changes in 5-HT1A receptor densities will be an important next step. Third, we can not be sure that the decrease in 5-HT1A mRNA represents somatodendritic autoreceptors because 5-HT1A receptors have been reported on a small number of non-5-HT neurons in the DRN (Kirby et al., 2003, Day et al., 2004). If the observed changes in 5-HT1A mRNA expression occurred mainly in this neuronal population, then intra-DRN circuits could be modulating 5-HT function independent of 5-HT1A autoreceptors. We maintain that this possibility is unlikely as somatodendritic autoreceptors on 5-HT neurons greatly outnumber 5-HT1A receptors on non-5-HT neurons in the DRN (Day et al., 2004).

A down-regulation of 5-HT1A autoreceptors would remove an important source of inhibition on DRN 5-HT neurons and might sensitize 5-HT neurons to future input. Maier and colleagues have proposed that the hyperactivity of DRN 5-HT neurons that characterizes learned helplessness is mediated, at least in part, by a desensitization and/or down-regulation of 5-HT1A autoreceptors that occurs following an acute bout of inescapable shock (Maier and

Watkins, 2005). It is noteworthy that in the present study the change in DRN 5-HT1A mRNA following aggressive encounters appears to be due both to a reduction in losers and an increase in winners. Increased 5-HT1A mRNA in winners might lead to greater 5-HT1A autoreceptor-mediated inhibition and protection against future stressors that activate the DRN. The benefits of winning aggressive encounters might be similar to exercise insofar as voluntary wheel running elevates 5-HT1A mRNA in rats and prevents the development of learned helplessness (Greenwood et al., 2003, Greenwood et al., 2005).

In general, low 5-HT activity is associated with greater impulsivity and aggression (Coccaro and Kavoussi, 1997, Westergaard et al., 1999, Chiavegatto et al., 2001, Parsey et al., 2002). In Syrian hamsters, 5-HT acts in the anterior hypothalamus and ventromedial hypothalamus to inhibit aggression while vasopressin stimulates it (Delville et al., 1996, Ferris et al., 1997, Ferris et al., 1999). In our study, the increased message for DRN 5-HT1A autoreceptors found in winners might enhance autoreceptor-mediated inhibition of DRN 5-HT neurons and predispose winners to future aggression. In fact, after hamsters gain experience winning they more rapidly attack novel opponents in future aggressive encounters (Hebert et al., 1994). A predisposition for future aggression should be distinguished from the initiation and display of aggressive behavior because ongoing aggression has also been associated with the acute activation of 5-HT neurotransmission (Delville et al., 2000, van der Vegt et al., 2003, Haller et al., 2005). Although we did not find increased c-Fos expression in the 5-HT neurons of winners, in the dorsal region of the mid-rostral DRN we found a slight trend toward increased activation of 5-HT neurons in both winners and losers (see Figure 2b).

We have shown that losing aggressive encounters increases c-Fos expression in 5-HT and non-5-HT neurons in discrete subregions of the DRN relative to winners and controls and reduces 5-HT1A mRNA expression throughout the DRN relative to winners. Our results are consistent with other research showing that stressful events can activate 5-HT neurons in discrete subregions of the DRN (e.g. Grahn et al., 1999, Gardner et al., 2005), and they provide some of the first evidence that an acute stressor can alter 5-HT1A mRNA expression in the DRN. Our finding that losing aggressive encounters can decrease 5-HT1A mRNA expression in the DRN suggests that 5-HT neurons in losers might be more prone to future activation because of reduced 5-HT1A autoreceptor-mediated inhibition. Although losing aggressive encounters is known to alter future agonistic behavior in hamsters (Huhman et al., 2003), it is unclear whether differences in 5-HT1A mRNA expression between winners and losers can explain defeat-induced changes in behavior. In any case, our studies suggest that losing aggressive encounters can alter 5-HT neurotransmission in the DRN, which is a critical node in the neural circuitry controlling the formation of conditioned defeat. These findings may help elucidate how brief exposure to social stress can lead to long lasting changes in social and emotional behavior and may, in turn, help us understand the mental health consequences of social stress.

ABBREVIATIONS

BNST, bed nucleus of the stria terminalis
 CRF, corticotropin-releasing factor
 DAB, 3,3'-diaminobenzidine
 DEPC, diethylpyrocarbonate
 DRN, dorsal raphe nucleus
 EDTA, ethylenediamine tetraacetic acid
 HPA, hypothalamic-pituitary-adrenal
 LS, lateral septum
 MRN, median raphe nucleus
 mRNA, messenger ribonucleic acid

PBS, phosphate-buffered saline
 PSL, photo stimulus luminescence
 SCC, saline sodium citrate
 5-HT, serotonin
 VMH, ventromedial hypothalamus

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Figure 1.

Photomicrographs (10× magnification) of coronal slices through rostral (A), mid-rostral (B), mid-caudal (C), and caudal (D) dorsal raphe nucleus (DRN). Sections are approximately 5.2, 5.4, 5.7, and 6.0 mm posterior to bregma, respectively (Morin and Wood 2001). Sections were labeled for serotonin immunoreactivity and are presented here to delineate the dorsal, ventral, and lateral aspects of the DRN used for immunohistochemistry and *in situ* hybridization quantification. Aq, cerebral aqueduct; mlf, medial longitudinal fasciculus.

Figure 2.

A representative photomicrograph (40X magnification) of a coronal slice through the rostral dorsal raphe nucleus showing c-Fos (small black particles) and 5-HT (larger, light brown particles) double immunolabeling. Single-headed arrows point to 5-HT-labeled cells, double-headed arrows point to c-Fos-labeled cells, and triple-headed arrows point to c-Fos/5-HT-labeled cells.

Figure 3.

The number of c-Fos/5-HT-labeled cells in the rostral (A), mid-rostral (B), mid-caudal (C), and caudal (D) dorsal raphe nucleus are shown for winners, losers, and controls. Animals experienced a 15-min aggressive interaction and were classified as winners (N = 10) and loser (N = 10) based on the outcome. Control animals (N = 10) were exposed to an empty cage for 15 minutes. We collected brains 75-min following treatment and processed them for c-Fos and 5-HT immunoreactivity. Values represent the mean number of serotonergic c-Fos-positive cells $\pm$ SE. * indicates that losers are greater than both winners and controls ($p < .01$).

Figure 4.

The number of single c-Fos-labeled cells in the rostral (A), mid-rostral (B), mid-caudal (C), and caudal (D) dorsal raphe nucleus are shown for winners, losers, and controls. Animals experienced a 15-min aggressive interaction and were classified as winners ($N = 10$) and loser ($N = 10$) based on the outcome. Control animals ($N = 10$) were exposed to an empty cage for 15 minutes. We collected brains 75-min following treatment and processed them for c-Fos and 5-HT immunoreactivity. Values represent the mean number of non-serotonergic c-Fos-positive cells $\pm$ SE. * indicates that losers are greater than both winners and controls ($p < .01$).

Figure 5.

Representative autoradiographic signal in coronal sections through the rostral, mid-rostral, mid-caudal, and caudal dorsal raphe nucleus (DRN) of a winner and loser. We labeled 5-HT1A autoreceptor messenger ribonucleic acid (mRNA) using *in situ* hybridization and quantified relative 5-HT1A mRNA levels in the DRN.

Figure 6.

Expression of 5-HT1A messenger ribonucleic acid (mRNA) in the total dorsal raphe nucleus (DRN) is shown for winners, losers, and controls. Animals experienced four 5-min aggressive encounters at one hour intervals and were classified as winners ($N = 10$) and losers ($N = 10$) based on the outcome. Control animals ($N = 9$) were exposed to an empty cage at each treatment. We collected brains immediately following the last encounter and processed them for 5-HT1A mRNA *in situ* hybridization. Values represent mean photo stimulus luminescence $\pm$ SE. * indicates that winners are greater than losers ($p < .05$).

Table 1Total number of cells counted across all subdivisions of the DRN (mean $\pm$ SE)

Counts	Winner	Loser	Control	p
c-Fos+/5-HT+	17.8 $\pm$ 3.3	17.5 $\pm$ 3.8	11.7 $\pm$ 3.5	ns
c-Fos+/5-HT-	108 $\pm$ 18.3	132.7 $\pm$ 17.7	83.2 $\pm$ 19.0	ns
Total 5-HT+	824.4 $\pm$ 14.6	876.6 $\pm$ 27.9	863.8 $\pm$ 39.7	ns

c-Fos+/5-HT+: c-Fos-immunopositive/5-HT-immunopositive neurons; c-Fos+/5-HT-: c-Fos-immunopositive/5-HT-immunonegative cells; total 5-HT+: 5-HT-immunopositive neurons with and without c-Fos staining. (ns: $p > .05$)

Table 2

Relative 5-HT1A mRNA levels

Brain Region	Winner	Loser	Control	p
Hippocampus (CA1)	47.8 ± 3.5	52.1 ± 2.7	44.4 ± 4.6	ns
Lateral septum	18.1 ± 1.7	16.4 ± 1.2	20.3 ± 1.7	ns
Ventromedial hypothalamus	19.7 ± 1.8	18.6 ± 1.1	21.4 ± 1.2	ns

Values represent mean photo stimulus luminescence ± SE. Winners (N = 10), losers (N = 10), and controls (N = 9) did not significantly differ in 5-HT1A mRNA levels in the brains regions shown (ns, $p > .05$).