REGULAR RESEARCH ARTICLE

Sex Differences in Serotonin 5-HT 1A Receptor Responses to Repeated Restraint Stress in Adult Male and Female Rats

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Abstract

Background: Male and female rats were exposed to repeated restraint to determine how changes in serotonin (5-hydroxytryptamine; 5-HT) 1A receptors associate with stress hypothalamic-pituitary-adrenal (HPA) axis habituation.
Methods: In response to 2-hour episodes of restraint, repeated daily for 5 consecutive days, males and females displayed reliable declines in HPA output, indicated by diminished adrenocorticotropin and corticosterone secretory responses. Using the 5-HT 1A receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) as a pharmacological challenge for inducing hypothermia and elevations in plasma corticosterone, males displayed sensitized hypothermal responses after repeated restraint, whereas corticosterone responses to 8-OH-DPAT were enhanced in both sexes following single or repeated exposure.
Results: Only males showed elevations in 5-HT 1A receptor G-protein coupling responses in the dorsal raphe after repeated restraint, whereas only females showed an increase in 5-HT 1A receptor responses in the hippocampus following single or repeated exposure. G-protein coupling responses within both regions correlated positively with 5-HT 1A receptor binding capacity. Thus, despite expressing similar capacities for stress HPA axis habituation, males and females emerged from repeated restraint to show region-specific changes in 5-HT 1A receptor function that may be explained, at least in part, by changes in receptor availability.
Conclusions: Based on the hypothermal and corticosteroid responses to 8-OH-DPAT, the present data suggest that stress habituation is met by an increase in the sensitivity of presynaptic 5-HT 1A receptors in males and by an increase in the sensitivity of a population of postsynaptic receptors in both sexes.

Keywords: Stress habituation, sex differences, corticosterone, serotonin, 5-HT 1A receptor

Introduction

Males and females display different neuroendocrine and behavioral responses to acute and chronic stress, often interpreted as informing the bases for sex differences in stress vulnerability. Between these 2 paradigms, however, exists a rather huge gap in our understanding of stress habituation. Understanding this process is important, considering that maladaptive coping and impaired expression of stress hypothalamic-pituitary-adrenal (HPA) axis habituation have been proposed as leading causes for mood, metabolic, and cardiovascular disorders (Hänninen and Aro, 1996; Pico-Alfonso et al., 2007).

Exposure to the same (homotypic), non–life-threatening stimulus that is repeated in a predictable manner results in the diminished responsiveness of the HPA axis (Grissom and Bhatnagar, 2009), reflected by decreases in the stress-induced

Significance Statement

The ability to learn from and adapt to repeated stress is critical for survival. We previously reported that the adaptive declines in glucocorticoid responses to repeated stress is met by sex-specific changes in serotonin (5-HT) 1A receptor mRNA levels in the central nervous system. To determine the size and extent to which these alterations reflect changes in receptor availability and function, here we tested male and female rats leaving repeated restraint by assessing their physiological responses to a challenge dose of the 5-HT 1A agonist 8-OH-DPAT in addition to measuring 5-HT 1A receptor binding and G-protein coupling responses in brain tissue slices. The overall results suggest that stress habituation involves sex-specific changes in the sensitivity of the 5-HT 1A receptor at both pre- and postsynaptic sites. In addition to opening new avenues for understanding stress resilience, these findings may help reconcile the bases for sex disparities in stress-related pathology.

activation of the paraventricular nucleus of the hypothalamus (PVH) and consequently decreased anterior pituitary adrenocorticotropin and adrenal corticosteroid secretory responses (Viau and Sawchenko, 2002). Stress HPA axis habituation is considered adaptive, as the remaining corticosterone response continues to meet the metabolic demands of the familiar challenge, all the same minimizing the potential damaging effects of glucocorticoid excess. This process is also accompanied by long-term changes in neuronal structure and function in the brain, particularly within HPA axis regulatory regions, in addition to the biosynthetic capacity of neuroendocrine neurons in the PVH (Ulrich-Lai and Herman, 2009). These alterations likely account for another hallmark feature of homotypic stress exposure, whereby HPA axis responses become sensitized to subsequent novel (heterotypic) challenges (Bhatnagar and Dallman, 1998), affording an additional means of adapting to changing environments.

Challenges that facilitate HPA axis responses or require sustained increments in glucocorticoid secretion in the male rodent, including repeated immobilization, food restriction, chronic variable stress, and chronic social stress, are accompanied by increases in serotonin (5-hydroxytryptamine [5-HT]) turnover as well as reductions in 5-HT 1A receptor expression and function at both pre- and postsynaptic sites (Korte et al., 1995; Grippo et al., 2005; Bambico et al., 2009; Li et al., 2009). Decreases in 5-HT 1A receptors at presynaptic (somatodendritic) sites, which normally act to diminish raphe neuron excitability, could provide a mechanism for enhancing 5-HT turnover and availability to forebrain target sites (Blier and Ward, 2003). This would also provide a means for maintaining glucocorticoid secretory responses in the face of chronic stress, given the central, stimulatory effects of serotonin on the HPA axis (Lanfumey et al., 2008). Sustained periods of glucocorticoid exposure and/or 5-HT turnover may come at a price, however, as animals exposed to chronic stress show alterations in affective behaviors, decreases in 5-HT 1A receptor binding in the hippocampus, and blunted adrenocortical responses to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), signifying a global impact of chronic stress and/or glucocorticoid excess to decrease 5-HT 1A receptor responsiveness at postsynaptic sites (Meijer and de Kloet, 1998; Chaouloff, 2000; Flügge et al., 2004).

Based on the stimulatory effects of serotonin on the HPA axis, we propose that normal declines in adrenal responsiveness should, in the very least, be reflected by increases in 5-HT 1A receptor binding and/or function at presynaptic sites, given the influence of somatodendritic 5-HT1A receptors to decrease cell firing of serotonin-producing cells in the dorsal raphe and 5-HT availability at postsynaptic sites. Our previous studies indicate that stress habituation is associated with an increase in 5-HT 1A receptor mRNA expression in the dorsal raphe nucleus of male but not female rats. Thus, we hypothesize that males, but not females, would emerge from repeated restraint to show increases in 5-HT 1A receptor availability and/or function in this region. Interestingly, transient removal of corticosterone during stress has been shown to promote behavioral adaptation, enhance postsynaptic responses to 5-HT, as well as facilitate the corticosterone response to 8-OH-DPAT (Kennett et al., 1985; Cassano and D'Mello A, 2001). Thus, in the face of stress habituation, we hypothesize that animals show sensitized 5-HT 1A receptor mediated-adrenocortical activation after repeated restraint. Moreover, because males and females can show adaptive declines in glucocorticoid responses, we expect this sensitized response to occur in both sexes.

To test these hypotheses, we compared in male and female rats the effects of 5 days of repeated restraint exposure to alter hypothermal and corticosterone responses to a low challenge dose of 8-OH-DPAT (0.05 mg/kg, s.c.) taken as indices of pre- and postsynaptic 5HT 1A receptor function, respectively (McAllister-Williams et al., 2001; Blier and Ward, 2003). Brain tissue slices obtained from animals exposed to repeated restraint were then used to measure 5-HT 1A receptor binding capacity using a [³H]8-OH-DPAT autoradiographic approach. Changes in 5-HT 1A receptor function can also depend on receptor availability (Rossi et al., 2006, 2008). Thus, additional tissue series were used to measure 8-OH-DPAT–induced [³⁵S]GTPγS binding to provide an index of 5-HT 1A receptor-mediated G-protein coupling, representing the first step in the intracellular cascade and functional readout of the receptor (Sóvágó et al., 2001). The findings suggest that males and females emerge from repeated restraint to show sex-specific alterations in 8-OH-DPAT–induced hypothermal and neuroendocrine responses as well as alterations in G-protein coupling of the 5-HT 1A receptor in different brain regions. The extent to which these changes contribute to or otherwise respond to stress HPA axis habituation is discussed.

MATERIALS AND METHODS

Animals and Procedures

Male and female Sprague-Dawley rats (Charles River, Kingston, NY, USA) were approximately 60 days of age on arrival. All animals were socially housed in unisex pairs in the same room, pair-housed in Optirat Plus cages (Animal Care Systems, Inc.; Centennial, CO, USA) under controlled temperature (22°C±2°C) and lighting conditions (12:12-hour-light-:-dark cycle, lights on at 7:00 AM), with food and water available ad libitum. Commencing 7 days after arrival, rats were acclimatized to daily handling and body weight assessments and/or mock injections commencing for 3 consecutive days (9:00 AM to 11:00 PM) prior to stress testing.

To test the reliability of the repeated restraint paradigm as a model of stress HPA axis habituation, male and female rats were placed in Plexiglass restrainers for 120 minutes, repeated daily (9:00 AM to 11:00 PM) for 5 consecutive days. On both the first (Acute) and fifth (Repeat) day of restraint, serial blood samples

(300 μL) were obtained from individual animals (<3 minutes) via a lateral tail vein incision, starting at 0, 30, 60 and 90 minutes of restraint exposure (see Figure 1). Animals were approximately 77 days of age at the start of this experiment, with males and females weighing 380.6±5.1 and 257.2±3.3, respectively, on the first day of stress testing. Because of this body weight difference, different-sized restrainers were employed to achieve comparable levels of forced confinement: inner dimensions: male=19 cm (L)×10.0 cm (W) ×4.9 cm (H); female=15.2 cm (L) ×8.2 cm (W) ×3.8 cm (H).

To test the nature and extent to which repeated restraint is reflected by sex differences in 5-HT 1A receptor availability and function, a separate cohort of male and female rats was randomly assigned to the following 3 groups: (1) singly restrained animals; handled daily (presented to, but not placed within, restrainers) for 4 days prior to stress testing, which involved placing them in Plexiglas restrainers for a single 120-minute period; (2) repeatedly restrained animals; similarly handled and subjected to 5 consecutive days of 120-minute restraint exposure; and (3) naïve-control; treated comparably with their experimental counterparts but without experiencing restraint. See Figure 1 (bottom) for treatment groups and experimental paradigm. Animals were approximately 70 days of age at the start of this experiment, with males and females weighing 358.4±3.0
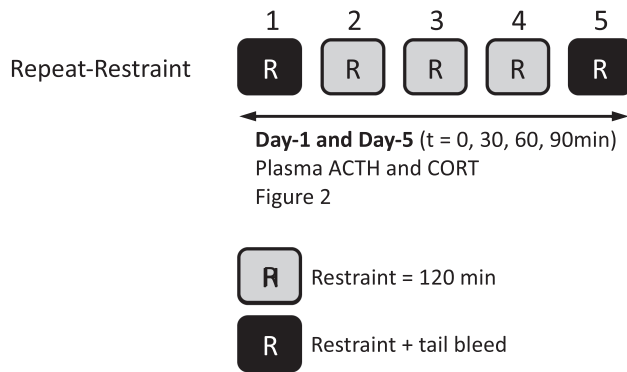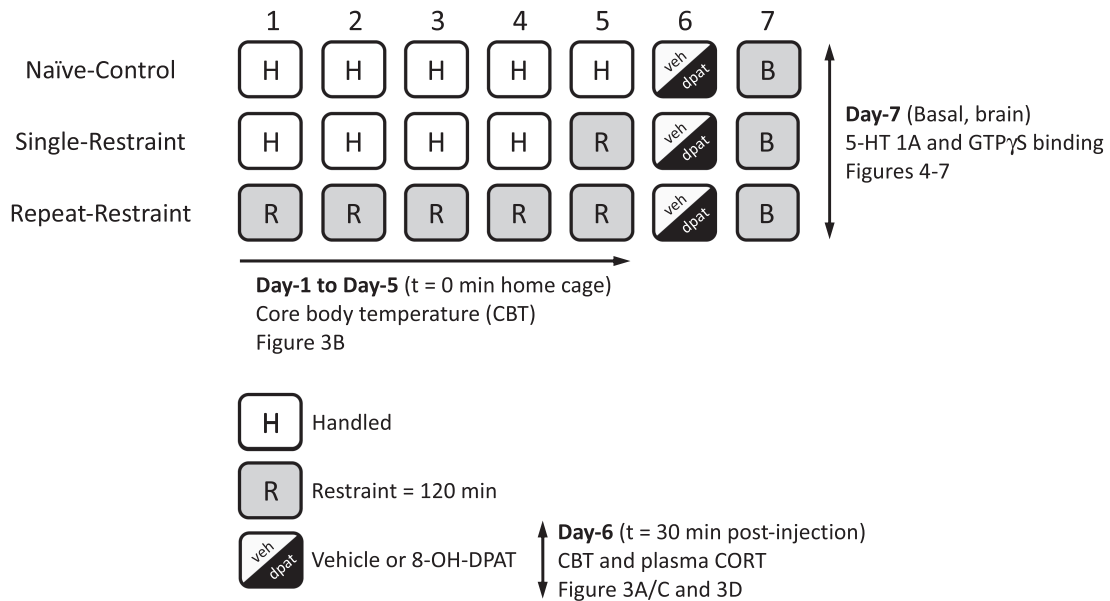

Figure 1. Repeated restraint paradigm for assessing stress HPA axis responses (top). Groups of male and female rats (n=6/group) were restrained in Plexiglass restrainers for 2 h/d for 5 consecutive days, repeatedly tail bled for adrenocorticotropin (ACTH) and corticosterone (CORT) on the first day of restraint, and then again on the last day of exposure. Experimental paradigm for assessing serotonin (5-HT) 1A receptor responses to repeated restraint (bottom). New cohorts of male and female rats were either singly restrained, exposed to repeated restraint, or treated comparably with their experimental counterparts, but without experiencing restraint. Day 1 to day 5: pre-stress (basal) core body temperatures were recorded daily in stress-naïve male and female rats (n=44/group and 32/group, respectively) and in repeatedly restrained male and female rats (n=17/group and 15/group, respectively). Day 6: 24 hours after stress testing, subsets of control, singly and repeatedly restrained animals were then either treated with vehicle or 8-OH-DPAT for testing 5-HT 1A receptor function: Vehicle=6/group; 8-OH-DPAT=12-16/group per sex/stress condition. Day 7: 24 hours later, brain tissue samples obtained under basal conditions were then randomly selected (n=6/sex/stress condition) for 5-HT 1A receptor binding/coupling analysis.

and 246.4±2.5, respectively, on the first day of stress testing, employing the same size restrainers described above.

As we hypothesized that repeated restraint is met by an increase in 5-HT 1A receptor responses, the effects of 2 doses (0.05 and 0.2 mg/kg) of 8-OH-DPAT (Sigma-Aldrich, Oakville, ON, Canada) to induce hypothermia and plasma corticosterone levels were first piloted in stress-naïve and repeatedly restrained male rats. Although both responses to 8-OH-DPAT were potentiated by repeated restraint exposure, the largest differences between naïve and stressed animals occurred among those bearing the lower, 0.05-mg/kg dose of 8-OH-DPAT (data not shown).

Based on this preliminary finding, separate cohorts of animals were exposed to single and repeated restraint and 24 hours after stress exposure given a challenge dose of 8-OH-DPAT (0.05 mg/kg s.c.) or vehicle (0.9 % saline). Based on previous time course studies (Haleem et al., 1989; Uphouse et al., 1991), core temperature readings (Thermalert device, Physitemp; Clifton, NJ, USA) were taken 30 minutes after vehicle or 8-OH-DPAT administration, and tail vein blood samples (200 μL) were obtained immediately thereafter. Twenty-four hours after 8-OH-DPAT testing, brain tissue was obtained via decapitation under basal conditions (9:00 AM to 11:00 PM). All protocols were approved by the University of British Columbia Animal Care Committee.

Hormone Assays

Blood samples were collected in ice-chilled tubes containing ethylenediaminetetraacetic acid and aprotinin. Plasma obtained by centrifugation (3000 g) was stored at −80°C until assayed for HPA output, including adrenocorticotropin (ACTH) and corticosterone, in addition to sex steroid hormones. Estrous cycle phase in females was not determined in the current study; however, gonadal status (testosterone in males and estradiol in females) was assessed from trunk blood samples obtained during tissue harvesting. Plasma ACTH, testosterone, and estradiol were measured using commercial radioimmunoassay (RIA) kits (MP Biomedicals, Solon, OH, USA). Corticosterone was measured by RIA as previously described (Viau and Meaney, 1991). Briefly, equal volumes of plasma sample, [³H]corticosterone (3 nmol/l PerkinElmer, Waltham, MA, USA), and primary antiserum (1:2500, Ab1297, Sigma, Boston, MA, USA) were incubated overnight at 4°C. Dextran-coated charcoal was then added to the incubates at 4°C, after which bound ligand was obtained by centrifugation (3000 g) for 10 minutes. Intra- and inter-assay coefficients of variation were 6% and 12%, respectively, for ACTH; 8% and 10% for corticosterone; 3% and 8% for testosterone; and 6% and 11% for estradiol. Detection limits were 10.0 pg/mL (ACTH), 10 ng/mL (corticosterone), 0.1 ng/mL (testosterone), and 1.5 pg/mL (estradiol).

[³H]8-OH-DPAT Autoradiography

Brains were removed, flash frozen in isopentane (−50°C), and stored at −80°C. Multiple series of 20-μm-thick sections through each region of interest were stored at −80°C until time of assay and processing for 5-HT 1A binding using [³H]8-OH-DPAT, as previously described (Mendelson and McEwen, 1991; Pompeiano et al., 1992). For nonspecific and total binding, sections were incubated in a Tris-HCl (pH 7.4) solution containing 2 nM of the 5-HT 1A agonist [³H]8-OH-DPAT (PerkinElmer) either in the presence or absence of the 5-HT 1A antagonist, WAY100635 (1 μM, Sigma-Aldrich Canada), respectively. Following 1-hour incubation, sections were rinsed, air-dried overnight, and then opposed to BioMax MS film and intensifying screen (Sigma, Canada) alongside ¹⁴C microscale standards for 4–6 weeks for

optimal signal detection. Captured images were analyzed using NIH Image J v.1.52d software to generate mean optical density levels, assisted by redirected sampling of adjacent Nissl-stained material. Specific binding was determined by subtracting nonspecific from total binding, expressed as calibrated binding units (nCi/mg) according to the microscale standard curve.

[³⁵S] GTPγS Autoradiography

Agonist-stimulated [³⁵S] GTPγS binding was performed as previously described (Sim et al., 1997; Waeber and Moskowitz, 1997; Happe et al., 2001). Briefly, tissue series were first pre-incubated for 30 minutes in glycylglycine assay buffer containing GDP. For stimulated and basal binding, tissue slices were then incubated in assay buffer containing 0.05 nM [³⁵S]GTPγS (PerkinElmer) in either the presence or absence of 1 μM (±)8-OH-DPAT, respectively. For nonspecific binding, adjacent tissue sections were incubated in assay buffer containing 0.05 nM [Sulfur-35]guanosine 5'-O-[gamma-thio]triphosphate ([³⁵S]GTPγS) in the presence of 1 μM GTPγS, without (±)8-OH-DPAT. Following 1-hour incubation at 30°C, sections were rinsed, air-dried overnight, and then opposed to BioMax MR film alongside ¹⁴C microscale standards for 1–3 days, depending on the region of interest. Captured images were analyzed using NIH Image J v.1.52d software to generate mean optical density levels, calibrated to nCi/mg units. Nonspecific binding was subtracted from binding in the presence or absence of 8-OH-DPAT to determine agonist-induced [³⁵S]GTPγS binding, expressed as percentage of basal binding. Densitometric data gathered for the zona incerta adjoining the lateral hypothalamic area, hippocampus subfields, and raphe nuclei were further assisted by redirected sampling of corresponding Nissl-stained sections, with reference to the anatomical atlas of Swanson (Swanson, 2018).

Statistical Analysis

HPA output (ACTH and corticosterone) data obtained from the same animals on day 1 and day 5 of stress testing were compared using 3-way ANOVAs (sex as between-subject variable, time, and stress/day as repeated measures). Sex hormone levels were compared using 1-way ANOVAs (stress as a between-subject variable). 8-OH-DPAT–induced hypothermal and corticosterone responses were compared using 3-way ANOVAs (sex, stress, and treatment as between-subject variables). Pre-stress, baseline temperatures were compared using a 3-way ANOVA (sex and stress as between-subject variables and stress/day as repeated measure). Receptor-based data were compared using 2-way ANOVAs (sex and stress as between-subject variables). Where main effects and interactions were found to be significant ($P < .05$), additional comparisons were made using Tukey's honestly significant difference (HSD) test. Because alterations in 5-HT 1A receptor coupling responses can be accounted for by changes in receptor availability, Pearson's correlations were used to determine relationships between these variables of interest.

RESULTS

Hormone Responses to Repeated Restraint

ACTH and corticosterone responses under acute (day 1) and repeat (day 5) restraint conditions are shown in Figure 2. For ACTH, 3-way analyses indicated significant effects of sex ($F_{(1,10)} = 39.7$; $P < .0001$), stress ($F_{(1,18)} = 48.3$; $P < .0001$), and time ($F_{(3,30)} = 44.1$; $P < .0001$) as well as significant interactions between stress and
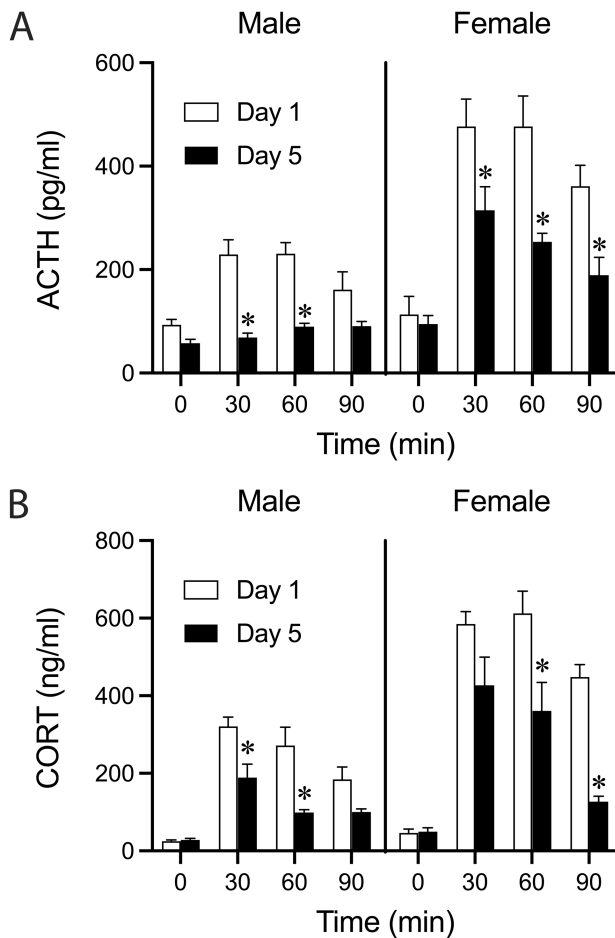
Figure 2. Male and female rats show repeated restraint-induced declines in HPA axis responsiveness. Mean±SEM plasma ACTH (A) and corticosterone (B) responses during acute and repeated restraint stress exposure. *$P < .05$ vs acute; n=6/group.

time ($F_{(3,30)} = 6.3$; $P = .002$). For corticosterone, 3-way analyses likewise indicated significant effects of sex ($F_{(1,10)} = 96.9$; $P < .0001$), stress ($F_{(1,10)} = 70.1$; $P < .0001$), and time ($F_{(3,30)} = 58.8$; $P < .0001$) as well as significant interactions between stress and time ($F_{(3,30)} = 8.4$; $P = .0003$). Post-hoc analysis confirmed higher plasma ACTH and corticosterone concentrations in females overall ($P < .01$), whereas ACTH and corticosterone responses were reliably reduced ($P < .05$) after repeated restraint in both males and females (Figure 2).

One-way comparison of gonadal status in naïve, singly and repeatedly exposed animals revealed no significant effects of restraint on plasma testosterone in males ($P > .1$) and plasma estradiol in females ($P > .5$).

8-OH-DPAT–Induced Hypothermia and Corticosterone Responses

To address the repetitive nature of the stressor, subsequent analysis of 8-OH-DPAT–induced hypothermal and corticosterone responses was expanded to include comparisons made between groups of stress-naïve animals and those exposed to either single or repeat restraint. Three-way analysis of core body temperature revealed significant effects of sex ($F_{(1,96)} = 59.7$; $P < .0001$), stress ($F_{(2,96)} = 7.4$; $P = .001$), and treatment ($F_{(1,96)} = 188.7$; $P < .0001$) and a significant sex × stress × treatment interaction ($F_{(2,96)} = 4.0$;

$P = .021$). Post-hoc analysis confirmed higher core temperatures in females regardless of treatment ($P < .01$). Relative to naïve controls, post-hoc analysis confirmed greater 8-OH-DPAT–induced hypothermal responses in the repeat condition in males but not in females (Figure 3A). Additional assessment of baseline temperatures revealed a significant sex × stress × day interaction ($F_{(4,416)} = 15.5$; $P < .0001$) in which only males showed small but reliable decreases in pre-stress temperature levels over the course of repeated restraint (Figure 3B). Even after taking the average of these pre-stress, baseline measures into account, males in the repeat condition continued to show greater responses to 8-OH-DPAT relative to their naïve and singly stressed counterparts (Figure 3C).

For corticosterone, 3-way analysis revealed main effects of stress ($F_{(2,96)} = 38.8$; $P < .0001$), sex ($F_{(1,96)} = 68.2$; $P < .0001$), and treatment ($F_{(1,96)} = 143.4$; $P < .0001$) and a significant sex × stress × treatment interaction ($F_{(2,96)} = 11.9$; $P < .0001$). Post-hoc analysis confirmed higher plasma corticosterone concentrations in females overall ($P < .01$). Relative to naïve controls, post-hoc analysis confirmed greater corticosterone responses after single and repeated restraint exposure in both males and females (Figure 3D).

Repeated Restraint and [³H] 8-OH-DPAT Binding

Consistent with previous studies (Pazos and Palacios, 1985; Chalmers and Watson, 1991), [³H] 8-OH-DPAT binding density was highest in the hippocampus and lateral septum (see Figure 4), relatively lower in anterior cingulate and medial prefrontal cortex, with the lowest densities encountered in the amygdala and posterior hypothalamus, including the lateral hypothalamus and dorsomedial/ventromedial nuclei. Specific binding accounted for almost all the total binding, as nonspecific binding of [³H] 8-OH-DPAT defined in the presence of either 5-HT or the 5-HT 1A antagonist WAY100635 was practically indistinguishable from film background.

As with previous surveys (Pompeiano et al., 1992), partitioning of binding in the hippocampus revealed the highest density of 5-HT 1A receptors in the molecular layer of the dentate gyrus as well as within the strata oriens and radiatum of Ammon's horn, particularly in the CA1, and in the CA3 field, biased towards the part of the CA3 entering the limbs of the dentate gyrus (Amaral et al., 2007). For analyzing these subfields, we did not distinguish the pyramidal cell layer, given the low number of binding sites in this region. Based on the relative lack of binding in the CA2, this hippocampal subfield was not analyzed.

Similar to previous in situ hybridization histochemical surveys (Chalmers and Watson, 1991; Pompeiano et al., 1992; Wright et al., 1995), [³H] 8-OH-DPAT binding was not detected in the PVH but localized to a discrete bundle of cells occupying the medial part of the zona incerta (Figure 4). Between raphe nuclei of interest, by far the highest density of 5-HT 1A receptors was found in the dorsal raphe nucleus (Figure 7). Relatively little to no [³H] 8-OH-DPAT binding was evident within the median raphe and raphe magnus, respectively, and these nuclei were excluded from analyses.

Based on 2-way analyses, there were no main effects of sex and stress and no significant interactions between sex and stress for the PFC, septum, zona incerta, and amygdala (Figure 4; all $P > .05$) in addition to the remainder of the diencephalon, including the lateral hypothalamic area and adjoining dorsomedial and ventromedial nuclei. The only forebrain region showing reliable alterations in 5-HT 1A binding occurred with the hippocampus, specifically in the CA3. For this subfield, there was a main effect
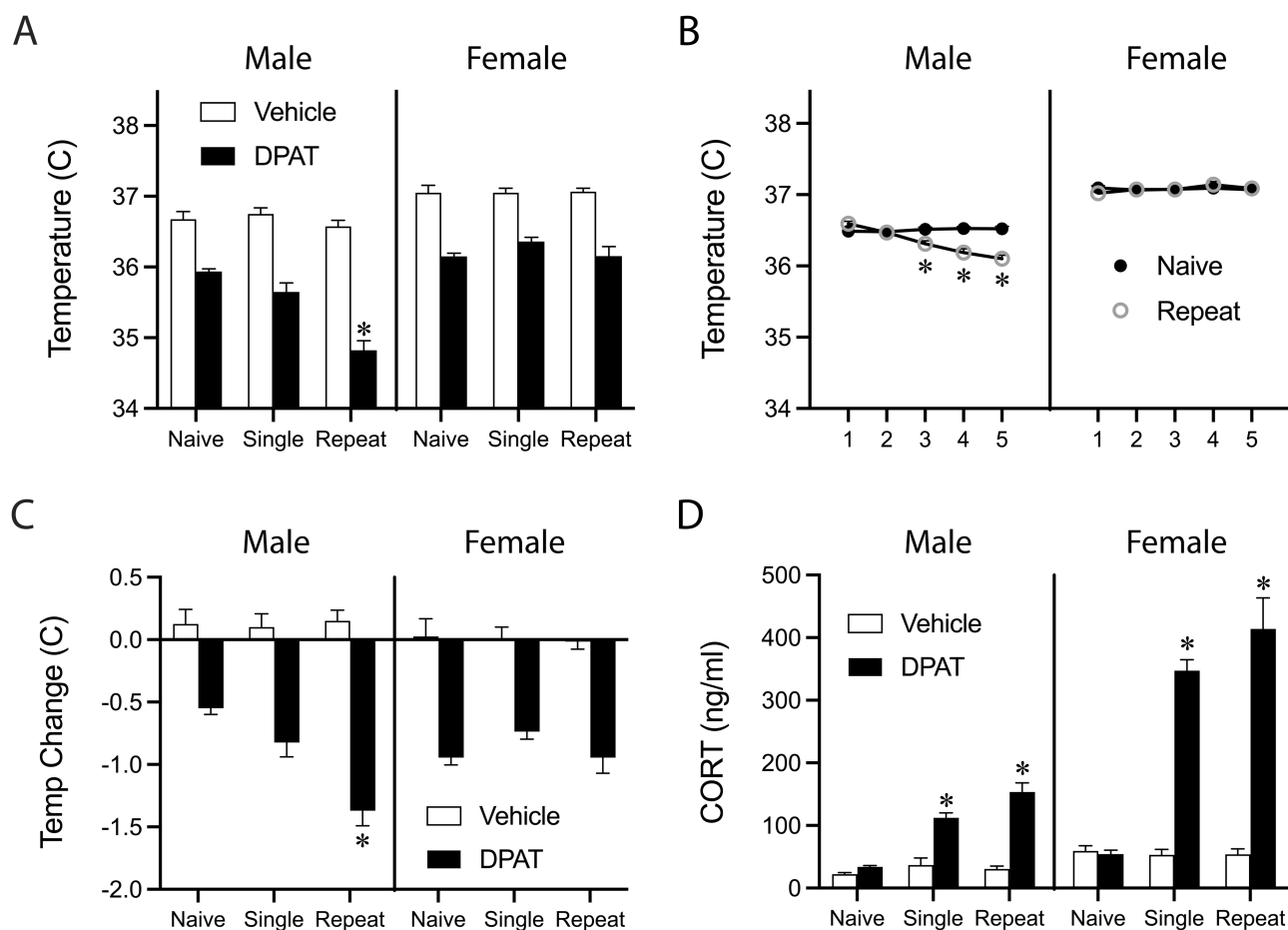
Figure 3. Sex-specific effects of repeated restraint exposure on 8-OH-DPAT–mediated hypothermia and corticosterone responses. (A) Mean±SEM absolute core (rectal) temperature levels following vehicle or 8-OH-DPAT administration in stress-naïve male and female rats and 24 hours after their only (single) and final (repeat) restraint exposure. *$P < .05$ vs naïve and singly exposed counterparts; $n = 6$ (vehicle) and 12–16 (8-OH-DPAT) per sex/stress condition. (B) Mean±SEM baseline and pre-stress core temperatures in naïve and repeatedly restrained animals, respectively; *$P < .05$ vs day 1; $n = 44$ and 17 (male); $n = 32$ and 15 (female) per stress naïve and repeated restraint conditions, respectively. Note that the naïve group, in this case, includes basal and pre-stress temperatures recorded in control animals and those yet to be exposed to acute restraint, respectively. (C) Mean±SEM hypothermal responses corrected for changes in pre-stress, baseline temperatures; *$P < .05$ vs naïve and singly exposed counterparts; $n = 6$ (vehicle) and 12–16 (8-OH-DPAT) per sex/stress condition. (D) Mean±SEM plasma corticosterone concentrations following vehicle or 8-OH-DPAT administration in stress naïve male and female rats and 24 hours after their only (single) and final (repeat) restraint exposure. *$P < .05$ vs naïve counterpart; $n = 6$ (vehicle) and 12–16 (8-OH-DPAT) per sex/stress condition.

of stress ($F_{(2,30)} = 5.47$; $P = .009$) attributed to overall higher 5-HT 1A binding levels in both single and repeat restraint conditions (Figure 4), no main effect of sex ($F_{(1,30)} = 0.03$; $P = .85$), and no significant interaction between sex and stress ($F_{(2,30)} = 0.73$; $P = .49$). Finally, for the dentate gyrus and subfield CA1, there were no main effects of sex and stress and no reliable interactions between sex and stress (all $P > .05$). For the dorsal raphe, there was a main effect of stress ($F_{(2,30)} = 3.50$; $P = .043$) attributed to overall higher 5-HT 1A binding levels in the repeat restraint condition (Figure 7), no main effect of sex ($F_{(1,30)} = 2.28$; $P = .141$), and the interaction between sex and stress tended to be significant ($F_{(2,30)} = 3.14$; $P = .058$).

Repeated Restraint and [35S] GTPγS Binding

In addition to, or independent of changes in binding capacity, alterations in serotonin signaling can occur as a consequence of changes in the efficacy of 5-HT 1A receptors to activate G proteins. Thus, we measured 8-OH-DPAT–stimulated [35S] GTPγS binding on tissue sections adjacent to those used to describe [3H] 8-OH-DPAT binding. Most of the brain regions surveyed showed reliable increments in [35S] GTPγS binding induced by

8-OH-DPAT, ranging on average from approximately 50% to 150% of basal levels, depending on the region of interest and [35S] GTPγS binding in the absence of 8-OH-DPAT. Stimulation of [35S] GTPγS binding was completely abolished in the presence of the selective 5-HT 1A receptor antagonist WAY100 635 (1 μM). Two-way analysis, in all cases, failed to show reliable effects of stress and sex on basal [35S] GTPγS binding (data not shown).

Like the findings discussed above for the 5-HT 1A receptor, there were no main effects of sex and stress and no significant interactions between sex and stress for the PFC, septum, amygdala, and posterior hypothalamus (all $P > .05$). Likewise for the hippocampus, no significant alterations in 8-OH-DPAT–stimulated [35S] GTPγS binding were revealed for the dentate gyrus and CA1 region. However, 2-way analyses revealed significant interactions for the CA3 (Figure 5). For this hippocampal subfield, there was a main effect of stress ($F_{(2,30)} = 10.20$; $P = .004$), no main effect of sex ($F_{(1,30)} = 1.86$; $P = .19$), and a significant interaction between sex and stress ($F_{(2,30)} = 7.03$; $P = .003$). Relative to stress-naïve animals, post-hoc analysis confirmed reliable effects of both acute and repeated restraint to increase [35S] GTPγS binding stimulated by 8-OH-DPAT only in females (Figure 5).
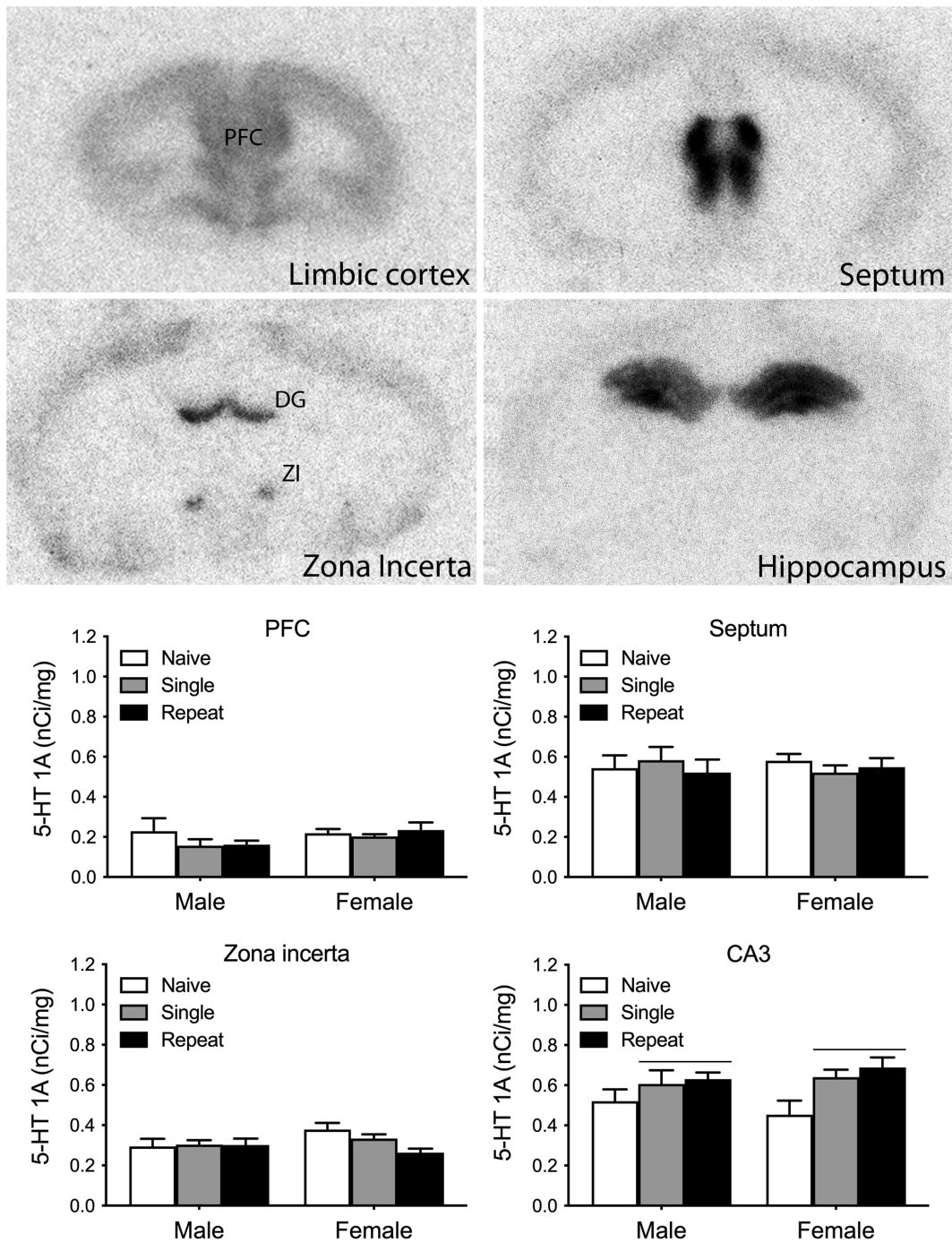
Figure 4. Distribution and impact of restraint stress on 5-HT 1A receptor binding sites in the forebrain. Top panels: representative autoradiographs of [³H]8-OH-DPAT binding in limbic cortex, lateral septum, hippocampus, and in the zona incerta. Abbreviations: DG, dentate gyrus; PFC, prefrontal cortex; ZI, zona incerta. Bottom panels: mean ± SEM 5-HT 1A receptor binding in stress naïve male and female rats, and 24 hours after their only (single) and final (repeat) restraint exposure. Horizontal bars signify main effects of single and repeated restraint to increase receptor binding; *$P < .05$ vs naïve; n = 6/group.

Whereas the pattern of [³⁵S] GTPγS binding in the hippocampus followed the distribution of the 5-HT 1A receptor, [³⁵S] GTPγS binding in the zona incerta was not as circumscribed as that observed for [³H] 8-OH-DPAT binding (see Figures 4 and 6). Nonetheless, GTPγS binding stimulated by 8-OH-DPAT was reliably attenuated ($P < .01$) in this region after repeated restraint but only in females (Figure 6). Note that immediately ventral to the zona incerta, including within the area occupied by the

dorsomedial and ventromedial hypothalamic nuclei (Figure 6), there were no main effects of stress and sex and no interaction between sex and stress (all $P > .05$).

Moderate to high levels of basal [³⁵S] GTPγS binding were detected in the brainstem, including within the periaqueductal gray and dorsal raphe (Figure 7), in addition to the median raphe and raphe magnus. Nonetheless, the raphe nuclei responded to show, on average, comparable degrees of 8-OH-DPAT–stimulated
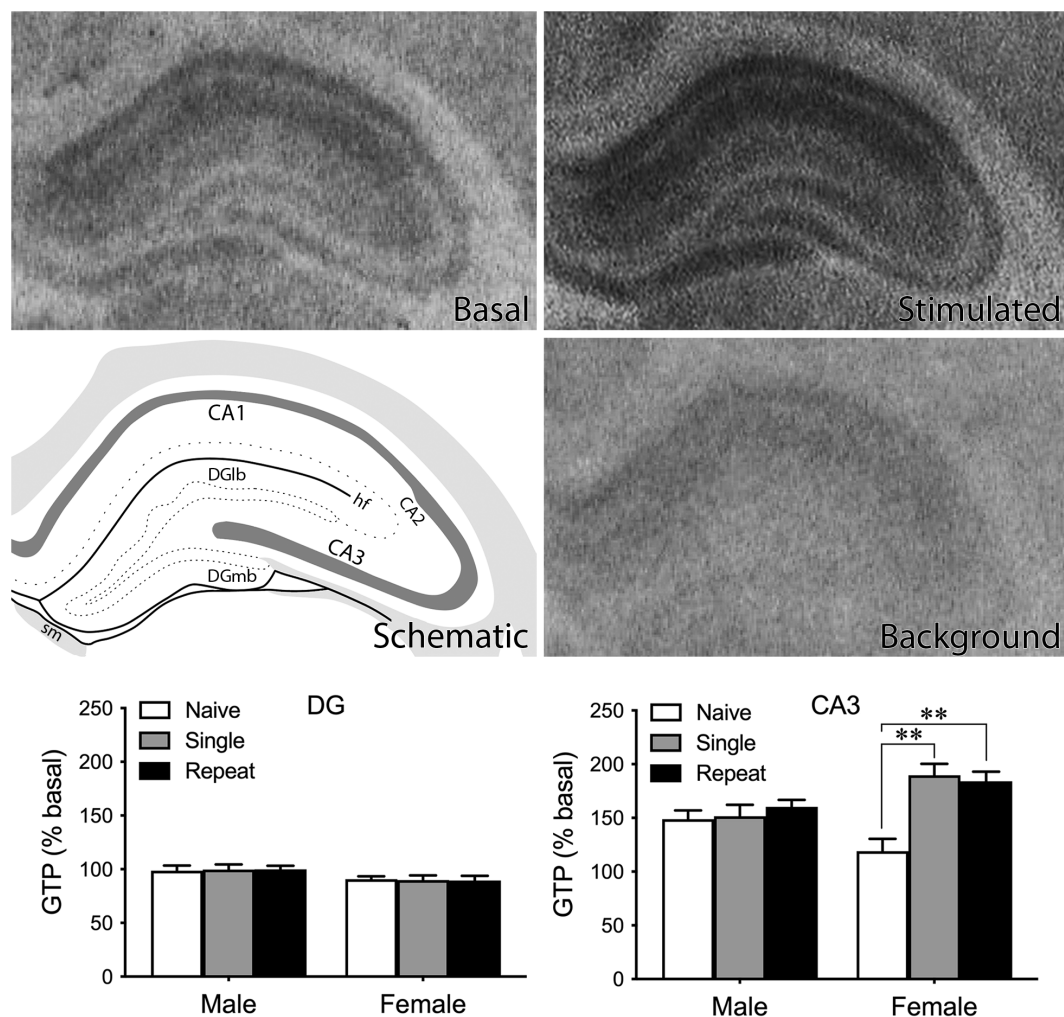
Figure 5. 5-HT 1A-mediated, G-protein coupling responses in the hippocampus. Top panels: representative autoradiographs of basal, 8-OH-DPAT stimulated, and background [^{35}S] GTPγS binding in the dorsal hippocampus. Structures (schematic) labeled for reference. CA1-CA3, subfields, Ammon's horn; DGlb and DGmb, dentate gyrus and lateral and medial blades; hf, hippocampal fissure; sm, stria medullaris. Bottom panels: mean ± SEM 5-HT 1A receptor-stimulated [^{35}S] GTPγS binding in stress-naïve male and female rats and 24 hours after their only (single) and final (repeat) restraint exposure. **$P < .01$ vs naïve; n = 6/group.

[^{35}S] GTPγS binding: 73.6 ± 12.2, 75.3 ± 2.5, and 80.8 ± 2.4 (percentage of basal), respectively. Two-way analyses revealed, however, significant interactions only for the dorsal raphe. For this nucleus, there were main effects of stress ($F_{(2,30)} = 18.52$; $P < .0001$) and sex ($F_{(1,20)} = 33.86$; $P < .0001$) and a significant interaction between sex stress ($F_{(2,30)} = 7.02$; $P = .0031$). Relative to stress-naïve and singly exposed animals, post-hoc analysis confirmed a reliable effect of repeated restraint to increase [^{35}S] GTPγS binding stimulated by 8-OH-DPAT in the dorsal raphe only in males (Figure 7).

Because changes in GTPγS responses could be accounted for by alterations in 5-HT 1A receptor availability, we analyzed the correlation between GTPγS stimulated binding and 5-HT 1A receptor binding in the hippocampus and zona incerta of females and in the dorsal raphe of males. There was no significant correlation between these variables for the zona incerta ($r^2 = 0.19$; $P = .07$). There were, however, positive correlations for the CA3 region of the hippocampus ($r^2 = 0.48$; $P = .0014$) and dorsal raphe ($r^2 = 0.52$; $P = .0007$), suggesting that the stress-induced increases in G-protein coupling responses in these regions were matched by increments in 5-HT 1A receptor density (Figure 8). Thus, alterations in 5-HT 1A receptor-stimulated [^{35}S] GTPγS binding in the dorsal raphe and hippocampus of males and females, respectively, may be explained, at least in part, by changes in receptor availability.

Discussion

Both male and female rats showed reliable declines in the magnitude of the corticosteroid response between the first and last day of restraint exposure, affording an opportunity for understanding how the 5-HT 1A receptor may come to associate with this adaptive process. While several studies have made important inroads on the glucocorticoid basis for maladaptive changes in 5-HT 1A receptor function during chronic stress, it remains surprising that such interactions are not as equally considered alongside stress-HPA axis habituation, given the vital role of this process in stress coping.

Our previous studies suggested sex differences in HPA axis output responses to 5-HT 1A receptor antagonism, at least under acute stress conditions (Goel et al., 2014a), as well as for sex-specific effects of repeated restraint to alter the expression of 5-HT 1A mRNA in the dorsal raphe, zona incerta, and
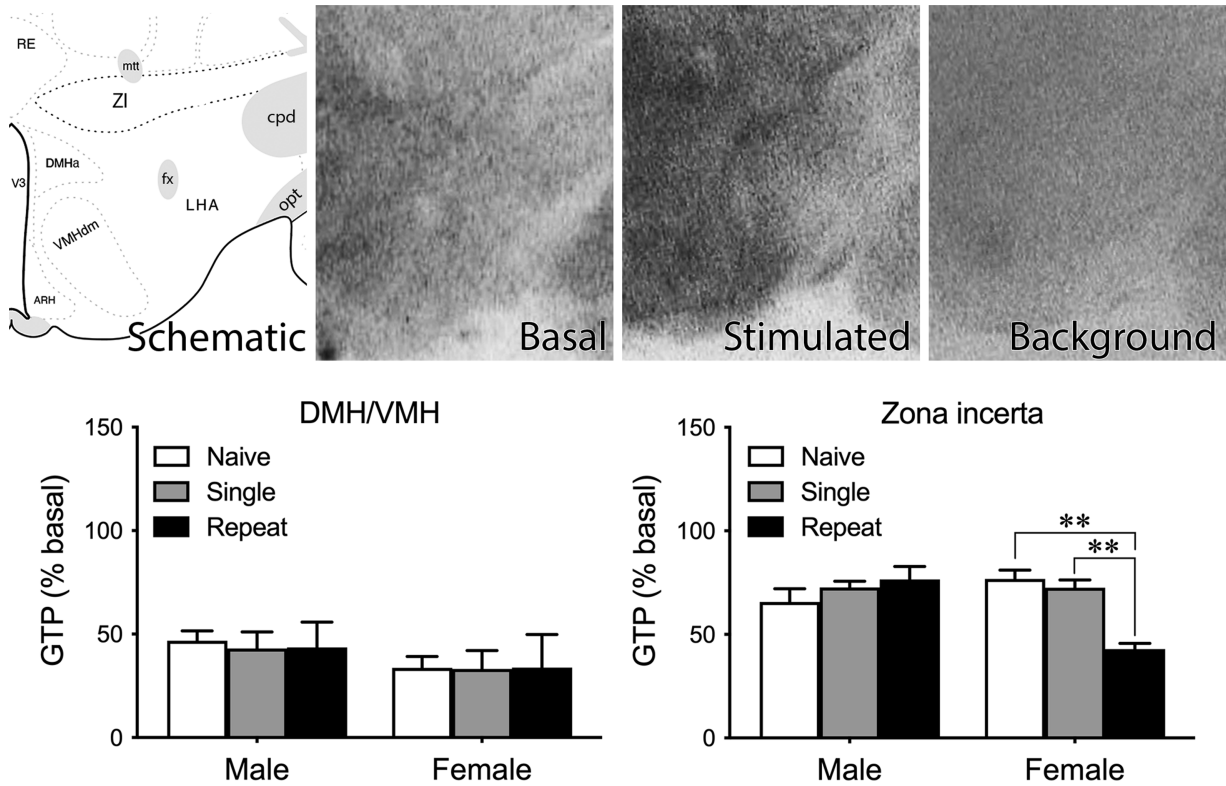
Figure 6. 5-HT 1A–mediated, G-protein coupling responses in the zona incerta. Top panels: representative autoradiographs of basal, 8-OH-DPAT stimulated, and background [^{35}S] GTPγS binding in the zona incerta and adjoining posterior hypothalamus, including the dorsomedial and ventromedial nuclei. Structures (schematic) labeled for reference. ARH, arcuate nucleus hypothalamus; cpd, cerebral peduncle; DMH, dorsomedial nucleus hypothalamus; fx, columns of the fornix; LHA, lateral hypothalamus area; mtt, mammillothalamic tract; opt, optic tract; RE, rhomboid nucleus; VMH, ventromedial nucleus hypothalamus; Z1, zona incerta. Bottom panels: mean±SEM 5-HT 1A receptor-stimulated [^{35}S] GTPγS binding in stress-naïve male and female rats and 24 hours after their only (single) and final (repeat) restraint exposure. ** $P < .01$ vs naïve; n=6/group.
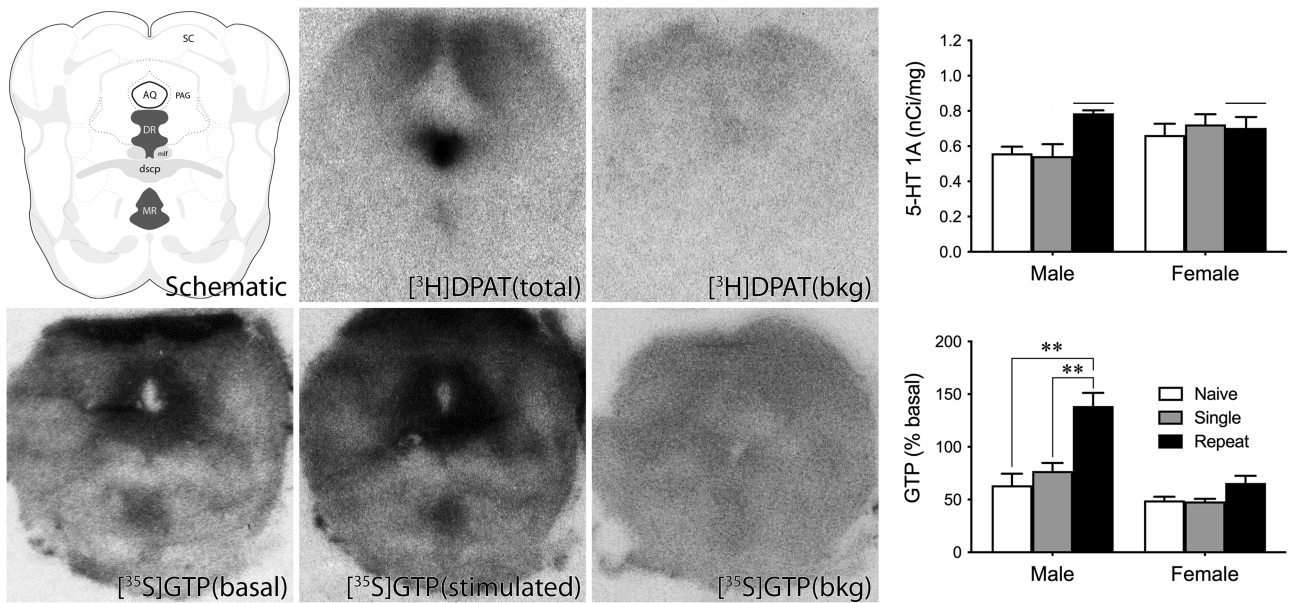

Figure 7. 5-HT 1A receptor binding and G-protein coupling responses in the dorsal raphe. Top panels: representative autoradiographs of total and background [^{3}H]8-OH-DPAT binding. Structures (schematic) labeled for reference: AQ, cerebral aqueduct; DR, dorsal nucleus raphe; dscp, decussation of the superior cerebellar peduncle; mlf, medial longitudinal fascicle, MR, median nucleus raphe; PAG, periaqueductal gray; SC, superior colliculus. Mean±SEM 5-HT 1A receptor binding in stress naïve male and female rats, and 24 hours after their only (single) and final (repeat) restraint exposure. Horizontal bars signify main effect of repeated restraint to increase receptor binding; *$P < .05$ vs naïve; n=6/group. Bottom panels: representative autoradiographs of basal, 8-OH-DPAT–stimulated, and background [^{35}S] GTPγS binding. Mean±SEM 5-HT 1A receptor-stimulated [^{35}S] GTPγS binding in stress-naïve male and female rats and 24 hours after their only (single) and final (repeat) restraint exposure. **$P < .01$ vs naïve; n=6/group.
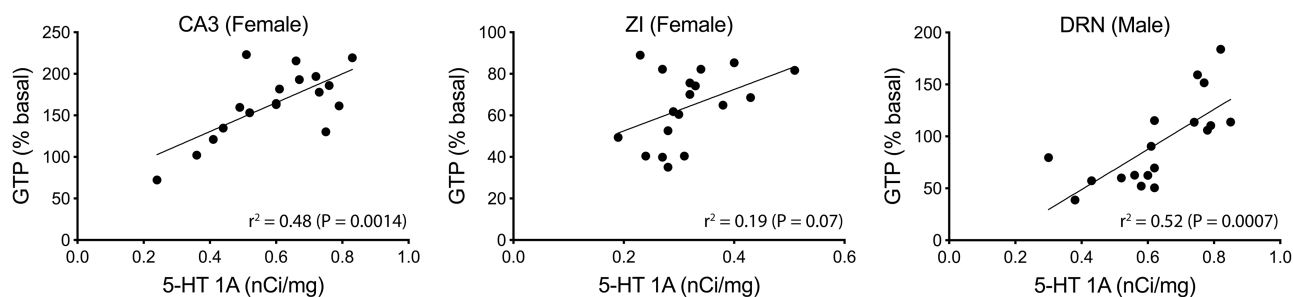
Figure 8. Correlational analysis of 5-HT 1A receptor and 5-HT 1A receptor-stimulated GTPγS binding in the CA3 region of the hippocampus and zona incerta (ZI) in females, and dorsal raphe (DRN) in males (n = 18/region).

hippocampus (Goel et al., 2022). Based on these findings to suggest that stress habituation involves both pre- and postsynaptic processes, here we tested animals leaving repeated restraint by measuring (1) hypothermic and corticosteroid responses to a submaximal dose of the 5-HT 1A agonist 8-OH-DPAT taken as indices of pre- and postsynaptic 5-HT 1A receptor function, respectively, and from a random subset of these animals; and (2) the density and G-protein coupling of the 5-HT 1A receptor in brain slices.

As hypothesized, males, but not females, emerged from repeated restraint to showed potentiated hypothermal responses to 8-OH-DPAT, recapitulated by increases in dorsal raphe 5-HT 1A receptor binding and G protein responses. Importantly, no alterations in 5-HT 1A receptor responses occurred in singly restrained animals, suggesting that the changes observed in the repeat condition were caused by the repetitive nature of the stressor and not its physical attributes per se. Previous dose-response studies in the rat indicate that the sexual dimorphism in 8-OH-DPAT–induced hypothermia, higher in females, is least apparent using quantities ≤0.1 mg/kg and that responses in females do not vary over the estrous cycle (Haleem et al., 1989; Uphouse et al., 1991). Thus, the apparent lack of any stress-related shift in hypothermic responses detected in females in the present study cannot be explained by a ceiling effect or compromised by underlying variations in ovarian hormone status. Large, single doses of 8-OH-DPAT can desensitize dorsal raphe but not hippocampal 5-HT 1A receptor responses (Riad et al., 2001; Hensler, 2003). However, this effect in the raphe is rather short-lived (<24 hours) and opposite to what occurred in repeatedly exposed males, making it unlikely that the preceding 8-OH-DPAT challenge was a cause for the changes in 5-HT 1A receptor regulation observed.

Whether 5-HT 1A receptors in the dorsal raphe are directly responsible for the differential thermal responses to 8-OH-DPAT remains to be seen, as several studies favor a postsynaptic site of action for this ligand in the rat (Martin and Heal, 1991; Blier et al., 2002). Nonetheless, hypothermia in the rat can be triggered by intra-raphe injections of either 5-HT or 8-OH-DPAT (Hillegaart, 1991). Moreover, functional studies suggest that the dorsal raphe is an obligatory relay for 5-HT 1A–sensitive, hypothalamic effectors of thermogenesis, including within the preoptic area (Werner and Bienek, 1985). Thus, the changes in hypothermal responses observed in the current study may be attributed, at least in part, to an upregulation in 5-HT 1A receptor function in the dorsal raphe.

Interestingly, in the repeat condition males showed small but reliable decreases in absolute, basal (pre-stress) temperature levels on each successive day of restraint exposure, as noted by others (Chen and Herbert, 1995). Restraint-induced elevations

in body temperature, like many stressors, are thought to reflect an increase in sympathetic tone, mediated by a complex of immune, peptide, and neurotransmitter systems in the brain, including serotonin (Myers, 1981; Vinkers et al., 2008). Given the roles proposed for the 5-HT 1A receptor in thermoregulatory cooling and defense against hyperthermia (Hale et al., 2011), this hypothermic response in males, evidently anticipatory, would make sense teleologically and, as argued elsewhere (Dallman, 2007; Herman, 2013), would assist in reducing the metabolic cost of the stressor. This anticipatory response could form the basis for habituated corticosterone and hyperthermic responses to repeated restraint, at least in males (Barnum et al., 2007), although it remains to be seen if females are equally capable of showing such adaptive changes in thermal responses.

As we predicted, both singly and repeatedly exposed males and females showed increased corticosterone responses to 8-OH-DPAT. Although the substrate mediators for this effect remain to be determined, the stimulatory effect of systemic 8-OH-DPAT on the HPA axis is generally accepted as reflecting a central, postsynaptic site of action (Blier and Ward, 2003). Moreover, because increments in plasma corticosterone concentrations typically follow the intra-hypothalamic administration of the ligand (Lanfumey et al., 2008), interactions between stress and 8-OH-DPAT are presumed to signify a change in 5-HT 1A receptor function at the level of the PVH. However, based on previous connectivity and hybridization-histochemical studies, including our own, 5-HT input to the PVH is rather scarce compared with the surrounding neuropil (Williamson and Viau, 2007), and the nucleus is practically devoid of the 5-HT 1A transcript. The most proximal candidate mediator in this regard is represented by a discrete bundle of 5-HT 1A mRNA-expressing cells within the zona incerta (Goel et al., 2014a) and consistent with the pattern of [³H]8-OH-DPAT binding identified here, located along the dorsal perinuclear zone of the PVH.

To the extent that single and repeated restraint enhanced 5-HT 1A receptor-mediated adrenocortical activation in males and females, we would have expected, therefore, a positive shift in receptor density and/or function in the zona incerta in both sexes. By contrast, only females responded to show a reduction in 5-HT 1A G-protein coupling responses in this region, restricted to the repeat condition. While this would argue against a role for the zona incerta in this context, a reduction in 5-HT signaling in this region may nonetheless provide a mechanism for regulating stress habituation in females.

Consistent with 1 previous report, increases in the density and G-protein coupling of the 5-HT 1A receptor occurred within the CA3 region of the hippocampus in females but not in males after repeated restraint (Mendelson and McEwen, 1991). As

argued elsewhere, stress habituation heavily depends on the context, predictability, and ability for recognizing whether a stressor is familiar (Grissom et al., 2007), and there is ample evidence to suggest that 5-HT 1A receptors in the hippocampus play a role in these dimensions (Stiedl et al., 2015). Transient elevations in corticosterone secretion and occupancy of the glucocorticoid receptor during repeated restraint could account for the increased expression and/or responsivity of the 5-HT 1A receptor in the hippocampus (Meijer and de Kloet, 1998). However, as with the dorsal raphe and zona incerta, the sex-specific nature of this response continues to predict an involvement of the gonadal steroids in organizing these receptor responses (Goel et al., 2014b, 2022) and perhaps other stress-related factors (Mo et al., 2008). Thus, although we found no differences in plasma testosterone and estradiol concentrations with repeated restraint, this does not preclude possible interactions between the gonadal steroids and putative modulators of 5-HT 1A receptor coupling efficacy (Bethea et al., 2002; Mize et al., 2003; Le Saux and Di Paolo, 2005).

Beyond the hippocampus and zona incerta, we found no interactions in the remainder of the forebrain to explain where and how animals show sensitized adrenocortical responses to 8-OH-DPAT. Previous studies examining antidepressant actions, as well as other agents, frequently observe variations in 5-HT 1A receptor G-protein coupling and/or electrophysiological properties independent of changes in receptor number/availability, which could be explained by alterations in receptor affinity and reserve, different types of G proteins, as well as by changes in downstream ion channel and second messenger effector systems (Hensler, 2003; Albert, 2012; Albert and Vahid-Ansari, 2019). Nevertheless, although the relevant substrate mediating the positive shift in adrenocortical responses to 8-OH-DPAT remains to be determined, it is reasonable to propose that an increase in the activity of a population of postsynaptic 5-HT 1A receptors underlies facilitation of the HPA axis to novel stress, as seen in various repeated stress paradigms (Bhatnagar and Dallman, 1998; Radley and Sawchenko, 2015).

At this point of our studies, we have only begun to understand the nature by which alterations in 5-HT 1A receptor expression and/or coupling responses associate with repeated restraint exposure. Although both males and females can express stress HPA axis habituation, the results imply that they do so by employing different serotonin-based substrates. Whereas the changes observed in 5-HT 1A receptor responses in females appear restricted to the hippocampus and zona incerta, changes in receptor responses in the dorsal raphe in males may have broader implications. The dorsal raphe nucleus projects to a wide array of limbic, cortico-limbic, thalamic, and hypothalamic sites (Lowry, 2002; Greenwood et al., 2005). Moreover, our previous studies suggest that stress habituation is associated with an increase in 5-HT 1A receptor mRNA expression through the rostrocaudal extent of the dorsal raphe in males (Goel et al., 2022). Thus, if the increase in 5-HT 1A receptor coupling within the dorsal raphe is no less pervasive, this would have a global influence to decrease serotonin availability at a multitude of postsynaptic sites, including those mediating adaptive behavioral, emotional, and neuroendocrine responses. In this regard, we likely underestimate the impact of repeated restraint in males, although we have yet to scrutinize other mechanisms and effectors of serotonin transmission in the context of stress habituation.

In summary, we have shown that repeated restraint differentially affects the G-protein coupling responses of the 5-HT 1A receptor, suggesting that the serotonergic underpinnings of stress habituation are remarkably different between males and females. With respect to HPA output responses, the present results are consistent with our previous findings (Goel et al., 2022) and those of others (Doremus-Fitzwater et al., 2009; Babb et al., 2014) showing similar capacities for stress HPA axis habituation in males and females, whereas other reports suggest that females are less if at all capable of showing dampened HPA axis responsiveness to repeated restraint (Bhatnagar et al., 2005; Grafe et al., 2017). Differences in the frequency and duration of stress exposure could account for this discrepancy, in addition to housing conditions, as the latter studies employed individually housed animals. Interestingly, social isolation has been shown to differentially alter 5-HT 1A receptor binding in a sex-dependent manner in addition to other receptors implicated in stress adaptation, including vasopressin V1A and oxytocin receptors (Liberzon and Young, 1997; Gray et al., 2014; Ross et al., 2019). Thus, in the context of stress habituation, where and how sex, social isolation, and repeated restraint interact on the 5-HT 1A receptor remains worthy of pursuit. Nonetheless, among the factors identified as contributing to impaired habituation to repeated restraint in females (Grafe et al., 2017), the hypothalamic orexins are also subject to serotonergic control through the 5-HT 1A receptor (Saito et al., 2018), further reinforcing a role for serotonin where sex differences in stress HPA axis habituation occur. Based on the utility of assessing hypothermic and corticosteroid responses to 8-OH-DPAT as determinants of stress habituation, future studies of varied habituation paradigms could take advantage of these tests to examine the potential role of the 5-HT system.

Although there is little dispute that serotonin is involved in stress, one of the more difficult problems in studies of this sort lies in the extent to which any organismal response to stress can be viewed as adaptive or maladaptive (Stanford, 1996; Herman, 2013). That we observed changes in the 5-HT 1A receptor against a backdrop of declining HPA axis responsiveness and glucocorticoid exposure provides some assurance that these responses are adaptive. To this end, note that the positive effects of repeated restraint on the 5-HT 1A receptor in the raphe and hippocampus observed here are opposite of what is commonly reported for chronic stress models. Thus, challenges promoting sustained, nonhabituating elevations in glucocorticoid secretion, including chronic social stress, chronic unpredictable stress, and repeated immobilization, have been shown to blunt adrenocortical responses to 8-OH-DPAT and/or decrease 5-HT 1A receptors at pre- and postsynaptic sites (Korte et al., 1995; Meijer and de Kloet, 1998; Flügge et al., 2004; Grippo et al., 2005; Bambico et al., 2009).

The 5-HT system is definitely involved in initiating HPA responses to acute stress. Whether this stimulatory component habituates alongside the HPA axis remains to be seen, although there are some indications that relative novelty of the stressor and the arousal it induces are important determinants of 5-HT release (Rueter and Jacobs, 1996; Rueter et al., 1997). Thus, in the repeat condition we anticipate a reduction in 5-HT release, at least in males, given the global influence of 5-HT 1A raphe autoreceptors in regulating serotonergic tone. Although this underscores a potential mechanism for adapting to the same or similar stressor, the extent to which males and females remain affixed to their respective changes in 5-HT 1A receptors remains an important question, given the growing interest in physical activity, behavioral therapy, and stress inoculation as treatments for resolving stress vulnerability and enhancing resilience (Parker et al., 2004; Greenwood et al., 2005; Fucich et al., 2018; Lee et al., 2021).

Acknowledgments

Interest Statement

The authors declare no conflict of interest.

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