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Research article

Local inhibition of uptake$_2$ transporters augments stress-induced increases in serotonin in the rat central amygdala

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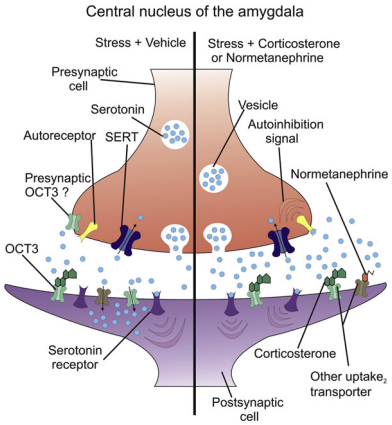
GRAPHICAL ABSTRACT



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ABSTRACT

Organic cation transporter 3 (OCT3) is a corticosterone-sensitive, low-affinity, high-capacity transporter. This transporter functions, in part, to clear monoamines, including serotonin (5-HT), from the extracellular space.

Abbreviations: 5-HT, 5-Hydroxytryptamine serotonin; aCSF, Artificial cerebrospinal fluid; CeA, Central nucleus of the amygdala; CORT, Corticosterone; GR, Glucocorticoid receptor; HPA, Hypothalamic-pituitary-adrenal; OCT3, Organic cation transporter 3 also referred to as Slc22a3 (Solute Carrier Family 22 (Organic Cation Transporter), member 3, and extraneuronal monoamine transporter (EMT); NM, Normetanephrine; PMAT, Plasma membrane monoamine transporter

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Normetanephrine
OCT3
Serotonin
uptake2

The central nucleus of the amygdala (CeA) is an important structure controlling fear- and anxiety-related behaviors. The CeA has reciprocal connections with brainstem nuclei containing monoaminergic systems, including serotonergic systems arising from the dorsal raphe nucleus, which are thought to play an important role in modulation of CeA-mediated behavioral responses. Organic cation transporter 3 (OCT3) is expressed in the CeA, but little is known about the role of OCT3 within the CeA in modulating serotonergic signaling. We hypothesized that inhibition of OCT3-mediated transport in the CeA during restraint stress would increase extracellular 5-HT. In *Experiment 1*, rats received unilateral reverse dialysis of either corticosterone or normetanephrine, which interfere with OCT3-mediated transport, into the CeA under home cage control conditions. In *Experiment 2*, rats received unilateral reverse dialysis of corticosterone, normetanephrine, or vehicle into the CeA, while undergoing a 40-min period of restraint stress. Infusion of these drugs had no effect on extracellular concentrations of 5-HT during home cage control conditions, but, in contrast, markedly increased extracellular concentrations of 5-HT during restraint stress, relative to vehicle-treated controls. These findings suggest a role for OCT3 in the CeA in control of serotonergic signaling during stressful conditions.

1. Introduction

Exposure to acute psychological or physical stressors activates the sympathomedullary system and the hypothalamic-pituitary-adrenal (HPA) axis, culminating in release of catecholamines and corticosterone (CORT), respectively, into the circulation. Corticosterone mediates adaptive responses to stressors, including termination of the HPA axis activity through negative feedback during acute stressors [11]. Activation of the HPA axis involves the paraventricular nucleus of the hypothalamus (PVN), which contains corticotropin-releasing factor-synthesizing neurons that signal release of adrenocorticotropic hormone from the anterior pituitary into systemic circulation, which, in turn, stimulates synthesis and release of CORT from the adrenal cortex [11].

When a real or potential threat is perceived, the activity of the PVN is modulated by multiple extrahypothalamic brain regions, including the central nucleus of the amygdala (CeA) [11]. The CeA projects to and receives projections from stress-related regions of the brain, such as the ventrolateral medulla, bed nucleus of stria terminalis, and dorsal raphe nucleus [16]. Output from the activation of the CeA is believed to contribute to the rapid integration and expression of phasic fear [16] and defensive behavioral responses [16].

In addition to the activation of glucocorticoid receptors (GRs) and mineralocorticoid receptors, CORT also blocks monoamine transport by organic cation transporters (OCTs) [9,17]. This class of transporters consists of high-capacity, low-affinity transporters belonging to the solute carrier subfamily 22 and includes OCT1 (Slc22a1), OCT2 (Slc22a2), and OCT3 (Slc22a3) [5]. Of these, OCT3 is widely distributed in the brain [5,17], including the CeA [5], and may alter behavioral expression through its role in the clearance of extracellular monoamines [1,9,10].

In this study, we hypothesized that the CORT-sensitive OCT3 transporter regulates extracellular 5-HT in the CeA under conditions where the serotonergic system is activated by a stressor, resulting in increased synaptic and extrasynaptic concentrations of 5-HT within the CeA. We measured extracellular concentrations of 5-HT in the CeA of rats subjected to acute restraint stress, while simultaneously delivering either CORT or the OCT inhibitor normetanephrine (NM) [10], an O-methylated metabolite of norepinephrine (NE), into the CeA using *in vivo* microdialysis.

2. Results

2.1. Effect of CORT or NM on extracellular 5-HT in the CeA

Fig. 1 shows representative placements of microdialysis probe membranes, drawn to scale, within the CeA from –2.56 mm to –3.30 mm caudal from bregma [15]. Probes were directed toward the CeA, but the 2.4 mm working length of the probe membrane sampled from the dorsal surround, ventral portions of the amygdaloid complex and, in some cases, medial portions of the basolateral amygdala. In these experiments, the mean baseline 5-HT levels calculated from the mean of three consecutive baseline samples in 5 μL of dialysate from the CeA (uncorrected for probe recovery) were 0.31 ± 0.05 pg, 0.35 ± 0.08 pg and 0.37 ± 0.09 pg in CORT-, NM-, and vehicle-treated animals, respectively. The mean 2:1 signal-to-noise ratio for these experiments was 0.07 ± 0.01 pg. Local perfusion of CORT or NM into the CeA had no effect on extracellular 5-HT within the CeA in unstressed, freely moving rats (Fig. 2). There were no effects of intra-CeA delivery of CORT or NM via reverse dialysis on concentrations of extracellular 5-HT ($F_{(1,78)} = 0.781$, $p = 0.396$), no effects of time on these measures ($F_{(8,78)} = 1.123$, $p = 0.364$), or interaction between drug and time ($F_{(8,78)} = 0.783$, $p = 0.62$) (Fig. 2). In *Experiment 2*, which tested the effets of acute restraint while simultaneously delivering either CORT or NM into the CeA, there were significant effects of

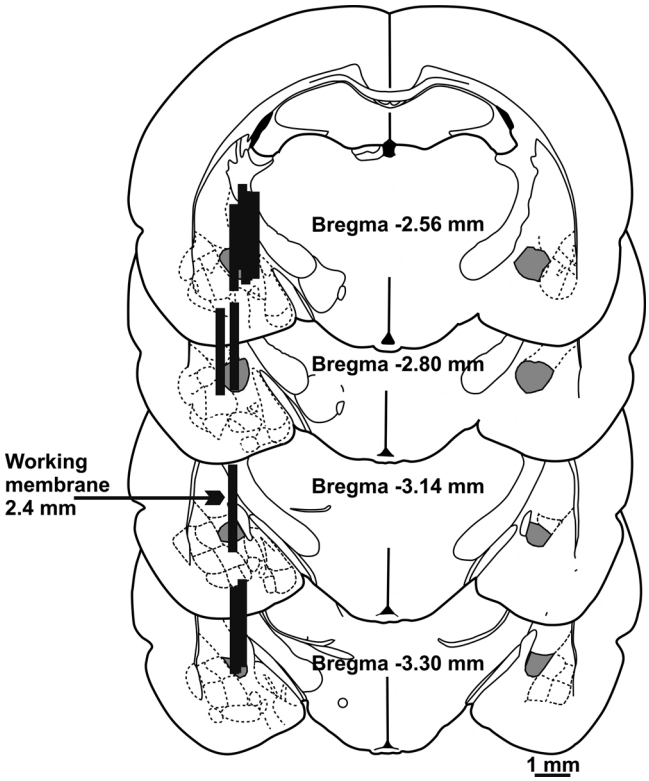

Fig. 1. Diagrammatic illustrations of placements of microdialysis probe membranes, drawn to scale, within the CeA from –2.56 mm to –3.30 mm caudal from bregma [15]. Probes were directed toward the CeA, but the 2.4 mm working length of the probe membrane sampled from the dorsal surround, ventral portions of the amygdaloid complex and, in some cases, medial portions of the basolateral amygdala. The black lines illustrate the portion of the probe that was sampling the extracellular fluid. The CeA is shaded in light gray.

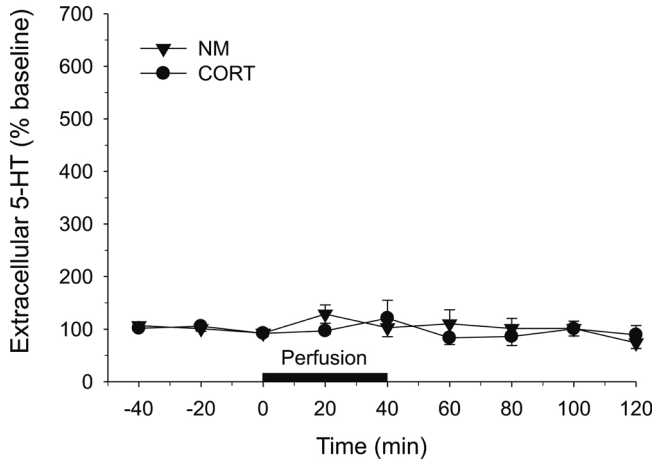

Fig. 2. Perfusion of either corticosterone (CORT) or normetanephrine (NM) into the central nucleus of the amygdala (CeA) did not significantly affect extracellular serotonin (5-HT) within the CeA (CORT, $n = 4$; NM, $n = 5$). The 40-min perfusion into the CeA was initiated at time 0(dark bar). Data are presented as the mean ± standard error of the mean.

treatment with CORT or NM ($F_{(2,18)} = 4.19$, $p < 0.05$), time ($F_{(9,143)} = 8.75$, $p < 0.001$), and a treatment x time interaction ($F_{(9,143)} = 2.57$, $p < 0.001$)(Fig. 3). Restraint stress increased extracellular 5-HT in the CeA by approximately 40% in rats treated with vehicle based on within-subjects comparisons using paired *t*-tests (Bonferroni, $p < 0.05$). This effect was only evident in the first sample collected after the conclusion of the stress period (i.e., the sample collected between the 20-min and 40-min time points). When intra-CeA CORT was superimposed on the stressor, extracellular 5-HT increased approximately 325%, relative to pretreatment values. This effect was present in the first sample collected after the conclusion of the stress period based on within-subjects comparisons using paired *t*-tests (Bonferroni, $p < 0.05$). Reverse dialysis of the inhibitor of OCT3-mediated transport, NM, into the CeA increased extracellular 5-HT relative to the −20-min pre-stress baseline. Extracellular 5-HT in the NM treatment group approached significance ($p = 0.0505$) at the 20-min time point with respect to the -20-min baseline value. In the first sample collected after the conclusion of the stress period, extracellular 5-HT increased approximately 640% and was significantly higher than pre-stress baseline values measured at –20 min *t*-based on within-subjects comparisons using paired *t*-tests (Bonferroni, $p < 0.05$). During the first two post-stress samples (i.e., 40- and 60-min time points), CeA 5-HT was significantly higher in rats treated with NM, when compared to vehicle- and CORT-treated rats as found with independent *t*-tests between-subjects comparisons (Holm-Sidak, $p < 0.05$). Intra-CeA 5-HT was also higher in CORT-treated rats, when compared to controls at the 40-min time point (Holm-Sidak, $p < 0.05$).

3. Discussion

Our results support the hypothesis that the corticosterone-sensitive, low-affinity, high-capacity serotonin transporter, OCT3, plays a role in control of extracellular 5-HT under stress conditions. Local infusions of inhibitors of OCT3-mediated transport, CORT and NM, had no effect on extracellular 5-HT in the CeA in rats exposed to home cage control conditions. However, intra-CeA administration of inhibitors of OCT3-mediated transport potentiated stress-induced increases in extracellular 5-HT in the CeA.

In studies using application of higher concentrations of CORT in mouse hippocampus, clearance rates of pressure-ejected 5-HT did not differ from pre-CORT values, suggesting that CORT does not have significant activity at the high-affinity, low-capacity, sodium-dependent serotonin transporter [1]. Additionally, higher concentrations of NM than were used in the current study were found to be ineffective in inhibiting monoamine uptake in the presence of low concentrations of substrate, but very effective at inhibiting monoamine uptake in the presence of high concentrations of substrate [9]. Together, these studies suggest that neither CORT nor NM have functional effects on the sodium dependent, high-affinity, low-capacity serotonin transporter.

Perfusion of either CORT or NM into the CeA did not affect extracellular levels of 5-HT under baseline conditions. In rats subjected to restraint and perfused with aCSF vehicle, a small (approximately 50%), but significant, increase in extracellular 5-HT was detected. This finding was consistent with earlier studies [12,14]. When CORT or NM perfusion into the CeA was combined with restraint stress, extracellular 5-HT in the CeA rapidly increased (~325% and ~640%, respectively). This response is similar to earlier work in which, among rats treated with a low dose of the OCT blocker decynium 22, but not in vehicle-treated controls, restraint stress increased extracellular 5-HT within the medial hypothalamus [8]. This is an interesting parallel, considering that, like the CeA, the dorsomedial hypothalamus expresses OCT3 [9] and is involved in the regulation of the HPA axis and behavioral responses to stress [11]. Further evidence of a role for OCT3 in control of 5-HT clearance within the hypothalamus is found in studies in which intra-hypothalamic administration of CORT increases and prolongs increases in extracellular 5-HT induced by systemic D-fenfluramine, a 5-HT-releasing drug [5]. Thus, the sensitivity of OCT3 to CORT, the presence of OCT3 in components of central stress circuits [5], and the effects of OCT3 blockade on monoamine clearance [1,8,10] suggest an important role for OCT3 (and/or other uptake$_2$ mechanisms) in modulating stress responses. Data from the present study suggest that OCT3 may contribute specifically to modulating the role of the CeA in stress-related behavioral responses, consistent with previous studies that demonstrate a role of the CeA in stress-related behavioral responses [14].

Restraint stress activates the HPA axis and increases plasma and brain concentrations of CORT, but few studies have quantified how much CORT reaches specific portions of the hypothalamic-limbic stress circuit. Although endogenous CORT concentrations in the CeA are not known, recent work showed that, under stress conditions, extracellular CORT levels in the hippocampus increase from baseline values of 1.8 ng/mL to 2.6 ng/mL following 20 min of restraint stress [4]. Based on the conditions used in that study (20 min collection at a flow rate of 0.7 µL/min), this translates to approximately 36 pg CORT under stress

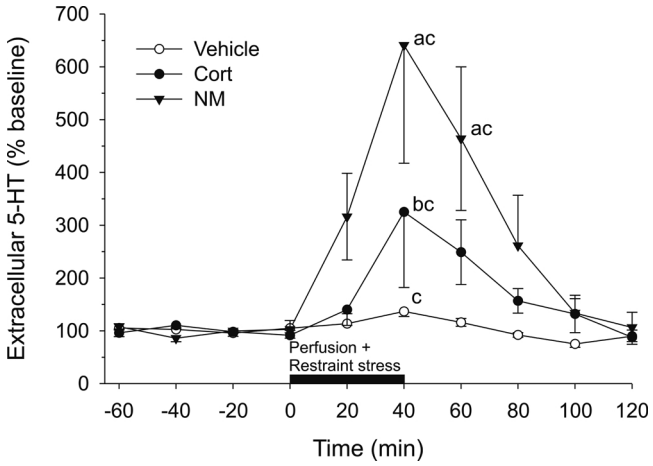

Fig. 3. Perfusion of either corticosterone (CORT) or normetanephrine (NM) into the central nucleus of the amygdala (CeA) superimposed during a 40-min restraint stress resulted in increases in extracellular serotonin (5-HT) within the CeA when compared to vehicle-treated controls ($n = 6$–7/group). The 40-min restraint was initiated at time 0, which coincided with the onset of either CORT, NM, or vehicle perfusion into the CeA (dark bar). Data are presented as the mean ± standard error of the mean. $^{a}p < 0.05$, NM vs vehicle; $^{b}p < 0.05$, CORT vs vehicle; $^{c}p < 0.05$, vs –20 min sample.

conditions. Similarly, CORT concentrations required to saturate OCT3 binding in the CeA *in vivo* are unknown. However, *in vitro* IC_{50} values of 4.9 μM from brain, based on tetraethylammonium uptake [17], and 0.03 μM, based on histamine transport [9] in hypothalamic minces, suggests that it is unlikely that CORT binding to OCT3 was saturated under the conditions used in our experiment. Additionally, there is the possibility that the drugs may have inhibited other high-capacity, low-affinity cation transporters. Both the plasma membrane monoamine transporter (PMAT) and organic cation transporter 2 (OCT2) have been localized in the amygdala [5,6]. High concentrations of CORT (100 μM) do not appear to inhibit PMAT-mediated monoamine transport [7]. The effects of NM on PMAT-mediated transport have not been evaluated. However, it is possible that some of the effects of CORT or NM on CeA 5-HT in restrained animals may have been through interference with OCT2-mediated transport. Corticosterone can effectively block OCT2-mediated transport at the concentration used in the current study [5] and OCT2 has been found within the lateral portion of the CeA [5]. Microdialysis probes used in the current study were directed at the CeA, and the 2.4 mm working length of the probe membrane sampled from the dorsal surround, ventral portions or the amygdaloid complex, and in some cases, medial portions of the basolateral amygdala. Inhibition of OCT2-mediated transport cannot be ruled out from contributing to results from the current study. We were not able to detect endogenous concentrations of NM, the other inhibitor of $uptake_2$-mediated transport used in this study, in the CeA. However, endogenous concentrations of NM within regions of the brain that receive monoamine input have been measured using microdialysis in other studies. For example, in the nucleus accumbens, extracellular concentrations of NM are very low (0.06–0.1 nM; [13]). To effectively inhibit OCT3, the NM concentrations required are unlikely to occur physiologically, even in brain regions with high noradrenergic innervation. Nevertheless, it is a useful tool for blockade of OCT3-mediated transport [10], and it remains possible that endogenous NM may contribute to modulating OCT3-mediated transport.

Our current findings are consistent with those reported in rats that were subjected to restraint stress following amphetamine withdrawal, which showed augmentation of extracellular 5-HT in the CeA, relative to controls [12]. In that study, local administration of the GR antagonist mifepristone into the CeA did not block stress-induced increases in extracellular 5-HT [12]. Thus, it appears unlikely that GR activation is responsible for the rapid stress-induced increases in extracellular 5-HT in the CeA. Our results show that intra-CeA administration of either CORT or NM augmented extracellular CeA 5-HT concentrations during restraint stress. These results suggest that both CORT and NM are increasing extracellular 5-HT through blockade of OCT3, considering 1) the CeA expresses OCT3, 2) extracellular 5-HT increased in a stress-dependent manner, and 3) GR activation has not been shown to increase extracellular 5-HT. To the best of our knowledge, there is no evidence that NM binds to GR.

In conclusion, the potentiation of stress-induced increases in CeA 5-HT in response to local infusion of inhibitors of $uptake_2$-mediated monoamine transport, i.e., CORT or NM, is consistent with a role for OCT3 in modulating 5-HT availability in the CeA during stressful conditions. Further research will be needed to determine if modulation of OCT3-mediated 5-HT clearance in the CeA alters the expression of stress-related behaviors.

4. Experimental procedures

4.1. Animals

Male Sprague Dawley rats (475 ± 16 g), obtained from Animal Resources Facility, University of South Dakota, were housed and maintained two per cage in plastic shoebox cages (46 cm length × 27 cm width × 23 cm height) at 22 °C and 25% humidity at the University of South Dakota animal facility. Lights were set at a 12 L/12D photoperiod, with lights on at 10:00 A.M. Experiments were conducted during the light period of the cycle, beginning at 11:00 A.M. Food (Teklad 2916, Envigo, Madison, WI, USA) and water were available *ad libitum*.

Experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals,* Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, D.C., 2011) and approved by the Institutional Animal Care and Use Committee of the University of South Dakota.

4.2. Surgical procedures

The stereotaxic surgeries for rats used in the initial microdialysis experiments in *Experiment 1* and *Experiment 2* were performed aseptically under xylazine-ketamine anesthesia (80 mg ketamine/10 mg xylazine, i.p.; Ketaset®, ketamine hydrochloride, Fort Dodge Labs Inc., Fort Dodge, IA, USA; xylazine, Vedco, Inc., St. Joseph, MO, USA). At the request of the IACUC of the University of South Dakota, subsequent stereotaxic surgeries were performed on rats anesthetized with 5% induction and 2–5% maintenance volume of isoflurane. Guide cannulae (C311GA; Plastics One, Inc., Roanoke, VA, USA) were unilaterally placed 2 mm directly above the CeA using the following coordinates from bregma: posterior 2.4 mm; lateral 4.2 mm [15] with a final probe cannula depth of 8.0 mm from the surface of the skull as describe earlier [12]. After surgery, the rats were treated with ketoprofen analgesic (5 mg/kg, i.m.; Fort Dodge Animal Health, Overland Park, KS, USA) twice at 12 h intervals, and allowed to recover for at least 3 days before undergoing further experimental procedures. At the conclusion of microdialysis experiments, rats were euthanized using an overdose of sodium pentobarbital (Fatal-plus® solution, Vortech, Dearborn, MI, USA, 0.5 mL, i.p.).

4.3. Drug preparation

Corticosterone (CORT) was dissolved in 100% ethanol and serially diluted with artificial cerebrospinal fluid (aCSF) to a final concentration of 1.73 μM and 0.02% ethanol. At this concentration, approximately 48 pg of CORT was delivered over the 40-min perfusion [2]. Normetanephrine (NM) was dissolved in an aCSF/ethanol mixture, using the same procedures as those used in the preparation of CORT in order to maintain the same vehicle across treatments, and was kept frozen at −80 °C until used at a concentration of 60 μM. Based on a probe recovery of approximately 18% and a flow rate of 5.2 μL/min, the concentration of NM delivered to the CeA was estimated to be approximately 25 ng over the 40-min perfusion. This concentration of NM has been shown to induce behavioral effects, presumably mediated by blocking OCTs, when injected into the nucleus accumbens [10].

4.4. Microdialysis procedure

Rats received a 0.2 mL intramuscular injection of 0.11 mL/100 g body weight xylazine/ketamine anesthetic prior to probe placement the night before sampling. Measurement of 5-HT in the extracellular fluid of the CeA was performed with concentric microdialysis probes. Probes were constructed with a working length of 2.4 mm, as described earlier [12]. After construction, the flexible external portion of the probe was reinforced with J-B Weld 8276 KwikWeld™ Quick Setting Steel Reinforced Epoxy (J-B Weld, Sulphur Springs, TX, USA) to prevent shearing of the silica tubing during restraint by increasing rigidity. A small piece of polyethylene tubing (PE-50) was glued on the 26 G barrel of the probe to act as a stopper and ensure proper depth of probe insertion. Probes were connected to a swivel (Instech Laboratories Inc., Plymouth Meeting, PA, USA), which enabled rats to move freely in a 10-gallon terrarium containing bedding. Flow rate of aCSF was held at 0.15 μL/min overnight prior to sampling to allow for probe

equilibration, after which, the flow rate was increased to 0.52 µL/min for the remainder of the experiment.

Serotonin (5-hydroxytryptamine; 5-HT) was separated with a 3 µm C_{18} microbore column (Bioanalytical Systems, Inc., West Lafayette, IN, USA) and detected with a glassy carbon electrode using an LC-4 potentiostat (Bioanalytical Systems, Inc.) set at an oxidation potential of +0.6 V, with respect to an Ag/AgCl reference electrode with the potentiostat sensitivity set at 1.0 nA/V. The mobile phase, consisting of 350 mg ethylenediaminetetraacetic acid, 3.2 g sodium hydrogen phosphate (NaH_2PO_4), 125 mg octanylsulfonic acid, 600 µL triethylamine, 89 mL methanol in 500 mL reverse osmosis water at pH 5.2, was delivered to the column using a pneumatic fluid displacement pump [3]. Dialysate was directly injected into the chromatographic system at 20 min intervals. Serotonin peak heights were measured using a CSW32 data program (DataApex Ltd., Prague, Czech Republic). Baseline 5-HT concentrations, uncorrected for probe recovery, were determined by comparison with peak heights obtained from the injection of 7.8 pg 5-HT standard.

After the collection of at least 3 consecutive 5-HT baseline samples, the rats were directly placed in a restraining tube (6 cm, i.d.; 27 cm, length), constructed from polyethylene tubes with a narrow channel cut in the top to allow passage of the lines attached to the microdialysis probe. Silica lines were designed so that it takes approximately 20 min for a drug to reach the brain and another 20 min before the first brain sample exposed to the drug was collected. Thus, time 0 in Figs. 2 and 3 represents the beginning of restraint stress and the estimated time at which the drug initially reaches the CeA. The rats remained in the restraining tubes for 40 min (the equivalent of 2 microdialysis sampling periods), before being released back into the testing chamber. In *Experiment 1*, delivery by dialysis of intra-CeA CORT or NM was tested in the absence of stress ($n = 4$, CORT; $n = 5$, NM) to determine if CORT or the NE metabolite NM affected extracellular 5-HT concentrations. In *Experiment 2*, treatment with intra-CeA CORT ($n = 7$), NM ($n = 7$), or vehicle ($n = 6$) was timed so that the drug delivery to the amygdala coincided with the onset of the restraint stress. At the end of the 40-min period of reverse dialysis of drug, aCSF was reintroduced into the probe.

4.5. Histology

Brains were removed and fixed in formalin for 3 days (Cat. No. 245-684, Fisher Scientific, Kalamazoo, MI, USA). The brains were frozen and serially cut into 60 µm-thick sections at −15 °C in a Jung 1850 cryostat (Leica Instruments, Heidelberg, Germany) and viewed under a microscope to determine placements of probes. Thirty-nine animals were excluded from the study due to infusion sites that either missed the CeA or punctured the base of the brain.

4.6. Statistical analysis

Extracellular 5-HT concentrations in the three baseline samples preceding treatment were averaged, and 5-HT concentrations were calculated as a percentage change from mean baseline concentrations for each rat. The effects of restraint stress and drug treatment on CeA extracellular 5-HT levels were determined by a two-way ANOVA with one repeated measure (time). In analyses that revealed significant effects of drug treatment or restraint stress, or a significant drug treatment or restraint stress x time interaction, effects of drug treatment at specific time points were identified using the Holm-Sidak *post hoc* between-subject pairwise comparisons. The Bonferroni *post hoc* correction for multiple comparisons versus a single control (where the control was the –20-min time point) was used for within-subjects comparisons. Statistical analysis was run using SigmaStat version 3.5 (Systat Software, Inc., San Jose California USA). Significance levels for all statistical tests were set at $p < 0.05$.

Author contributions

KJR, CAL, MO and JEH Jr designed the studies; JEH Jr, VEC, HL, JTR, RCA and CV performed the experiments; JEH Jr and KJR did the statistical analysis; CAL, JEH Jr, and KJR wrote the manuscript. KTN created the graphical abstract and provided critical evaluation of the manuscript. All authors have approved the final article.

Declaration of conflicts of interest and sources of funding

The authors report no conflicts of interest. Dr. Christopher A. Lowry is supported by the National Institute of Mental Health (grant number 1R21MH116263), Department of the Navy, Office of Naval Research Multidisciplinary University Research Initiative (MURI) Award (grant number N00014-15-1-2809), Department of Veterans Affairs Office of Research and Development (VA-ORD) RR&D Small Projects in Rehabilitation Research (SPiRE) (I21) (grant number 1 I21 RX002232-01), the Colorado Department of Public Health and Environment (CDPHE; grant number DCEED-3510), and the Alfred P. Sloan Foundation (grant number G-2016-7077).

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