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Research report

Stress-evoked increases in serotonin in the auditory midbrain do not directly result from elevations in serum corticosterone

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ABSTRACT

Neurochemicals such as serotonin convey information about behavioral context to sensory processing. In the auditory system, serotonin modulates the responses of neurons in the inferior colliculus (IC) to acoustic stimuli, including communication vocalizations. Levels of extracellular serotonin in the IC can change rapidly in response to stressful situations such as social challenge and limited movement. Since activation of the hypothalamo–pituitary–adrenal (HPA) axis can influence serotonin in other brain regions, we examined the relationship between serum corticosterone and serotonin release in the IC. We used voltammetry to measure extracellular serotonin in the IC of male CBA/J mice during restriction of movement, a low-intensity restraint stress. Enzyme immunoassay (EIA) was used to measure the concentration of corticosterone circulating in the blood serum as an indicator of the activation of the HPA axis. Changes in serotonin and corticosterone were also compared with behavioral performance. Restriction stress caused increases in serotonin in the IC and circulating corticosterone, and changes in behavior. Changes in serotonin and corticosterone were not correlated with each other across individuals. Individual behavioral performance was correlated with elevations in corticosterone, but not in serotonin. We further explored the relationship between physiological pathways by directly manipulating serum corticosterone. Injections of corticosterone elevated circulating levels beyond normal physiological ranges, but had no effect on serotonin in the IC. These findings suggest that, within the auditory system, serotonin is released during stressful events, but this is a correlate of behavioral arousal, rather than a direct response to elevations in serum corticosterone.

1. Introduction

Exposure to stress induces a broad set of physiological and behavioral changes selected to increase the probability of survival. Perhaps the most well characterized physiological response to stress is the activation of the hypothalamo–pituitary–adrenal (HPA) axis, which results in the secretion of glucocorticoids into the blood stream and the activation of glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in tissues throughout the body. These mechanisms ultimately induce suites of metabolic, endocrine, and neural changes that facilitate performance in stressful environments [1–5]. The activation of the HPA axis is variable, such that the magnitude and time course are often correlated with both the exact nature of the stressor and the subsequent behavioral response of the individual [6,7]. The behavioral response to a stressor can also be influenced or facilitated by other physiological changes that are coincident with the activation of the HPA axis, including the alteration of sensory processing [8,9]. In this way, stress-induced changes in sensory processing could have evolutionary consequences by providing the advantage of enhanced perception of salient stimuli in situations of duress, for example. Though the amygdala and prefrontal cortex have been the focus of much of the research in this area, a growing body of evidence suggests that some stress-related changes in perception can occur at earlier stages of sensory processing [10,11].

The relationship between stress and sensory processing is evident in the auditory system. Stress and the subsequent changes in glucocorticoid levels are correlated with changes in the timing and amplitude of auditory evoked potentials in both humans and mice [8,12]. Stress can also induce auditory hypersensitivity

Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; CV, coefficient of variation; DOPAC, 3,4-dihydroxyphenylacetic acid; EIA, enzyme immunoassay; GLM, general linear model; GR, glucocorticoid receptor; HPA, hypothalamo–pituitary–adrenal axis; IC, inferior colliculus; i.p., intraperitoneal; MR, mineralocorticoid receptor; OCT3, organic cation transporter 3; s.c., subcutaneous; s.e.m., standard error of the mean.

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[13] or influence auditory pathologies such as tinnitus or Meniére's disease [14,15]. Further, exposure to restraint stress prior to acoustic trauma has a protective effect against noise-induced hearing loss [16]. These impacts of stress on the auditory system may be due to direct or indirect effects of the activation of the HPA axis. MRs are found in the inner ear [17,18] and GRs are distributed throughout the peripheral and central auditory system [19] but the activation of GRs elsewhere in the brain can also have strong effects on multiple modulatory systems, including the serotonergic system, to facilitate a coordinated response to stress at the sensory level [2].

In the inferior colliculus (IC), a midbrain auditory nucleus, the response properties of auditory neurons are influenced by the presence of serotonin [20,21]. Serotonin receptors modulate aversive behavior produced by direct stimulation of the IC [22,23]. Moreover, extracellular serotonin in the IC increases to varying degrees when mice are exposed to noise, restriction of movement or social intrusion, all potentially stressful stimuli [24,25]. It is unclear, however, whether serotonergic increases in the IC directly result from activation of the HPA axis. The serotonergic response in the IC may be part of a separate pathway, independent of the HPA axis, which modulates auditory processing in a variety of contexts that are not necessarily stressful [25–27].

To investigate the relationship between indicators of global physiological stress and the local serotonergic response to the same stressors in the IC, we exposed mice to restriction stress and monitored their physiological and behavioral responses. Restriction of movement, a non-auditory stressor, was used in these experiments to avoid any potential confounding effects of acoustic overstimulation. In addition, in our hands, restraint stress has induced larger and more reproducible increases in serotonin in the IC than acoustic stimulation [25]. We measured circulating levels of corticosterone in the serum before and after restriction as a measure of the activation of the HPA axis, a commonly used indicator of the intensity of a stressor. We also monitored changes in serotonin in the IC during restriction using *in vivo* voltammetry. An individual's corticosterone and serotonergic responses to restriction of movement were compared to each other and to behavior to determine whether any of these responses were correlated. Further, we examined the possibility of a causal link between elevations in peripheral levels of corticosterone and changes in serotonin in the IC by artificially elevating levels of circulating corticosterone via subcutaneous (s.c.) injection.

2. Materials and methods

2.1. Animals

Data were obtained from 15 male CBA/J mice (*Mus musculus*, Jackson Laboratory, Bar Harbor, ME, USA). This strain shows behavioral and physiological responses to stress that are comparable to other strains of mice [28]. Mice ranged in age from 8 to 35 weeks and averaged 21 ± 2.2 (s.e.m.) weeks old. Mice were housed individually on a 14/10 light-dark cycle and supplied with food and water available *ad libitum*. All protocols were approved by the Bloomington Institutional Animal Care and Use Committee.

2.2. Voltammetry

2.2.1. Carbon fiber electrode construction

Carbon fiber electrodes were prepared from pulled glass capillary tubes. The glass tip was broken and a single carbon fiber (11 μm in diameter, Thornell P25; Cytec Industries Inc., West Paterson, NJ) was threaded through the tube until it protruded from the end of the glass 100–150 μm. The carbon fiber was sealed in place with an epoxy resin (Miller-Stephenson Chemical Company, Inc.; Danbury, CT) and soldered to a length of wire that protruded from the back of the electrode using a low melting-temperature bismuth alloy (Small Parts; Miramar, FL).

Carbon fiber electrodes were electrically and chemically treated prior to use in order to increase the sensitivity to and selectivity for serotonin [24]. To increase sensitivity and to separate the electrochemical signal of serotonin from that of other oxidizable molecules, all electrodes were electrically treated in phosphate–citrate buffered saline. An e-Corder controlled by Chart software (EDAQ; Denistone East, Australia) was used to produce a triangular wave form (70 Hz, 0–3 V vs. calomel auxiliary electrode) for 40 s followed by a 1.5 V constant potential for 10 s, a −0.5 V constant potential for 5 s and a 1.5 V constant potential for 8 s. All electrodes were then coated with Nafion, an ion exchange resin, by dipping them in a 5% solution of Nafion (Sigma–Aldrich, St. Louis, MO) 3 times and allowing them to air dry for 3 min between each dip. Nafion coating increases electrode specificity for serotonin; this was confirmed by testing each electrode prior to use in a solution of 50 μM ascorbic acid, 5 μM 3,4-dihydroxyphenylacetic acid (DOPAC) and 1 μM serotonin (Sigma–Aldrich, St. Louis, MO). Electrodes were used in experiments if they measured a serotonin signal at approximately +250 mV (vs. Ag/AgCl) and showed little or no response to DOPAC and ascorbic acid.

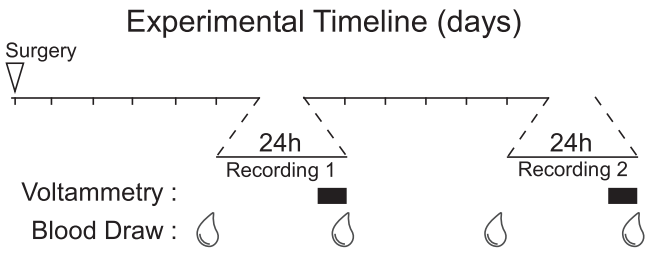

Fig. 1. Experimental timeline. Coordinated measurements of serotonin during limited movement (black bars) and serum corticosterone (drop symbols) were performed twice over a 2-week period in the same mice following the implantation of voltammetric hubs (inverted triangle). Baseline blood samples were taken 24 h prior to samples after the restriction of movement.

2.2.2. Surgery

Head stages were surgically mounted on the skull overlying the IC to permit voltammetric recording in behaving mice (Fig. 1, inverted triangle). Prior to surgery, mice received metacam [1 mg/kg, s.c.]. After at least 1 h, mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (120 mg/kg) and xylazine (5 mg/kg). Anesthetic state was determined by lack of response to tail pinch.

Once mice were fully anesthetized, the hair on top of the head was removed with depilatory cream and mice were placed in a stereotaxic device for aseptic surgery (Stoelting; Wood Dale, IL). An incision was made to expose the skull from bregma to the musculature of the neck. Bregma and lambda were placed in the horizontal plane and holes approximately 1.5 mm in diameter were drilled over the left and right IC (1.1 mm posterior, 1.6 mm lateral to lambda). Custom-made Teflon hubs [29], designed to mate with micro-drives containing the recording and reference electrodes, were placed above each hole at an angle of 15° from vertical. Hubs were secured to the skull with two stainless steel bone screws (PlasticsOne Inc.; Roanoke, VA) and dental cement. Between experiments, the hubs were plugged with custom-made Teflon screws. On the day following surgery, mice received metacam (1 mg/kg, s.c.). Mice were allowed to recover from surgery for at least 7 days prior to recording.

2.2.3. Recording

Immediately prior to voltammetric recording, mice were anesthetized with ketamine (90 mg/kg) and xylazine (1 mg/kg) for the placement of the electrodes. Both the carbon fiber recording electrode and Ag/AgCl reference electrodes were mounted in custom-made, Teflon microdrives that screwed into the hubs mounted on the skull. After the microdrives were secured in the hubs, the recording electrode was lowered into the IC on one side of the brain and the reference electrode was lowered until it came into contact with the cerebrospinal fluid above the IC on the other side of the brain. Immediately after electrode placement, mice were returned to their home cage, which was then placed in a Faraday chamber. Mice were allowed to recover from anesthetic on a 10 cm^2 piece of laboratory tissue in order to separate them from the bedding material and were considered to have recovered once they voluntarily walked off of the tissue. A light-weight, flexible tether connected the recording and reference electrodes to a bipotentiostat (EI-400; Cypress Systems, Chelmsford, MA) through an electric swivel (PlasticsOne Inc.; Roanoke, VA) which allowed the mouse to move freely around the cage. Voltammetric recording began as soon as the electrodes were connected to the tether and were performed continuously throughout the recording session. A staircase waveform (−300 mV to +600 mV to −300 mV, 10 mV steps, 30 mV/s, 1 min duration) was applied with 5 min between each waveform, so that one serotonin measurement was made every 6 min. All manipulations were performed more than 1 h after the mice recovered from anesthesia. Throughout the recording session, the cage was surrounded by an opaque barrier, 40 cm tall, in order to decrease interference from external visual stimuli and prevent mice from escaping.

Voltammetric recording took place in two sessions, one in each IC, with at least a 7-day recovery period between sessions (Fig. 1, black boxes). Whether the first session recorded from the left or right IC was chosen randomly. For each mouse, both sessions occurred at the same time of day, from 09:00 h to 12:00 h or 13:00 h to 16:00 h. Over the two sessions, mice were exposed to a restriction stressor and a control manipulation, or a corticosterone injection and a control injection. Whether the control manipulation occurred in the first or second recording session was determined randomly. In order to facilitate the rapid collection of blood after the

termination of the experiment and minimize the handling required to disconnect the mouse from the voltammetry equipment, no lesions were made after recording. Instead, the placement of the recording electrode within the IC was confirmed post-mortem by the placement of the hubs on the skull and/or a small red blood clot on the dorsal surface of the IC at the site of electrode penetration, easily visible with the naked eye. In previous studies using identical surgical methods and electrodes of the same dimensions, all electrolytic lesions were within the IC [24,25]. Although we are confident of the placement of our recording electrodes in the IC, the length of the active surface of the electrode precludes selective measurement of serotonin within different subnuclei of the relatively small IC in mice. We can therefore make no statements on whether the serotonergic response differed among the central IC and the dorsal or external cortices.

2.3. Restriction of movement

The restriction manipulation was modeled after restraint-stress paradigms and has been used in previous studies [24]. One hour prior to the end of the recording session, mice ($n = 11$) were contained within a small, circular arena (8.5 cm in diameter) within their home cage for 30 min (6 measurements). This was achieved by surrounding the animal with an opaque plastic tube that had high, smooth walls to prevent jumping or climbing but no floor and an open top to accommodate the recording tether. The size of the arena limited the lateral range of travel without preventing other types of behavior. After the restriction manipulation, mice remained in the recording chamber undisturbed for another 30 min to allow for the accumulation of corticosterone in the serum [30]. Blood was collected 60 min after restriction onset.

Changes in circulating corticosterone measured after restriction experiments could have been due to the handling and manipulation performed in order to prepare the mouse for voltammetric recording. To control for the effect of the recording procedure on the glucocorticoid response, the recording session in the contralateral IC contained no restriction manipulation. Mice received electrodes and underwent voltammetric recording for the same amount of time, but no stressor was applied. At the end of the recording session, blood was collected at the same time as the other session. Control and restriction experiments were performed one week apart and in random order.

2.4. Behavior

Mouse behavior was recorded using a digital video camera, DVR PCI card and software (Q-See, Digital Peripheral Solutions Inc., Anaheim, CA, USA). The duration of all behaviors was scored by an observer naïve to the results of voltammetric recording using ODLog software (Macropod Software).

The behaviors scored were:

Rearing: the head and forepaws were elevated off the bedding or the forepaws were placed on the side wall of the tube.
Immobility: no obvious movement except breathing.
Digging: disruption of bedding with forepaws.
Locomotion: demonstrated turning movement inside the restricted arena, or demonstrated horizontal displacement throughout the cage when not in the restricted arena.

2.5. Corticosterone injection

To examine whether direct manipulation of the glucocorticoid signaling pathway influenced serotonin in the IC, anesthetized mice ($n = 4$) were injected with corticosterone (30 mg/kg, s.c.) in sesame oil. Control injections consisted of sesame oil alone. Since anesthetic state can influence serotonin in the IC [24], mice received a supplemental dose of anesthetic [ketamine (40 mg/kg) and xylazine (1 mg/kg), i.p.] after electrode implantation to maintain anesthesia for at least 60 min (average 90.8 ± 9.9 min) after the initiation of recording. Voltammetric recording was conducted for 15–25 min to ensure a stable base line prior to the s.c. injection. Recordings were considered stable if they varied less than 10% between measurements. Of the 4 mice in this group, 2 received the control vehicle injection during the first recording session and 2 received it during the second. Serum collection occurred approximately 130 min after corticosterone or vehicle injections to allow adequate time for any effect on short-term changes in extracellular serotonin in the IC.

2.6. Serum collection

Total serum corticosterone was assayed from blood collected the day before and 30 min after restriction, or the comparable time during control experiments (Fig. 1, drops). Placing the baseline measurement the day before the restriction stress minimized the adverse effects of collecting this blood volume from the mice. To collect blood, mice were lightly anesthetized with isoflurane and a small (~80 μl) blood sample was obtained from the retro-orbital sinus. Samples were allowed to clot at room temperature for 1 h. After the clots were removed, the samples were centrifuged (at 4 °C) for 30 min at 5000 × *g*. Serum aliquots were aspirated and stored in microcentrifuge tubes at −20 °C.

Blood serum was collected before and after experiments to determine the effect of the manipulation on circulating corticosterone. Base line samples were collected 24 h before experimental samples to control for circadian variation in the concentration of corticosterone in the blood. Samples from individual mice were always collected at the same time, 12:00 h or 16:00 h, for both recording sessions. In order to minimize the effects of handling during blood collection on the circulating glucocorticoid levels, samples were taken as rapidly as possible. Most samples (58/60) were collected in less than 3 min. Within this time period, there was no relationship between the time it took to collect the serum and base line circulating corticosterone level (Pearson's $r = -0.083$, $p = 0.714$). The two samples that took longer than 3 min to obtain were within one standard deviation of the mean of the samples in the same treatment group and were therefore not excluded from analysis.

2.7. Corticosterone assay

Total serum corticosterone was assessed using a commercial enzyme immunoassay (EIA) kit (Correlate-EIA™, Assay Designs, Ann Arbor, MI). Cross-reactivity was 100% for corticosterone, 28.6% for deoxycorticosterone, 1.7% for progesterone, 0.28% for tetrahydrocorticosterone, 0.18% for aldosterone, 0.13% for testosterone, 0.046% for cortisol and <0.03% for pregnenolone, β-estradiol, cortisone, or 11-dihydrocorticosterone acetate (kit instructions). Samples were diluted to 1:40 and run in duplicate on 2 plates. The sensitivity of the assay was 26.99 pg/ml. The average intra-assay coefficient of variation (CV) for samples was 4.13%; no individual sample had a CV that was greater than 13% and none were excluded from analysis. The average inter-assay coefficient of variation, calculated from 14 samples run on both plates, was 3.58%.

2.8. Data analysis

The concentration of extracellular serotonin was measured as the height of the serotonin oxidation peak in the first derivative of the voltammetric current measurement. Deriving the current trace increases the ability to detect small changes in signal amplitude which are correlated with changes in the concentration of serotonin in the extracellular fluid [24,31]. To account for differences in the sensitivities of individual electrodes, serotonin peak values were normalized to the average of two measurements taken just prior to the administration of the restriction stressor. During control recording sessions, mice were not exposed to a stressor but, in all other respects, the data were treated the same way: i.e. measurements from the control group were normalized to the two measurements taken just prior to the time when the stressor would have been applied. For voltammetric recordings, differences between groups were evaluated using a general linear model (GLM) with Bonferroni post hoc tests. Correlations between serotonin, corticosterone, age, mass and behavior were investigated using multivariate general linear models with the total duration of the performance of different behaviors as dependent variables. All error bars in figures represent the standard error of the mean (s.e.m.).

3. Results

3.1. Physiological responses to restriction of movement

To explore the effects of stress on serotonin release in the auditory system and on circulating levels of corticosterone, mice ($n = 11$) were exposed to restriction of movement for 30 min by placing them in a small tubular arena (Fig. 2A, shaded area). Measurements of serotonin were made before, during, and after the period of restriction at intervals of 6 min. By the second measurement, within 7–12 min of restriction, the concentration of extracellular serotonin in the IC increased by 14.7% relative to pre-restriction baseline (Fig. 2A, crosses, black line; GLM, Bonferroni $T = 4.581$, $p = 0.001$). When compared to control experiments performed on the same mice, in which no restriction of movement occurred (Fig. 1A, broken gray line), extracellular serotonin in the IC remained elevated throughout the restriction period and even until the first measurement after restriction removal (Fig. 2A, asterisks; GLM, Bonferroni post hocs, all p-values < 0.05). By the second measurement after restriction removal, however, serotonin in the IC returned to control levels.

Corticosterone levels were measured before and after the restriction stress. To control for circadian fluctuations in circulating corticosterone, baseline measurements were made from blood taken from the same mice, 24 h before the post experiment blood collection (open boxes, Fig. 1B). All blood samples from a single mouse were taken at the same time of day, 12:00 h or 16:00 h, and stored at −20 °C until analysis. Time of day had a significant effect

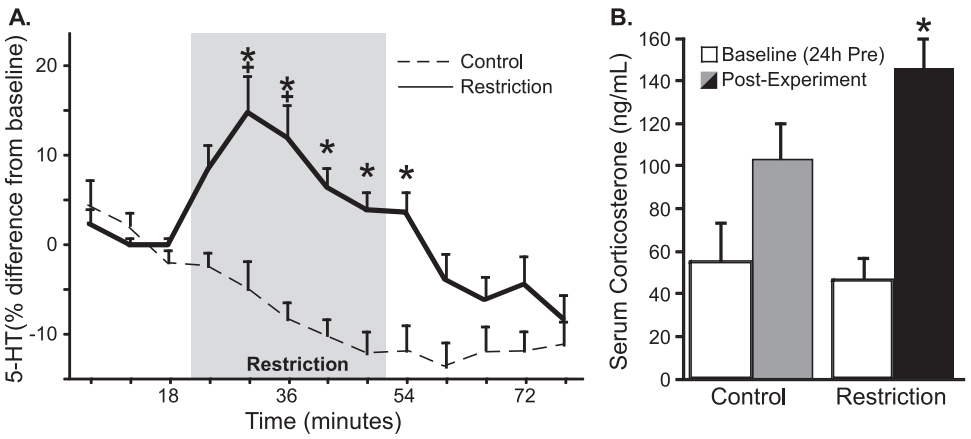

Fig. 2. Extracellular serotonin in the IC and serum corticosterone increase during restriction stress. (A) Male mice ($n = 11$) showed an increase in serotonin during the restriction of movement (shaded region: black line), but not during control experiments without restriction (broken gray line). Crosses: values significantly different from pre-restriction measurements; asterisks: values significantly different from controls. Blood collection occurred 30 min after the restriction of movement, within 3 min on the termination of voltammetric recording. Error bars are s.e.m. (B) Corticosterone levels from samples taken from the 11 mice in (A), 24 h prior to restriction (open bars) and 30 min after the end of restriction (filled bars). Serum corticosterone levels were elevated relative to baseline after restriction (black) experiments (asterisk) but not after control (gray) experiments.

on total corticosterone measured in baseline samples; 12:00 h samples contained less corticosterone than 16:00 h samples (Student's t-test, $p = 0.002$). However, time had no effect on the corticosterone response to restriction of movement or injections (Student's t-tests, both p-values > 0.748). Corticosterone was measured 30 min after the end of restriction of movement to allow for the accumulation of corticosterone in the serum [30]. At this time, the concentration of circulating corticosterone was significantly elevated relative to baseline measurements (Fig. 2B, asterisk; GLM, Bonferroni $T = -4.488$, $p = 0.007$). Although somewhat elevated, circulating corticosterone was not significantly increased after control experiments, even after accounting for time of day and the order of the experiments (Fig. 2B; GLM, Bonferroni $T = -2.192$, $p = 0.201$). Thus, handling and manipulation did not significantly increase levels of circulating corticosterone, but the addition of restriction of movement did.

Restriction of movement caused elevated corticosterone and serotonin in the same population of mice, so we investigated whether these two types of physiological response were correlated within individuals. The amplitude of the serotonergic response to restriction of movement for an individual mouse was calculated as the average percent change from pre-restriction baseline across the 5 measurements that took place during the manipulation. The amplitude of the serotonergic response in the IC was not correlated with an individual's circulating corticosterone level after restriction (Pearson's $r = -0.327$, $p = 0.327$), nor was it correlated with the change in circulating corticosterone relative to baseline measurements taken 24 h before (Fig. 3; 24 h pre–post restriction, Pearson's $r = -0.470$, $p = 0.144$).

3.2. Behavioral responses to restriction of movement

We investigated correlations between the behavioral performance and physiological responses to the restriction of movement manipulation. Locomotion, immobility, rearing and digging were scored both during and after the restriction period and during the complementary period in control experiments. Locomotion was measured as duration of horizontal displacement before and after the restriction of movement, and as the time spent circling during the restriction of movement. The proportional times (s/min) of most behaviors were similar during and after restriction. Locomotion, however, significantly increased from 8.04 ± 1.67 s/m during restriction of movement to 14.12 ± 1.41 s/m after the restriction was removed (paired t-test, $t = -3.860$, $p = 0.003$).

Individual behavioral performance was not correlated with the amplitude of the serotonergic change measured within the IC, but was correlated with corticosterone. Across the 5 measurements that took place during the restriction of movement, the average serotonergic response of an individual mouse was not correlated with the performance of locomotion, immobility, rearing and digging (GLM, $p > 0.05$). Further, there was no correlation between serotonin and behavior after restriction (GLM, $p > 0.05$). In contrast, there was a strong relationship between circulating glucocorticoid levels and behavioral performance after, but not during, the restriction of movement. The circulating level of corticosterone was correlated with the overall performance of immobility, locomotion, rearing, and digging behavior during the 30 min between the end of restriction and prior to blood collection (GLM, $F(4,6) = 7.265$, $p = 0.017$), though not with any behavior individually. The change in corticosterone from the previous day's

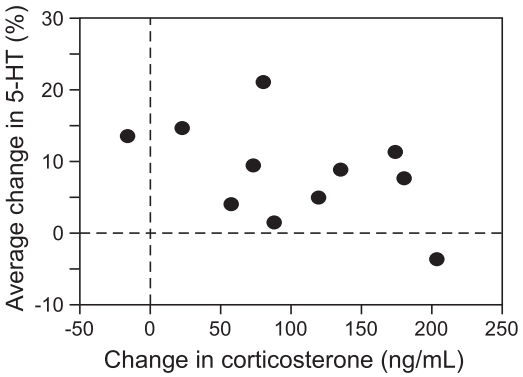

Fig. 3. Changes in serum corticosterone were not correlated with changes in serotonin in the IC. The amplitude of an individual's serotonergic response in the IC to the restriction of movement was not correlated with their change in serum corticosterone after the same manipulation (Pearson's $r = -0.470$, $p = 0.144$) The average change in serotonin was calculated as the difference between the average of the 5 measurements taken during the restriction of movement and the average on the two measurements take just before restriction onset. Change in serum corticosterone was calculated as the difference between serum corticosterone concentration assayed from blood collected after restriction and blood collected 24 h before. Dashed lines on each axis represent no change.

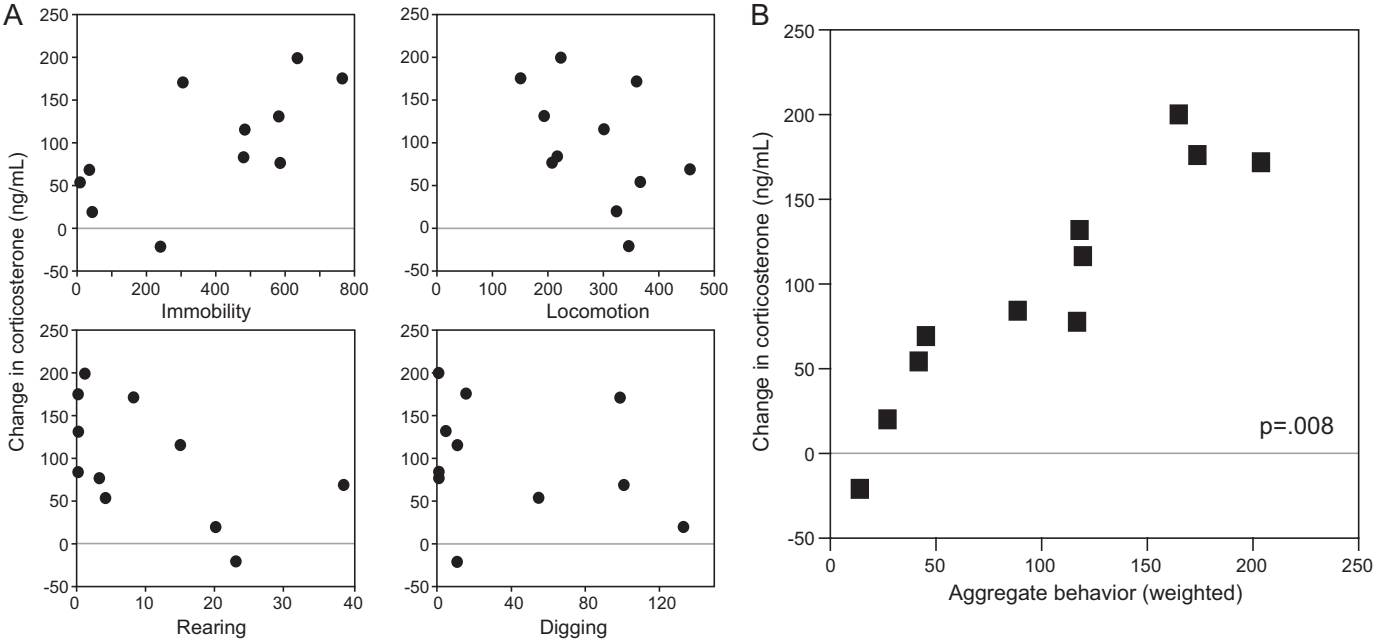

Fig. 4. Behavior was correlated with corticosterone. (A) The change in serum corticosterone after the restriction of movement relative to the baseline measured 24 h earlier was not correlated with the total duration (in s) of immobility, locomotion, rearing, or digging behaviors across subjects. The correlation of behavioral durations to changes in corticosterone assessed individually for each behavior in a GLM. (B) The change in serum corticosterone was correlated with aggregated behavioral performance during the 30 min between the end of restriction and blood collection. A weighted behavioral index (aggregate behavior) was calculated for each mouse using the equation describing the relationship between behavior and corticosterone: Change in Corticosterone (ng/ml) = (−351) + (0.976 × Locomotion) + (0.481 × Immobility) + (0.964 × Digging) + (−4.17 × Rearing) (GLM, $F(4,6) = 10.198$, $p = 0.008$). The weighted index was plotted versus the change in serum corticosterone to visually demonstrate that the aggregate behavior of each mouse is correlated with corticosterone measured in the serum after restriction (Pearson's = 0.93, $p < 0.001$).

baseline was also correlated with an aggregate index of these four behaviors, but not individual behaviors, in the post-restriction period (Fig. 4; GLM, $F(4,6) = 10.198$, $p = 0.008$).

3.3. Correlation of age and mass with responses to restriction of movement

Because larger mice may experience greater confinement when placed in the restriction tube and because we have previously observed correlations between serotonin and the age or mass of individual mice [25], we examined whether the physiological responses to restriction correlated with the age and mass of the mice used in this study. The serotonin response in the IC during the restriction of movement was negatively correlated with a mouse's mass ($\beta = -0.703$, $t = -2.969$, $p = 0.016$), such that smaller mice showed larger serotonergic responses. Neither mass nor age was correlated with behavioral performance during restriction of movement. Similarly, there were no significant correlations between circulating corticosterone sampled after restriction and the age or mass of the individuals used in this study.

3.4. Pharmacology

Though not quantitatively correlated, both serotonin and corticosterone increased in response to the restriction of movement. To determine whether a change in circulating corticosterone was sufficient to induce a change in serotonin in the IC, anesthetized mice ($n = 4$) were injected with corticosterone (30 mg/kg, s.c.) and sesame oil vehicle (s.c.), each in a separate voltammetric recording session. After injections, voltammetric recording was continued until the time of blood collection, 120 min after the injection. The injection of corticosterone elevated serum corticosterone above the reliable detection limits of the assay in all 4 cases (Fig. 5A). Therefore, all values were rounded down to the highest concentration on the standard curve of the assay, 800 ng/ml. Though the exact concentrations are uncertain, the injection produced levels of circulating corticosterone in the serum that are an order of magnitude greater than the baseline values measured from samples taken 24 h before (Fig. 5A, open boxes). In contrast, when the same mice were injected with vehicle, circulating corticosterone levels averaged 97.5 ± 22.9 ng/ml, which are no different from baseline samples take 24 h before (Fig. 5A, gray bars; GLM, Bonferroni $T = -1.622$ $p = 0.8352$) and comparable to the 102.9 ± 17.1 ng/ml measured after the control experiments in Fig. 2B (filled gray bar).

The injection of corticosterone had no effect on serotonin in the IC. Fig. 5B illustrates the serotonergic signal measured during the time following the injection of corticosterone and the injection of vehicle. The signal in both groups decreased over time (GLM, $F(21,175) = 5.6$, $p < 0.001$) which is typical for carbon fiber electrodes and an indicator of a loss of electrode sensitivity with use [24,32,33]. Over the same time period that the concentration of circulating corticosterone in the serum increased beyond 800 ng/ml, the serotonergic responses of mice injected with corticosterone were no different from the responses of those injected with vehicle (GLM, $F(1,175) = 0.30$, $p = 0.583$). Therefore, the elevation of circulating corticosterone to supraphysiological levels had no effect on extracellular serotonin in the IC.

4. Discussion

The physiological response to stressors consists of multiple interacting components. There are components that globally influence somatic and neural systems such as glucocorticoids, and components that are more localized within the brain such as the serotonergic raphe system. These chemical signals are highly relevant to the operation of sensory systems, since they influence sensory processing and may optimize responses to cues about potential threats. Our previous work in an auditory midbrain

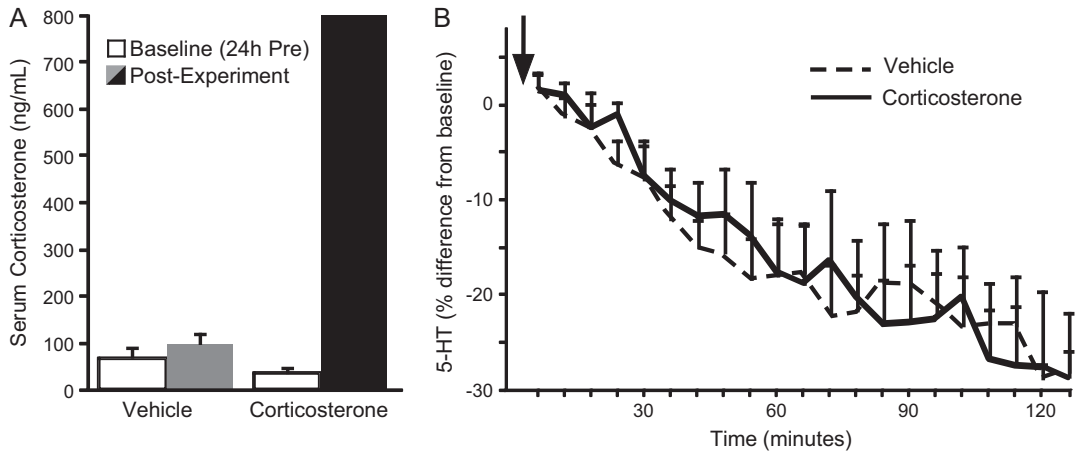

Fig. 5. Direct elevation of serum corticosterone had no effect on serotonin in the IC. (A) Injection of 30 mg/kg corticosterone (black bar) elevated serum corticosterone, but injection of vehicle (gray bar) did not relative to baseline measurements (open bars, $n = 4$). Injections of corticosterone (filled black bar) resulted in circulating levels of corticosterone that were beyond the limits of reliable quantification in this assay, thus all values were estimated as the highest point on the standard curve, 800 ng/ml. (B) Voltammetric measurements were taken from the same 4 mice in (A). In separate experiments, the injection of corticosterone (arrow: solid line) did not alter serotonin in the IC relative to injection of a vehicle (broken line). The serotonergic signal decreased in both cases due to electrode rundown. Blood collection occurred within 3 min of the termination of voltammetric recording. Error bars are s.e.m.

nucleus, the IC, suggested that stress might be a common feature of the stimuli that evoke local increases in serotonin: the presentation of broadband noise, the restriction of movement, and the presentation of a novel intruder [24,25]. In multiple regions of the brain, stressful situations can increase serotonergic activity within minutes or less [24,34–36]. Further, previous studies have linked the serotonergic system and the HPA axis as co-responsive systems during stressful events [7,34,36–38]. In the current study, we therefore addressed whether corticosterone directs the local serotonergic response to a stressor in the IC.

4.1. Corticosterone and serotonin: coincident or causative?

The restriction stressor we presented to the mice in our study caused significant increases in both circulating corticosterone and local serotonin in the IC relative to controls. This coincident increase suggested that the two could be causally related, with the activation of the HPA axis directly triggering serotonin release in the IC. There are multiple potential pathways through which this could be achieved. Stress and corticosterone can influence serotonin synthesis in the raphe [39], the activity patterns of some serotonin-releasing neurons [40,41] and the release or reuptake of serotonin in target regions throughout the brain [36,42–44]. The increases in serotonin in response to stressors that we have observed in the past were relatively rapid, occurring on the order of minutes. Consistent with this time scale, glucocorticoids may have rapid non-genomic effects through receptors localized to cell membranes, in addition to causing changes in gene expression through cytosolic receptors [30,45]. Indeed, peripheral infusion of corticosterone increases serotonergic activity in the hippocampus and amygdala within 20 min [46]. In addition to rapid effects of glucocorticoids, corticotropin releasing hormone (CRH), the hypothalamic peptide hormone that triggers the secretion of adrenocorticotropic hormone (ACTH) from the pituitary and consequent increase in circulating glucocorticoids via the adrenal cortex, also has direct and rapid effects on neurons [47,48].

Our experimental design allowed a comparison between circulating corticosterone and serotonin in the IC in the same mice. Both of these measurements showed variability among individuals. For the corticosterone measurements, inter-individual variability was significantly correlated with the variability in behavioral performance, which supports the contention that variability in the corticosterone response was behaviorally relevant. In contrast, circulating corticosterone levels were not significantly correlated with serotonin in the IC, suggesting that there was not a direct relationship between the two physiological responses. In considering this result, it is useful to place our measurements in the context of spatial and temporal profiles of changes in corticosterone. We measured corticosterone in the serum, an indicator of the global activation of the HPA axis. It should be noted, however, that peripheral measures of circulating corticosterone can be [49,50], but are not always [51], higher than levels of corticosterone in the central nervous system and that measuring central levels may have produced a different result. Additionally, it is possible that the timing of blood sampling, 1 h after restriction onset and 30 min after offset, could have missed the peak of circulating corticosterone.

Additional evidence that the local increase in serotonin was not directly triggered by corticosterone is that the peripheral injection of corticosterone had no effect on serotonin compared to an injection of vehicle. The injection elevated the level of corticosterone in the serum past the reliable detection limits of the assay. Although we are unable to report the exact concentration of circulating corticosterone in the blood, the injection of corticosterone elevated circulating levels far more than the restriction of movement and this increase in circulating corticosterone had no effect on serotonin in the IC. An important caveat to this conclusion is that dose response curves for glucocorticoids may be non-linear or have an inverted U-shape, such that supraphysiological doses can have little effect compared to lower doses [49,52,53]. Whether U-shaped functions are observed following administration of corticosterone depends on the physiological factor of interest, and also varies substantially among brain regions [46,49]. If there were such a U-shaped dose–response relationship between corticosterone and serotonin in the IC, our conclusion that corticosterone does not trigger stress-related increases in serotonin in the IC might apply only to supraphysiological levels such as those induced by our injection. For studies comparable to ours that measure different features of the serotonergic system following acute administration of corticosterone, results have varied. In one study of *Anolis* measuring serotonin in brain micropunches, neither serotonin nor its metabolite, 5-HIAA, changed following the injection of corticosterone, but the ratio between the two showed an inverted dose–response relationship in the amygdala and hippocampus, such that a lower dose

increased serotonin turnover but a higher dose did not [46]. In a related study administering corticosterone by direct perfusion, however, measurements of serotonin overflow using microdialysis showed a positive dose–response relationship over two orders of magnitude, with larger doses triggering higher levels of serotonin [54].

Though there was no relationship between serum corticosterone and serotonin in the IC during restriction in awake mice or after corticosterone injection in anesthetized mice, the effect of direct injection of corticosterone could have been influenced by anesthesia. Ketamine anesthesia could have influenced the activity of serotonin-releasing cells or their sensitivity to pharmacological manipulation and thus may have been responsible for the lack of an effect of the corticosterone injection on extracellular serotonin. We argue against this hypothesis with two points. First, ketamine injection increases extracellular serotonin by blocking serotonin reuptake [55–59]. If corticosterone were responsible for increasing serotonin release in the IC, then any effect of corticosterone injection should be exaggerated by the presence of ketamine. Second, the mice in this treatment group received no supplemental anesthetic after they were placed in the voltammetric recording chamber, at least 15 min before the injection of corticosterone. The prolonged time without supplemental anesthetic allowed all mice to recover motor function by the end of the recording session. Even though no mice were fully anesthetized at the end of the recording session, there was no difference between corticosterone and vehicle treatment. The observation that corticosterone injection in anesthetized animals does not increase serotonin in the IC does not, however, exclude the possibility that, in awake animals, changes in circulating corticosterone may influence a number of other neurochemical pathways or behaviors and, through those mechanisms, influence the serotonergic response in the IC.

As a whole, we observed no evidence that serum corticosterone directly influences serotonin in the IC. The lack of a linear correlation or a U-shaped relationship between serotonin and corticosterone in the physiological range evoked by restriction of movement, combined with the lack of effect produced by a supraphysiological dose of corticosterone, is not consistent with a direct effect of corticosterone on serotonin in the IC. We conclude that the increases in serotonin and corticosterone measured here were two parallel components of the physiological response to our restriction of movement stressor.

Despite our lack of evidence that corticosterone directly triggers serotonin release, the HPA axis and serotonergic system may interact with each other in ways that our studies did not address. There are multiple mutual links between these two systems, including serotonergic regulation of the release of CRH through 5-HT1A receptors, and regulation of 5-HT1A receptors by activation of MRs and GRs [38,59,60]. Receptors for serotonin and products of the HPA pathway, such as CRH and glucocorticoids, are located within the auditory system [13,17,19,20,61,62]. Therefore, the two stress-activated physiological responses could also potentially influence the same targets. An additional level of complexity arises from the existence of neural projections from the paraventricular nucleus of the hypothalamus to other regions of the brain. Several studies have documented a projection of moderate strength from the paraventricular nucleus to the dorsal and median raphe nuclei [63–66], which provide the bulk of serotonergic innervation to the IC [67]. These direct projections provide a potential avenue for a rapid regulation of serotonin in the IC during stress that bypasses the HPA axis. Although we found no evidence that corticosterone directly triggers increases in serotonin in the IC within minutes of a stressor, these two systems could interact in other ways to influence sensory processing within the auditory system on a longer time scale.

4.2. What does serotonin signal in the IC?

The serotonergic system regulates a range of behaviors and behavioral states including mood, aggression, social dominance, appetite, and stress [37,68–70]. The relationship of these factors to serotonin in the IC has not been definitively established, but can be clarified by observing the range of situations that trigger changes in serotonin, and by comparing serotonin to behavioral responses to these situations. Features of our current and previous work are consistent with some link between serotonin in the IC and stress. Restriction of movement increased serotonin in the IC; this manipulation also influenced behavior and increased serum levels of corticosterone in the same mice and can therefore be considered to be stressful. We have previously observed serotonergic increases in the IC in response to other, potentially stressful situations, including the presentation of broadband noise or an intruder of the same sex [24,25]. Although we did not observe significant correlations between serotonin and behavior in our current study, in other paradigms we have observed correlations between serotonin and behaviors including the overall level of inactivity and social investigation of an intruder [25]. Multiple potentially stressful situations therefore cause serotonin increases in the IC, with associated changes in behavior.

Not all evidence unequivocally supports serotonin as a neurochemical signal that is exclusive for stress in the IC. In the current study, although the levels of serum corticosterone varied among individuals, interindividual changes in serotonin did not match this variation. In our previous work, some potential stressors, such as the presentation of a component of predator urine, did not trigger increases in serotonin, even though they evoked strong behavioral responses [24]. Furthermore, our data are also consistent with competing hypotheses on the role of serotonin in the IC that generate overlapping predictions regarding increases in serotonin and changes in behavior. For example, the novelty of stimuli can influence serotonin release or synthesis [71,72]. Novelty could account for the serotonergic increases we observed in the current study, since the mice were not previously exposed to restriction of movement. However, the hypothesis of serotonin as a signal of novelty is not consistent with our previous findings, such as the lack of response to novel odor or food, or to facilitation of the serotonergic response across repeated social interactions [25]. We have also previously considered additional hypotheses that serotonin in the IC is related to the overall level of movement, or with the establishment of dominance status [25], with similarly equivocal outcomes. Given the lack of correlation with a peripheral measure of corticosterone, a standard indicator of stress [73], and the existence of unresolved competing hypotheses, the most conservative general conclusion may be that serotonin in the IC is associated with situations demanding a high level of alertness or behavioral arousal [26,74–76]. This would enable serotonin to adaptively modulate sensory processing during important behavioral events.

4.3. Implications for auditory processing

Increases in corticosterone and serotonin can both influence auditory processing. Although serotonin and glucocorticoid receptors are each found peripherally and centrally in the auditory system [17,57,77–79], the physiological effects of glucocorticoids have been better characterized peripherally, and those of serotonin have been better characterized centrally. Multiple types of stressors that increase circulating glucocorticoids, including restraint stress, heat stress, and acoustic stress, are well-known to protect the cochlea from subsequent acoustic or metabolic trauma [16,80–82]. During acute stress, however, the thresholds of auditory brainstem responses and distortion products are lowered, so that measurable auditory activity occurs at lower sound intensities

[13,16]. This suggests that stressful events are associated with an acoustic hypersensitivity that may be adaptive [13].

In the central auditory system, serotonin has a generally suppressive effect on spontaneous and evoked responses of single auditory neurons, at the level of both the cochlear nucleus and the IC [83,84]. However, this general decrease in activity is seldom accompanied by an increase in threshold for favored stimuli [84]. Instead, the suppression of spikes is associated with an increase in selectivity for a variety of stimuli, including species-specific vocalizations [21,85]. There is also circumstantial evidence that corticosterone mediates the facilitation of such serotonergic effects. The cochlear nucleus and IC possess high levels of a non-specific and low-affinity monoamine transporter, the organic cation transporter 3 (OCT3), which is inhibited by corticosterone [44,86]. By blocking this uptake transporter under conditions in which serotonin is already high, such as during a stressful event, corticosterone could increase the levels of serotonin even further. This effect might not have been observed during the direct injections of corticosterone in our experiments, because the baseline levels of serotonin release would have been very low.

Serotonin also modulates the behavioral response to stimulation of the IC. In addition to being a central site for the processing of acoustic stimuli, the IC is an important locus in the neural network regulating aversive behaviors, as well as in associative learning related to aversive stimuli [87–89]. Direct electrical or chemical stimulation of the IC evokes behaviors such as vigilance, freezing, and running or jumping in rats [90,91]. Manipulating the serotonergic system locally within the IC can attenuate some of these types of aversive responses. Preventing the reuptake of endogenous serotonin and activating several types of serotonin receptors decreases conditioned aversive responses and anxiety in the elevated plus maze [22]. The local manipulation of serotonin in the IC does not, however, alter the thresholds of directly stimulated aversive behaviors [23].

A model that emerges from these reported effects is one in which the non-auditory stressor of limiting movement activates the parallel physiological pathways of the HPA axis and the serotonergic system. This could result in an increase in both the sensitivity and selectivity of auditory processing, optimizing analysis of the acoustic environment and decreasing some types of aversive behaviors.

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