

Serotonin in the inferior colliculus fluctuates with behavioral state and environmental stimuli

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SUMMARY

Neuromodulation by serotonin (5-HT) could link behavioral state and environmental events with sensory processing. Within the auditory system, the presence of 5-HT alters the activity of neurons in the inferior colliculus (IC), but the conditions that influence 5-HT neurotransmission in this region of the brain are unknown. We used *in vivo* voltammetry to measure extracellular 5-HT in the IC of behaving mice to address this issue. Extracellular 5-HT increased with the recovery from anesthesia, suggesting that the neuromodulation of auditory processing is correlated with the level of behavioral arousal. Awake mice were further exposed to auditory (broadband noise), visual (light) or olfactory (2,5-dihydro-2,4,5-trimethylthiazoline, TMT) stimuli, presented with food or confined in a small arena. Only the auditory stimulus or restricted movement increased the concentration of extracellular 5-HT in the IC. Changes occurred within minutes of stimulus onset, with the auditory stimulus increasing extracellular 5-HT by an average of 5% and restricted movement increasing it by an average of 14%. These findings suggest that the neuromodulation of auditory processing by 5-HT is a dynamic process that is dependent on internal state and behavioral conditions.

Key words: serotonin, inferior colliculus, auditory, neuromodulation, voltammetry, sensory, stress.

INTRODUCTION

Both anatomical and physiological studies suggest that the neuromodulator serotonin (5-HT) influences neural processing in sensory systems, including the auditory system. Most nuclei in the auditory brainstem and midbrain receive robust 5-HTergic projections (Hurley and Thompson, 2001; Klepper and Herbert, 1991; Woods and Azeredo, 1999). The effects of 5-HT on auditory response properties in these regions have been characterized in multiple brainstem nuclei but have been most intensively studied in a midbrain nucleus, the inferior colliculus (IC) (Ebert and Ostwald, 1992; Fitzgerald and Sanes, 1999; Hurley et al., 2004). The IC is a center for the convergence of auditory and non-auditory input, including 5-HTergic inputs (Hurley and Thompson, 2001; Klepper and Herbert, 1991). The application of 5-HT to IC neurons produces widespread changes in the timing and frequency response profiles of IC neurons (Hurley et al., 2002; Hurley and Pollak, 2001; Hurley and Pollak, 2005b), leading to changes in the selectivity for species-specific vocalizations (Hurley and Pollak, 2005a). Findings such as these have led to the hypothesis that one of the functions of 5-HTergic neuromodulation is to selectively shape or filter auditory information (Hurley et al., 2004; Hurley et al., 2002). Little is known, however, regarding the conditions under which these changes in auditory filtering are likely to occur. This is because measurements of 5-HT in the auditory system have seldom been made in behaving animals.

Although the 5-HTergic system is centralized and projects extensively throughout the brain and spinal cord, 5-HT release and reuptake vary among different regions of the brain because of multiple presynaptic and postsynaptic regulatory mechanisms. Presynaptically, the IC receives 5-HTergic innervation from projections that arise mostly from neurons in the midline dorsal and median raphe nuclei (Klepper and Herbert, 1991). Subgroups of

raphe neurons, even within the same raphe nucleus, have different projection patterns as well as distinct morphological and electrophysiological characteristics (Hajos et al., 1995; Hensler, 2006; Molliver, 1987; Waterhouse et al., 1993; Waterhouse et al., 1986). These differences provide mechanisms for regional variation in the release of 5-HT into the extracellular fluid, where it binds with receptors. Postsynaptically, reuptake through several transporters and degradation by monoamine oxidase decrease the amount of 5-HT in the extracellular fluid. There is strong regional variation in the concentration of these proteins, which can lead to regional variation in the magnitude and time course of 5-HT profiles (Dahlin et al., 2007; Gasser et al., 2009; Gehlert et al., 2008). The variability in 5-HT regulatory mechanisms means that in order to determine the conditions in which 5-HT has an elevated effect on auditory processing, extracellular 5-HT must be measured directly within the auditory system.

Concentrations of extracellular 5-HT in the brain are not only region-specific but also context-specific, fluctuating in response to both behavioral state and environmental events. 5-HT-releasing neurons are themselves sensitive to changes in behavioral activation, like those that occur during the transition from sleep to waking (Jacobs and Fornal, 1999; Portas et al., 2000) or periods of stress and anxiety (Abrams et al., 2004; Grahn et al., 1999). In addition to behavioral transitions, the activity of some 5-HT-releasing neurons shows transient fluctuations with the presentation of simple environmental stimuli, like light and sound (Heym et al., 1982; Waterhouse et al., 2004). 5-HT release also varies in target regions during more complex behavioral paradigms or social interactions (Mas et al., 1995; Boutelle et al., 1990; Clement et al., 1998). As a result of both context- and region-specific influences on mechanisms that regulate 5-HT neurotransmission, 5-HT responses to a behavioral event can occur on a time course of minutes or less,

with magnitudes and temporal profiles tailored to specific brain regions (Inoue et al., 1994; Pum et al., 2008).

To investigate the behavioral and environmental conditions that influence the 5-HTergic filtering of auditory processing, we measured extracellular 5-HT in the IC using voltammetry. Voltammetry has been used in other regions of the brain to measure 5-HT or the 5-HT metabolite, 5-hydroxy-indole-acetic acid (5-HIAA), as indicators of 5-HT release (Bunin and Wightman, 1998; Crespi et al., 1990; Houdouin et al., 1991) and metabolism (Crespi, 1990; Lindefors et al., 1997). IC 5-HT was measured in conditions that have evoked either increases in the activity of dorsal raphe neurons or increases in 5-HT or 5-HIAA in previous studies, including during the presentation of simple sensory stimuli or stressors. In the IC, some of these stimuli increase extracellular 5-HT as predicted, but others do not. These findings suggest that the neuromodulation of IC neurons by 5-HT occurs during a specific set of conditions.

MATERIALS AND METHODS

Animals

Data were obtained from 52 male CBA/J mice (*Mus musculus* L.; Jackson Laboratory, Bar Harbor, ME, USA). Mice ranged in age from 5 to 22 weeks and averaged 11.3 ± 0.6 (s.e.m.) weeks old. Mice were housed individually on a 14h:10h light:dark cycle and supplied with food and water *ad libitum*. All protocols were approved by the Bloomington Institutional Animal Care and Use Committee.

Surgery

Head stages were surgically mounted on the skull overlying the IC to permit voltammetric recording in behaving mice. After a brief exposure to isoflurane fumes, mice were anesthetized with intraperitoneal (i.p.) injection of ketamine (120 mg kg^{-1}) and xylazine (5 mg kg^{-1}). Anesthetic state was determined by lack of response to tail pinch. The hair on top of the head was removed with depilatory cream, lidocaine was injected subcutaneously above the surgical site, and mice were placed in a stereotaxic device for surgery (Stoelting, Wood Dale, IL, USA). The skin was incised to expose the skull from bregma to the musculature of the neck. Bregma and lambda were placed in the horizontal plane, and holes $\sim 1.5 \text{ mm}$ in diameter were drilled over the left and right IC (1.1 mm posterior, 1.6 mm lateral to lambda). Custom-made Teflon[®] hubs (Rebec et al., 1993) were placed above each hole at an angle of 15 deg. from vertical and secured to the skull with two stainless steel bone screws (PlasticsOne, Inc., Roanoke, VA, USA) and dental cement. Hubs mated with custom-made micro-drives containing the recording and Ag/AgCl reference electrodes. Between experiments, the hubs were plugged with custom-made Teflon[®] screws. Mice were allowed to recover from surgery for at least 5 days prior to voltammetric recording.

Voltammetry

Carbon fiber electrode construction

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

Pretreatment

Carbon fiber electrodes were electrically and chemically treated prior to use. These treatments increase the sensitivity to and selectivity for 5-HT. To separate the electrochemical signal of 5-HT 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 , $0\text{--}3 \text{ V}$ 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. This treatment modifies the reactive surface of the carbon fiber and causes the 5-HT oxidation current to be produced at approximately $+250 \text{ mV}$ (vs Ag/AgCl) (Gonon et al., 1981). 5-hydroxy-indole-acetic acid (5-HIAA) and uric acid (UA) also oxidize at this voltage. To decrease the contribution of 5-HIAA and UA to the 5-HT oxidation signal, all electrodes were coated with Nafion[®] (Brazell et al., 1987; Rivot et al., 1995). Nafion[®] is an ion exchange resin that decreases the signal of negatively charged molecular species, such as 5-HIAA and UA. Electrodes were dipped in a 5% solution of Nafion[®] (Sigma-Aldrich, St Louis, MO, USA) 5–7 times and allowed to air dry for 5 min between each dip.

In vitro calibration

Electrodes were pretested *in vitro* in phosphate-citrate buffered saline containing $50 \mu\text{mol l}^{-1}$ ascorbic acid (AA), $5 \mu\text{mol l}^{-1}$ 3,4-dihydroxyphenylacetic acid (DOPAC) and $1 \mu\text{mol l}^{-1}$ 5-HT (Sigma-Aldrich). AA, DOPAC and 5-HT were used in the testing standard because they oxidize at different voltages and produce three distinct signals after the electrode is electrically treated. Also, the AA and DOPAC signals are dramatically reduced by coating the electrodes with Nafion[®]. A staircase waveform (-300 mV to $+600 \text{ mV}$ to -300 mV vs Ag/AgCl, 10 mV steps, 30 mV s^{-1}) was applied to the carbon fiber electrode to isolate the current produced by each of these molecules. This waveform was also used in all *in vivo* recordings. EChem software (EDAQ) was used to generate the voltammetric waveform and record the resulting signal. The electrode was used if there was no discernable signal from AA and DOPAC, indicating the integrity of the Nafion[®] coating, and a 5-HT signal at approximately $+250 \text{ mV}$, indicating the effectiveness of the electrical pretreatment. During some *in vitro* calibration procedures, 5-HIAA was added to the calibration solution after the initial measurements to demonstrate that it had no effect on the 5-HT signal measured at approximately $+250 \text{ mV}$.

In vivo assessment

In a subset of animals, to demonstrate that the signal measured *in vivo* was generated by 5-HT, two types of pharmacological manipulations were performed during voltammetric recording. One type of manipulation was to inject mice with a 5-HT precursor, L-5-hydroxy-tryptophan (L-5-HTP) (50 mg kg^{-1} ; Sigma-Aldrich), which increases the concentration of extracellular 5-HT (Blair et al., 1990; Shimizu et al., 1989). The major metabolite of 5-HT, 5-HIAA, which oxidizes at the same potential as 5-HT, was also manipulated. To confirm that 5-HIAA did not contribute to the *in vivo* voltammetric signal at 250 mV , its synthesis was blocked by i.p. injection of a monoamine oxidase inhibitor, clorgyline (10 mg kg^{-1} ; Sigma-Aldrich) (Celada and Artigas, 1993; Rivot et al., 1995). All injections took place while animals were anesthetized. After electrode placement, mice received a supplemental dose of anesthetic (30 mg kg^{-1} ketamine, 1 mg kg^{-1} xylazine) subcutaneously to maintain anesthesia for 1–2 h. 5-HT was monitored for 15–25 min prior to drug injection to ensure that baseline recordings were stable.

All pharmacological manipulations were the last manipulation performed prior to euthanasia. This was done to prevent the potential long-term effects that these drugs could have on the 5-HTergic system from influencing the effects of other manipulations.

Recording

Immediately prior to recording, mice were anesthetized with ketamine (90 mg kg⁻¹) and xylazine (1 mg kg⁻¹) for the placement of the electrodes. Both the recording and reference electrodes were mounted in custom-made Teflon[®] microdrives that screwed into the hubs mounted on the skull. The recording electrode was lowered into the IC, and the Ag/AgCl reference electrode was lowered until it came into contact with the cerebrospinal fluid. Immediately after electrode placement, mice were returned to their home cage in a sound-attenuated Faraday chamber. Mice were placed on a 10 cm² piece of laboratory tissue to separate them from the bedding material. A lightweight, flexible tether connected the recording and reference electrodes to a bipotentiostat (EI-400; Cypress Systems, Chelmsford, MA, USA) through an electric swivel (PlasticsOne, Inc.). This allowed the mouse to move freely around the cage during the experiment.

Voltammetric recording took place in two sessions, one from each IC, with at least a five-day recovery period between sessions. Mice were exposed to stimuli no less than one hour after recovering from anesthesia. After the first experiment, because electrode properties can change over time in the brain, the carbon fiber electrode was retested *in vitro* in the calibrating solution to confirm the separation and isolation of the 5-HT oxidation signal. After the second experiment, a current (~0.3 mA) was passed through the carbon fiber electrode for up to 5 s using a constant current lesion maker (Grass Instruments, Quincy, MA, USA) to make an electrolytic lesion and confirm localization of the electrode to the IC. Lesions were made only after the final experiment.

Behavioral state

To investigate changes in IC 5-HT over behavioral state, we recorded from mice as they recovered from a surgical dose of anesthetic. Mice were visually monitored during their recovery from anesthesia, and the time at which they recovered the ability to walk was recorded. A mouse was deemed to have recovered from anesthesia when it was able to walk off the 10 cm² piece of laboratory tissue upon which it was placed while anesthetized. These mice were compared to mice maintained in a constant anesthetic plane, as determined by tail pinch, with supplemental doses of anesthetic (30 mg kg⁻¹ ketamine; 1 mg kg⁻¹ xylazine) administered subcutaneously. In this experiment, we recorded with both Nafion[®] and non-Nafion[®]-treated electrodes to allow for the measurement of 5-HT and 5-HIAA. Voltammetric measurements were made every 6 min (1 min to record the trace, 5 min between each trace) for the duration of the recording session.

Stimuli

Mice were exposed to a range of stimuli, including an auditory stimulus, food presentation, an olfactory stimulus, a visual stimulus, and a movement restriction arena.

The auditory stimulus consisted of continuous broadband (0–60 kHz) noise presented at 94 dB SPL for 30 min (*N*=9). The broadband noise stimulus was generated using custom written BatCalls software (Dr Donald Gans, Kent State University, USA), passed through an amplifier (Servo 200; Samson Audio, Hauppauge, NY, USA) and delivered through a pair of speakers (Panasonic CJ-DVD137; Matsushida Electric Co., Ltd, Taiwan), one on either side

of the recording chamber. Noise intensity was calibrated with a 0.65 cm microphone placed in the center of the home cage (PS9200 kit, ACO Pacific, Belmont, CA, USA).

The food stimulus consisted of a piece of laboratory mouse chow (Rat Diet 5012; PMI Nutrition International, Richmond, IN, USA) approximately 3 cm long (*N*=5) or a novel food stimulus consisting of a small amount of peanut butter or piece of commercial snack chip placed in the center of the cage (*N*=4). The mouse was allowed to freely interact with the food. For all responses to the food stimulus included in the analysis, mice consumed the offered food. Presentation of novel foods did not cause a significantly different response from presentation of mouse chow (two-tailed unpaired *t*-test, *P*=0.421).

The olfactory stimulus (*N*=11) consisted of 25 μl of 99% solution of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) (Pherotech International, Delta, BC, Canada) placed on a tissue in a perforated 0.5 ml microcentrifuge tube. The olfactory stimulus control consisted of a similar arrangement using water instead of TMT. TMT is a component of red fox (*Vulpes vulpes*) urine and evokes a strong aversive response in rodents (Hayley et al., 2001). The visual stimulus (*N*=7) consisted of a 60 W light turned on approximately 1 m above the cage after at least 30 min of dark acclimation.

The restricted movement manipulation (*N*=10) was modeled after restraint–stress paradigms that are known to influence extracellular 5-HT in other regions of the brain (Bowers et al., 2007; Clement et al., 1998). Mice were contained within a small, circular arena (7.5 cm diameter) within their home cage. This was achieved by surrounding the animal with a 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 this behavioral arena limits the lateral range of travel without prohibiting other types of movement.

Most mice were exposed to a single stimulus. In some cases, a single mouse was exposed to multiple stimuli. For these mice (*N*=6), each stimulus was presented for 30 min or less, and 30 min was allowed for recovery. Only auditory, food or visual stimuli, in random order, were presented in this way.

Data analysis

The concentration of extracellular 5-HT was measured as the height of the 5-HT oxidation peak in the first derivative of the voltammetric current trace (Chen et al., 2007). Analyses of covariance (ANCOVA, $\alpha=0.05$) were used to determine if the 5-HT signal diverged from control over time. To account for differences in the sensitivities of individual electrodes, 5-HT peak values were normalized to the average of two measurements. For drug treatments, these were the measurements taken just prior to drug administration. For stimuli, these were the two measurements taken just prior to stimulus presentation. Measurements for the ‘anesthetized-to-wake’ transition were normalized to the average of the first two sequential measurements taken during a recording session. Error bars in figures represent s.e.m.

The effects of sensory stimuli were measured as changes in the pre- and post-stimulus 5-HT signal. For display purposes, changes in peak height are expressed as proportional changes. The magnitude of 5-HT change was determined by two-tailed repeated-measures *t*-tests comparing 5-HT peak values measured immediately before and after stimulus presentation. Using a power analysis based on control data collected during experiments where no stimulus was presented, we determined that the smallest significant change in 5-HT that we could measure in the stimulus group with the smallest

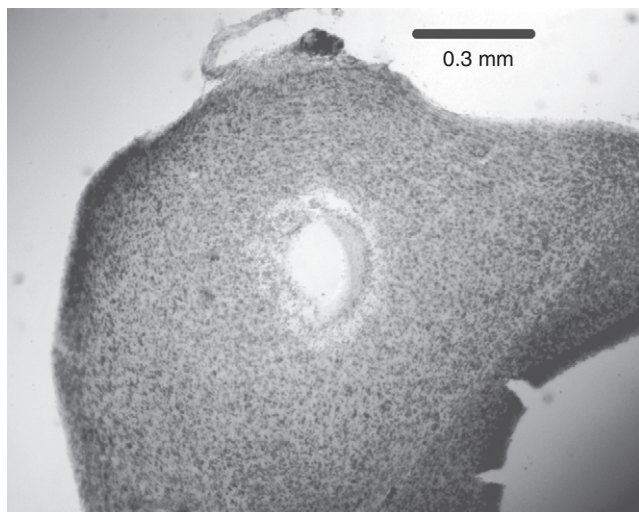


Fig. 1. Nissl stained coronal section of mouse brain showing a representative electrolytic lesion site within the IC created by a carbon fiber voltammetry electrode.

number of animals (5) was 7% and the change in 5-HT in the largest group (11) was 5%.

RESULTS

Pharmacology

Voltammetry with Nafion[®]-coated carbon fiber electrodes was used to measure 5-HT in the IC (Fig. 1). Changes in extracellular 5-HT were measured as the difference in the amplitude of the current produced at approximately +250 mV vs Ag/AgCl reference (Fig. 2, inset). We artificially manipulated the neurochemical environment of the IC to demonstrate the ability of the Nafion[®]-coated electrodes to selectively measure 5-HT *in vivo*. These manipulations were to (1) increase extracellular 5-HT with an i.p. injection of the 5-HT precursor L-5-htp (50 mg kg⁻¹, *N*=7) (Bluer et al., 1990; Shimizu et al., 1989) and (2) block the synthesis of the 5-HT metabolite, 5-HIAA, with the monoamine oxidase inhibitor clorgyline (10 mg kg⁻¹, *N*=7) (Celada and Artigas, 1993; Rivot et al., 1995). Fig. 2 presents the effects of these manipulations on the size of the 5-HT peak over time.

Values are normalized to the average of the two recordings taken prior to drug injection for each individual electrode to control for differences in electrode sensitivity. The time of injection is marked by the vertical line, and injections were made while mice were anesthetized. The data displayed were taken only while mice remained anesthetized, and the averages in Fig. 2 are only reported to time points at which half of the mice in our experiments woke from anesthesia. Values from awake mice were not included in these averages, so the values reported for later measurements of the treatment groups include between 50% and 100% of the animals in each population.

The injection of the saline vehicle (0.1 ml per 20 g mouse mass, depicted by crosses in Fig. 2, *N*=5) was used as a baseline for comparison for drug injection. The decrease in the size of the 5-HT peak is consistent with the loss of electrode sensitivity over time demonstrated by carbon fiber electrodes as current is repeatedly applied (Anastassiou et al., 2006; Marinesco and Carew, 2002). In contrast to this baseline, the injection of L-5-HTP significantly increased the concentration of extracellular 5-HT in the IC

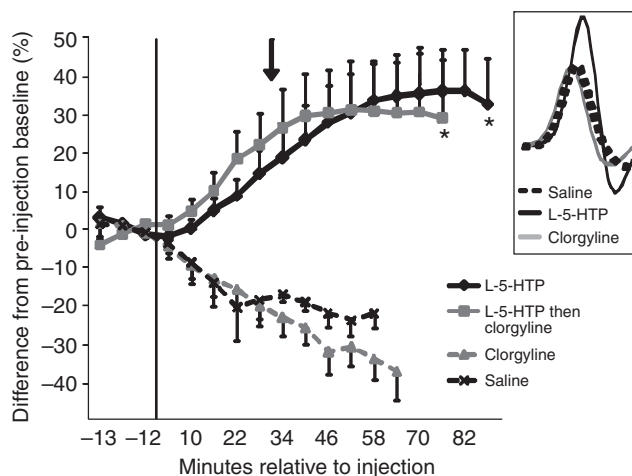


Fig. 2. Nafion[®]-coated electrodes selectively measure serotonin (5-HT) *in vivo*. Initial injections occurred at Time=0 (solid vertical line). The injection of L-5-HTP (*N*=7, black diamonds) increased the *in vivo* voltammetric signal above control levels (*N*=5, black crosses; ANCOVA, $F_{1,121}=18.26$, $P<0.001$). The injection of clorgyline prevented the synthesis of 5-hydroxyindole-acetic acid (5-HIAA) and had no effect on the baseline voltammetric signal when given alone (*N*=7, gray triangles; ANCOVA, $F_{1,77}=0.17$, $P=0.684$) or when given following an L-5-HTP injection (*N*=7, gray squares, L-5-HTP injection at line, clorgyline injection at arrow; ANCOVA, $F_{1,179}=0.97$, $P=0.325$). Inset: representative voltammetric current traces taken 46 min after the injection of L-5-HTP (solid black line), clorgyline (solid gray line) or saline vehicle (broken black line). Values are means \pm s.e.m.

(ANCOVA, $F_{1,121}=18.26$, $P<0.001$; diamonds in Fig. 2), which remained elevated for the duration of the recording session. This elevation in signal plateaued at 36% greater than pre-injection values at 70–76 min post-injection. The time of recording for mice injected with saline was shorter than other groups because these mice recovered from anesthesia more rapidly.

In other studies, the injection of L-5-HTP resulted in an increase in extracellular 5-HT and extracellular 5-HIAA (Nakatani et al., 2008; Okada et al., 1972). To demonstrate that the change in our *in vivo* signal is attributable to 5-HT alone, we inhibited the synthesis of 5-HIAA with clorgyline. Administration of clorgyline alone (Fig. 2, triangles) or after the injection of L-5-HTP (Fig. 2, arrow, squares) had no effect on the size of the 5-HT peak. This was the result expected if the Nafion[®] coating of our electrodes excludes contamination by 5-HIAA (Marinesco et al., 1996; Rivot et al., 1995). These results are all consistent with the selective measurement of 5-HT by our Nafion[®]-coated electrodes.

5-HT changes in behaving mice

To investigate the responsiveness of extracellular 5-HT in the IC to changes in behavioral state and environment, we monitored 5-HT in the ICs of mice transitioning from anesthesia to waking and, once awake, in response to different types of sensory stimuli.

Transition from anesthesia to waking

To determine whether the amount of 5-HT in the IC reflects the general level of behavioral arousal, as it does in other regions of the brain (Adell et al., 2002; Jacobs and Fornal, 1999), we recorded mice during waking from anesthesia (*N*=24). The waking mice were compared to mice that were maintained in a constant anesthetic plane for 90 min, as determined by tail pinch (*N*=4). Fig. 3 plots the

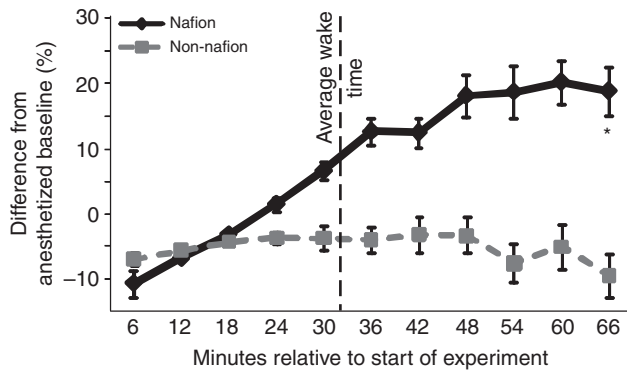


Fig. 3. Levels of inferior colliculus (IC) serotonin (5-HT) and 5-HT+5-HIAA in mice during waking. The voltammetric signal measured with Nafion[®]-coated electrodes increased as mice woke from anesthesia relative to a baseline of maintained anesthesia (black diamonds, Waking $N=24$, Anesthetized $N=4$; ANCOVA, $F_{1,316}=44.81$, $P<0.001$). When measured with non-coated (i.e. non-selective) electrodes, the voltammetric signal remained the same (gray squares, Waking $N=23$, Anesthetized $N=6$; ANCOVA, $F_{1,403}=0.27$, $P=0.606$). In the waking mice, the average waking time, determined by the recovery of motor function, was 33 ± 3 min (broken vertical line). Values are means \pm s.e.m.

difference between these two groups (diamonds). Mice allowed to recover from anesthesia showed significantly elevated extracellular IC 5-HT relative to anesthetized mice (ANCOVA, $F_{1,316}=44.81$, $P<0.001$). Extracellular 5-HT gradually increased with time, plateauing shortly after the average time of recovery of motor function (Fig. 3, vertical broken line), at about 25% greater than that measured in the anesthetized mice.

To assess whether the changes in extracellular 5-HT resulted in increased turnover of 5-HT, we additionally used electrodes that were not coated with Nafion[®] to measure the joint 5-HT+5-HIAA signal, which is mostly composed of 5-HIAA (Crespi, 1990). In contrast to the 5-HT, there was no difference in the joint voltammetric signal between mice under maintained anesthesia ($N=6$), and mice that woke from anesthesia ($N=23$; ANCOVA, $F_{1,403}=0.27$, $P=0.606$). Thus, the correlation with the level of behavioral arousal in the IC was specific to 5-HT and did not translate into changes in IC 5-HIAA, demonstrating the need to use Nafion[®]-coated, 5-HT-selective electrodes in our subsequent measurements.

IC 5-HT selectively responds to sensory stimuli

To examine the effects of external stimuli on extracellular 5-HT in the IC, we exposed awake animals to sensory stimuli of different modalities. Treatments included broadband noise, food, light and TMT (a component of fox urine). These treatments were chosen because visual and auditory stimulation can influence the firing of raphe neurons, and aversive stimuli, like TMT, can influence extracellular 5-HT in other regions of the brain. Mice exposed to these stimuli were compared to a control group with no stimulus presentation ($N=10$). Of all these treatments, only the broadband noise increased extracellular 5-HT significantly relative to the control group (Fig. 4, triangles, $N=9$, ANCOVA, $F_{1,51}=12.44$, $P=0.053$). Extracellular 5-HT increased sharply in the first measurement, taken 5 min after the onset of noise, and continued to increase gradually up to 17 min after the onset of noise. No such change in IC 5-HT was seen in response to bright light (Fig. 4, squares, $N=7$, ANCOVA, $F_{1,43}=1.36$, $P=0.249$) or the exposure to

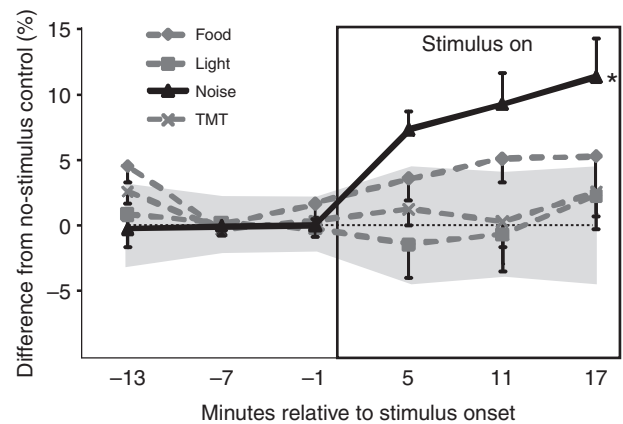


Fig. 4. Serotonin (5-HT) levels during the presentation of a variety of stimuli (boxed area). Mice exposed to noise (94 dB SPL, $N=9$, black triangles) demonstrated an increase in inferior colliculus (IC) 5-HT relative to control mice ($N=10$, black broken line, s.e.m. = shaded region; ANCOVA, $F_{1,57}=12.44$, $P=0.053$). Mice exposed to food ($N=9$, gray diamonds), light ($N=7$, gray squares) or TMT ($N=11$, gray crosses) showed no change in IC 5-HT relative to the no-stimulus control group. Values are means \pm s.e.m.

food, a non-aversive stimulus (Fig. 4, diamonds, $N=9$, ANCOVA, $F_{1,51}=0.68$, $P=0.413$). The lack of effect of TMT (Fig. 4, crosses, $N=11$, ANCOVA, $F_{1,63}=0.00$, $P=0.98$) was especially interesting because mice had a strong behavioral response to this chemical, often attempting to bury the microcentrifuge tube containing the chemical and distancing themselves from it. No such behavioral or 5-HTergic reaction ($N=2$, ANCOVA, $F_{1,9}=0.23$, $P=0.644$) was observed in response to the saline odor control.

A final behavioral manipulation assessed extracellular 5-HT in the IC during exposure to a stimulus designed to mimic restraint stress, because extracellular 5-HT in other regions of the brain change during exposure to stress paradigms (Clement et al., 1998; Mitsushima et al., 2006). Mice ($N=9$) were restricted in a small circular arena within their home cage using a high-walled tube that slid down the recording tether to surround the animal. Mice were not directly restrained, but their lateral movement in this arena was severely limited. After restriction, 5-HT in the IC significantly deviated from control measurements (Fig. 5; ANCOVA, $F_{1,51}=20.27$, $P=0.001$). Similar to the presentation of noise, the peak 5-HT response occurred within the first measurement after the onset of restriction. The initial 5-HT peak was followed by a gradual decline throughout the restriction period (Fig. 5, box). Interestingly, when the restriction tube was removed, a secondary peak occurred and extracellular 5-HT remained elevated even 22 min after tube removal.

Changes in IC 5-HT vary in amplitude

Fig. 6 compares the percent change in the 5-HT signal measured just before and after stimulus onset for all types of stimuli presented. This represents the effect of 5 min of stimulus exposure on IC 5-HT. The greatest immediate change in IC 5-HT was seen in response to the restriction stimulus. Extracellular 5-HT in the IC increased by an average of $14.2\pm 4.9\%$ within 5 min of restriction (paired t -test, $t=-5.44$, $P=0.001$). A smaller, but still significant, increase was seen in the IC of mice exposed to broadband noise at 94 dB. The IC 5-HT signal showed a $5.2\pm 1.5\%$ increase above pre-stimulus levels in these mice immediately after the onset of noise (paired t -

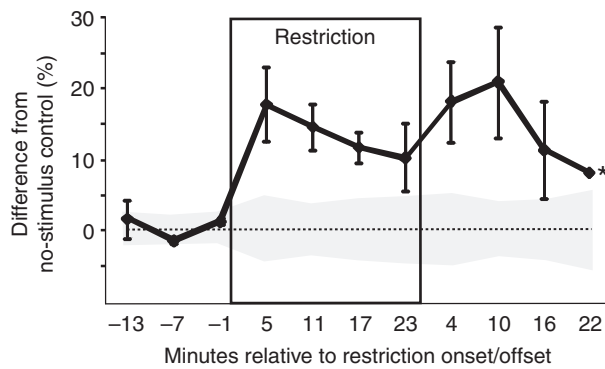


Fig. 5. Serotonin (5-HT) levels respond to restricted movement stress. Mice exposed to restricted movement ($N=9$, boxed area, black diamonds) displayed an increase in inferior colliculus (IC) 5-HT relative to control mice ($N=10$, black broken line, s.e.m. = shaded area; ANCOVA, $F_{1,51}=20.27$, $P=0.001$). Values are means \pm s.e.m.

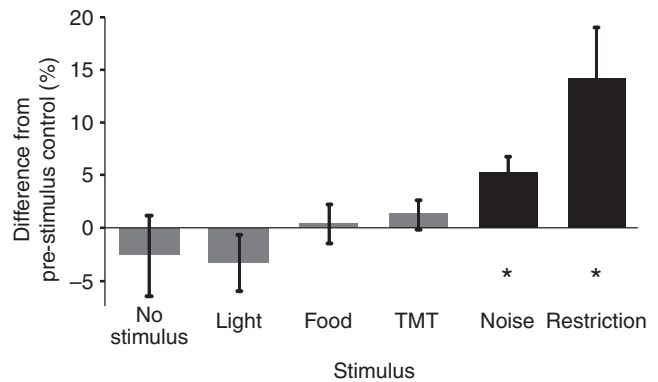


Fig. 6. Summary of changes in 5-HT during different stimulus paradigms. Only the noise (paired t -test, $t=-3.86$, $P=0.005$) and restriction (paired t -test, $t=-5.44$, $P=0.001$) stimuli induced significant changes in IC 5-HT relative to pre-stimulus values. Values are means \pm s.e.m.

test, $t=-3.86$, $P=0.005$), and values continued to increase during continual noise presentation. A power analysis based on the sample sizes of different groups indicates that an increase in extracellular 5-HT in the mice exposed to light would not be detected unless it was up to 2% greater than the response to noise, but this seems unlikely given the average in this group. In comparison to these values, mice showed an increase in extracellular 5-HT of approximately 25% from the anesthetized to awake states. Thus, the increases in extracellular IC 5-HT during behavioral arousal and in awake animals are capable of reaching a range of amplitudes in response to different types of stimuli.

DISCUSSION

We measured extracellular 5-HT in a midbrain auditory nucleus, the IC, to investigate the behavioral and environmental conditions under which 5-HTergic neuromodulation occurs in this nucleus. Our data are consistent with 5-HT serving as a relatively rapid and selective signal of some environmental events, as well as of increasing levels of internally driven behavioral arousal.

Measuring 5-HT with voltammetry

We measured extracellular 5-HT in the IC as an indicator of the level of 5-HTergic modulation of auditory processing in this region. Alternatively, voltammetric studies in other regions of the brain have measured extracellular 5-HIAA, which can potentially be used as an indicator of 5-HT's release, reuptake and then subsequent metabolism (Clement et al., 1998; Crespi et al., 1990; Houdouin et al., 1991). In the IC, however, we found that changes in the combined 5-HIAA+5-HT signal were not correlated with changes in the 5-HT signal during the recovery from anesthesia (Fig. 3). The lack of correlation between changes in extracellular 5-HT and 5-HIAA is not an uncommon finding (Auerbach et al., 1989; Crespi et al., 1990; Rivot et al., 1995). An underlying reason for this may be that the concentration of 5-HIAA in the extracellular fluid can be up to 1000 times greater than that of 5-HT (Stenfors and Ross, 2004). In voltammetry, the 5-HIAA oxidation signal occurs at the same potential as the 5-HT oxidation signal, so relatively small changes in 5-HT may not be detectable in the presence of a large 5-HIAA signal. Thus, we treated our electrodes with Nafion[®], which minimizes the detection of 5-HIAA and related interferants, to measure 5-HT selectively in the IC.

IC 5-HT fluctuates with behavioral state

To investigate the effect of behavioral state on 5-HT neurotransmission in the IC, we monitored 5-HT in the IC of mice as they woke from anesthesia. Compared to mice that were constantly anesthetized, levels of 5-HT in the IC progressively increased during the process of waking. Extracellular 5-HT stabilized in waking animals at approximately 25% greater than in anesthetized controls. This was larger than the average 5-HTergic increase in response to any of the external stimuli tested, suggesting that baseline changes in 5-HT with internal state are larger than those imposed by transient events in the IC. These state-related changes in extracellular 5-HT could be one mechanism contributing to anesthesia and sleep-related changes in IC processing (Astl et al., 1996; Morales-Cobas et al., 1995; Tortorolo et al., 2002).

The amplitude of the anesthesia-mediated decrease in 5-HT was relatively small, however, when compared to the change seen during the sleep cycle in other studies. In cats that are awake, for example, the 5-HT signal in the striatum is more than double that of cats in REM sleep (Trulson, 1985). In addition, although both sleep and anesthesia are correlated with a drop in the activity of 5-HT-releasing neurons, the decrease in firing rates during sleep, particularly REM sleep, is also larger than that observed during anesthesia (Heym et al., 1984; Trulson, 1985). Our measurement of 5-HT during recovery from anesthesia may therefore represent an underestimate of the maximum change in extracellular 5-HT during naturally occurring transitions in behavioral state in the IC.

5-HT fluctuations during environmental events

We investigated the conditions other than state that influence 5-HT neurotransmission in the IC. To do so, we exposed awake mice to stimuli that were strongest within a single modality, including broadband noise and predator odor, as well as stimuli that could be considered multisensory, including the presentation of food and a restricted area of movement. Though carbon fiber electrodes produce less injury than probes for microdialysis, the other option for measuring extracellular serotonin in behaving animals (Jaquins-Gerstl and Michael, 2009), the surgical manipulation required to implant the voltammetric electrodes and the tether through which we made our recordings could have an impact on the mouse's behavioral response to these stimuli. Although behavior was not quantified during these experiments, care was taken to minimize

the impact of experimental manipulations on mice, and each mouse demonstrated a range of behaviors typical to that of a non-manipulated mouse in response to these stimuli.

Using our methods, we measured significant increases in extracellular 5-HT in the IC following only exposure to restricted movement or broadband noise (Fig. 6). In another study, Cransac et al. found that IC 5-HT did not change after 45 min of exposure to broadband noise ranging up to 110 dB (Cransac et al., 1998). By contrast, we measured an increase in 5-HT after less than 5 min of exposure to 94 dB broadband noise. The disparity in the findings of the two studies could have resulted from a number of methodological differences; these include the technique used to measure 5-HT (voltammetry vs high-performance liquid chromatography on tissue homogenates), species differences (rats vs mice) or the duration of sound exposure. Recording for a longer time in our preparation could determine whether extracellular 5-HT increases and then subsequently decreases following noise presentation.

All the stimuli we tested were chosen for their ability to influence the concentration of extracellular 5-HT or 5-HIAA, or the activity of 5-HT-releasing neurons in studies of other brain regions. Light and sound influence the activity of 5-HT-releasing neurons and extracellular 5-HT across different areas of cortex (Heym et al., 1982; Pum et al., 2008). The ingestion of food increases c-fos immunoreactivity in the dorsal and median raphe nuclei, and the presence of food increases the amount of 5-HT released in response to predator exposure in hippocampus, corpus striatum, amygdala and prefrontal cortex (Rueter and Jacobs, 1996; Takase and Nogueira, 2008). Exposure to TMT increases extracellular 5-HT in the hippocampus, prefrontal cortex, central amygdala and frontal cortex (Hayley et al., 2001; Smith et al., 2006), and restricted movement increases extracellular 5-HT in frontal cortex, amygdala and hippocampus (Mitsushima et al., 2006; Smith et al., 2006; Vahabzadeh and Fillenz, 1994). Of all these manipulations, however, only the restriction of movement or broadband noise caused extracellular 5-HT to increase in the IC. These findings highlight the regional and contextual selectivity of neuromodulation by 5-HT.

Stimulus-specific selectivity in the 5-HT response to environmental stimuli has been demonstrated in frontal cortex and hippocampus (Beekman et al., 2005; Fujino et al., 2002). Multiple regulatory mechanisms, such as the local distribution of raphe projections or reuptake transporters, can influence this specificity, but there are several alternative explanations for the selectivity in the 5-HTergic response we observed within the IC. One possibility that could account for this finding is that the stimuli tested were not presented in a manner sufficient to elicit a 5-HTergic response in the IC as they did in other studies; perhaps the stimuli were not sufficiently intense. The light stimulus, for example, had no effect on IC 5-HT in the CBA/J mice used in this study. CBA/J mice are a well-established model system in auditory studies but they also have degenerate retinas, which may have led to a diminished perception of the light stimulus (Jackson Laboratory, Bar Harbor, ME, USA). Alternatively, 5-HT may represent some quality of the stimulus in the IC. Such a quality would be shared by the stimuli that evoked increases in extracellular 5-HT and would not be present in the stimuli that did not evoke increases. Two possible stimulus qualities are (1) whether a stimulus evokes a positive or aversive reaction and (2) modality or the particular sensory systems activated by a stimulus.

5-HT has been associated with stress in response to aversive stimuli in a number of brain regions, and biochemical interactions

occur at multiple levels between the 5-HTergic and glucocorticoid systems (Gasser et al., 2006; Lanfumey et al., 2008; Linthorst and Reul, 2007). Of the stimuli we presented, the two that did evoke significant increases in extracellular IC 5-HT were both potentially stressful. The restricted movement stimulus is a mild version of restraint, used to induce stress experimentally (Bowers et al., 2007; Clement et al., 1998). The broadband noise was relatively loud at 94 dB and so could also have created stress in the mice (Day et al., 2009; Rex et al., 2005; Vitale et al., 2005). What is not consistent with the hypotheses that 5-HT simply signals stressful events is the lack of response to the presentation of TMT, the component of predator urine, which has been used as a stressor in other studies (Hayley et al., 2001). Mice demonstrated a robust aversive behavioral response to this odor, burying and avoiding vials containing TMT but not responding to control vials containing saline. The stimulus-induced change in motor activity could also have influenced 5-HT release (Jacobs and Fornal, 1999), but the presence of TMT and the subsequent behavioral response did not evoke a significant increase in 5-HT in the IC, indicating that IC 5-HT is not purely a signal of stress or motor behavior.

A second way to explain the pattern of 5-HTergic increases in the IC is that they signal the activation of a particular sensory modality. Of the simple sensory stimuli, the most effective at causing an increase in 5-HT was the auditory stimulus. If 5-HTergic increases in the IC were specific to auditory stimuli, this would be somewhat surprising. This is because the 5-HTergic neurons that innervate the IC are located outside of the auditory system, mostly in the dorsal and median raphe nuclei (Klepper and Herbert, 1991). Although dorsal and median raphe neurons have been reported to respond to auditory stimuli, they are not auditory neurons *per se* and also respond to the stimulation of other sensory modalities (Bouwknicht et al., 2007; Jacobs and Fornal, 1999; Heym et al., 1982; Waterhouse et al., 2004). Within a single raphe nucleus, different subgroups of neurons do receive inputs from different sources and send projections to different sensory areas or even to different sub-compartments of single neurons (Abrams et al., 2004; Amargós-Bosch et al., 2004; Hornung, 2003), but whether this could serve as a basis for modality-specific modulation of sensory areas is unknown.

A specific argument against modality-specificity is that the restriction stimulus took place in the absence of auditory stimulation but the IC 5-HT response to this stimulus was even greater than the response to broadband noise. The 5-HT-releasing neurons that project to the IC can be responsive to stress and behavioral arousal (Abrams et al., 2004; Grahn et al., 1999). Restricted movement is not an auditory stimulus but it may induce a behavioral state change that could influence the activity of the raphe neurons and thereby the modulation of auditory processing in the IC. The change in IC 5-HT, therefore, appears not to be a response to auditory stimuli, but a response to the behavioral or environmental conditions during which the modulation of auditory processing occurs.

Our data do not support the idea that 5-HT increases in the IC exclusively signal aversive or auditory events. It seems more likely that a change in 5-HT accompanies complex and naturalistic multimodal events such as social interactions or foraging bouts. This hypothesis remains to be tested.

Functional implications

The regional and contextual selectivity of changes in 5-HT suggest functional differences in the behavioral salience of neuromodulation by 5-HT in different regions of the brain. Although the nature of the 5-HTergic signal in the IC must be characterized further, the

results presented here have identified features of 5-HT release in the IC that have implications for its function. One of these features is that the size of the 5-HT signal is variable across different types of stimuli. The largest increase in 5-HT observed occurred during waking from anesthesia, followed by 5-HT increases during restriction and in response to noise (Figs 4, 6). Extracellular 5-HT also fluctuated over time even within particular behavioral paradigms, declining during prolonged restriction and rebounding slightly after the removal of restriction. This variability suggests that 5-HT is not released in an all-or-nothing fashion, but instead in a variable way that can be fine-tuned according to ongoing changes in the behavioral context. Another characteristic of 5-HTergic fluctuations is that they could respond to stimuli relatively rapidly, in addition to slowly increasing during recovery from anesthesia. In response to both noise presentation and to restriction, significant increases occurred by the time of the first post-stimulus recording.

These features of 5-HTergic fluctuations match well with characteristics of 5-HTergic mechanisms in the IC. The effects of exogenously applied 5-HT and of selective 5-HT receptor agonists are dose-dependent (Hurley, 2006), such that variable levels of extracellular 5-HT would correspondingly create effects of variable magnitude. 5-HT receptors *in vivo* respond to application of agonists on a time scale of minutes (Hurley and Pollak, 1999), within the range we measured for 5-HT release. The preliminary model that emerges from these observations is one of 5-HT release that is capable of scaling to complement the intensity of a behavioral context, and in a time window that would allow it to influence auditory processing during many types of behavioral interactions. In addition, the differences in the conditions that trigger 5-HT release in the IC when compared to other regions support the concept of 5-HT as a selective physiological signal that mediates functional differences in the regulation of neural processing in sensory systems.

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxy-indole-acetic acid
5-HT	serotonin
AA	ascorbic acid
DOPAC	3,4-dihydroxyphenylacetic acid
IC	inferior colliculus
i.p.	intraperitoneal
L-5-HTP	L-5-hydroxytryptophan
REM	rapid eye movement
TMT	2,5-dihydro-2,4,5-trimethylthiazoline
UA	uric acid

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