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Serotonin modulates response properties of neurons in the dorsal cochlear nucleus of the mouse

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Abstract

The neurochemical serotonin (5-hydroxytryptamine, 5-HT) is involved in a variety of behavioral functions including arousal, reward, and attention, and has a role in several complex disorders of the brain. In the auditory system, 5-HT fibers innervate a number of subcortical nuclei, yet the modulatory role of 5-HT in nearly all of these areas remains poorly understood. In this study, we examined spiking activity of neurons in the dorsal cochlear nucleus (DCN) following iontophoretic application of 5-HT. The DCN is an early site in the auditory pathway that receives dense 5-HT fiber input from the raphe nuclei and has been implicated in the generation of auditory disorders marked by neuronal hyperexcitability. Recordings from the DCN in awake mice demonstrated that iontophoretic application of 5-HT had heterogeneous effects on spiking rate, spike timing, and evoked spiking threshold. We found that 56% of neurons exhibited increases in spiking rate during 5-HT delivery, while 22% had decreases in rate and the remaining neurons had no change. These changes were similar for spontaneous and evoked spiking and were typically accompanied by changes in spike timing. Spiking increases were associated with lower first spike latencies and jitter, while decreases in spiking generally had opposing effects on spike timing. Cases in which 5-HT application resulted in increased spiking also exhibited lower thresholds compared to the control condition, while cases of decreased spiking had no threshold change. We also found that the 5-HT₂ receptor subtype likely has a role in mediating increased excitability. Our results demonstrate that 5-HT can modulate activity in the DCN of awake animals and that it primarily acts to increase neuronal excitability, in contrast to other auditory regions where it largely has a suppressive role. Modulation of DCN function by 5-HT has implications for auditory processing in both normal hearing and disordered states.

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Keywords

auditory brainstem; 5-HT; DCN; fusiform cell; neuromodulation

1. Introduction

The serotonergic system contributes to a variety of important behavioral functions including arousal, stress response, and attention (Jacobs and Fornal, 1999; Kähkönen and Ahveninen, 2002). In addition, this system provides widespread input to numerous central auditory regions that allow serotonin (5-hydroxytryptamine, 5-HT) to modulate sound-evoked properties including response magnitude (Ebert and Ostwald, 1992), spike timing (Hurley and Pollak, 2005b), and frequency tuning (Hurley and Pollak, 2001). In addition to its role in normal auditory function, the serotonergic system has been implicated in a variety of behavioral disorders, some of which have a substantial auditory component (Lucki, 1998). For instance, there is evidence that disturbance of the serotonergic system contributes to the development of tinnitus, a condition marked by the perception of phantom sounds, typically following acoustic trauma (Simpson and Davies, 2000). Although the underlying mechanisms that lead to tinnitus are not entirely clear, trauma-induced neuronal hyperactivity, which is first observed in the central auditory system in the dorsal cochlear nucleus (DCN), may be a leading cause (Marriage and Barnes, 1995; Kaltenbach, 2007).

The DCN is a direct target of the auditory nerve and it receives additional inputs from interneurons that convey auditory and somatosensory information (Cant and Benson, 2003; Shore, 2005) and from 5-HT neurons of the raphe nuclei (Klepper and Herbert, 1991; Thompson and Thompson, 2001). Several functional roles for the DCN have been proposed that reflect its multimodal integration including sound localization in the vertical plane (Nelken and Young, 1996; Oertel and Young, 2004), suppression of self-generated sounds (Shore, 2005; Shore and Zhou, 2006), and sound identification (Young and Davis, 2002; Roberts and Portfors, 2015). The involvement of the DCN in the suppression of self-generated sounds may act to enhance the relative salience of sounds from external sources. Due to its roles in arousal and attention, the serotonergic system is positioned to impact the activity of DCN neurons by modulating responsiveness to incoming sounds that may have behavioral importance. The serotonergic system may also play a key role in hyperactivity, as local stimulation of 5-HT fibers in the DCN can induce increased spiking of postsynaptic neurons (Tang and Trussell, 2015), which resembles hyperexcitability reported in animal models of tinnitus (Brozoski et al, 2002; Kaltenbach, 2007). The output of the DCN is primarily transmitted by fusiform cells that target the inferior colliculus (IC). The IC is a prominent midbrain nucleus that subserves a multitude of auditory functions. Like the DCN, is heavily innervated by 5-HT fibers (Thompson et al., 1994; Hurley et al., 2002) and thus, may play an important role in auditory disorders marked by neuronal hyperexcitability (Jastreboff and Sasaki, 1986; Bauer et al., 2008). In this study, we examined whether exogenous application of 5-HT affects basic auditory response properties of DCN neurons in awake mice. We found that although 5-HT modulated responses in a heterogeneous manner, the predominant effect was an increase in neuronal excitability. We also found that the 5-HT_{2A} receptor is likely mediating serotonin effects. These findings demonstrate that

neuromodulation mediated by 5-HT can shape neural responses to sound at low levels of the ascending central auditory system.

2. Materials and Methods

2.1 Animals

Single unit responses were recorded in 19 awake CBA/CaJ mice aged three to six months (7 female, 12 male). Animals were housed with same-sex littermates on a reversed 12-hour light/dark schedule and had free access to food and water. All animal care and use procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Washington State University, an AAALAC-accredited institution.

2.2 Surgical procedures

Surgical procedures were similar to previous studies (Roberts and Portfors, 2015) and have been described in detail elsewhere (Muniak et al., 2012). Briefly, a subcutaneous injection of ketoprofen was given before surgery. Anesthesia was maintained with isoflurane (0.5–2.5%) inhalation while the mouse was in a stereotaxic frame. A lightweight metal headpost was attached to the skull using dental cement. To gain access to the DCN, a craniotomy approximately one square millimeter was performed, centered 5.9 mm caudal to Bregma and 2.30 mm lateral of the midline (Paxinos and Franklin, 2001). The craniotomy was covered with bone wax and the exposed tissue was treated with lidocaine (3%), as well as a triple antibiotic ointment that contained neomycin, polymyxin B, and bacitracin. The animal was returned to its home cage for a recovery period lasting at least one day. The bone wax overlying the craniotomy was removed prior to each recording session and reapplied at the conclusion of the session.

2.3 Acoustic stimulation

Stimulus generation and data acquisition were controlled by custom-written software (SSHF, Amy Boyle, Washington State University Vancouver). Acoustic stimuli were output through a 16-bit digital-to-analog converter (500,000 samples/s; National Instruments), sent to a programmable attenuator (PA-5, Tucker Davis Technologies), and routed to a ribbon tweeter (LCY K100, Ying Tai Corporation) placed 10 cm from the ear ipsilateral to the recording site. The speaker output was calibrated before each recording session over a range of 3–120 kHz using a ¼ inch calibrated microphone (model 4135, Brüel & Kjær) positioned at the location normally occupied by the animal's ear. The gradual roll-off of intensity measured at high frequencies was corrected online so that the sound pressure level for each frequency at a given intensity varied by less than 2 dB SPL. Distortion components of tonal stimuli were buried in the noise floor at least 50 dB below the signal level as determined by analyzing fast Fourier transforms of the digitized microphone signals.

2.4 Recording and drug application procedures

Experiments were conducted in a sound-attenuating chamber. The mouse was given a mild sedative (acepromazine, 2 mg/kg) at the beginning of each session that aided in placement of the animal in a foam body restraint. This small dose of acepromazine has no effects on basic

auditory response properties (Felix et al., 2012). The headpost was secured to a stereotaxic device to render the head immobile and maintain a consistent position during recordings. The animal was monitored frequently and the experiment was terminated if the animal appeared distressed. Experimental sessions lasted no more than four hours, and each animal was used in one to three recording sessions separated by at least one day.

Recordings and application of pharmacologic agents were conducted using piggy-back electrodes (Havey and Caspary, 1980) made by gluing a single-barrel recording pipette onto a three-barreled glass pipette (10–15 μm total tip diameter), such that the tip of the recording pipette extended 10–15 μm beyond the tip of the multibarrel pipette. The recording pipette (1–2 μm tip diameter) was filled with 1 M NaCl solution, which yielded a pipette impedance of 15–30 M Ω . Two of the drug delivery barrels were filled with one of the following agents dissolved in water (pH 4.5): 30 mM of serotonin creatinine sulfate (5-HT; Sigma), 30 mM of the 5-HT_{2A} receptor agonist ($\pm$)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; Tocris), or 10 mM of the 5-HT_{2A} receptor antagonist Ketanserin (KS; Tocris). The remaining barrel was filled with 1 M NaCl and was used as a sum channel to balance the currents of the drug barrels.

Electrodes were advanced in the brain using a hydraulic micropositioner (David Kopf Instruments) and neural activity was amplified (Multiclamp700B, Axon Instruments), bandpass filtered (300–6000 Hz; Krohn-Hite), and digitized with a 16-bit analog-to-digital converter (50,000 samples/s; National Instruments). Individual raw waveforms were viewed online, recorded, then stored for offline analysis. Single-unit responses were isolated by advancing the electrode slowly through the DCN (located at a depth approximately 2.5–3.5 mm from the surface of the brain) while presenting a broadband noise search stimulus (70 dB SPL). Upon isolation of a unit, the characteristic frequency (CF) was determined, defined as the frequency that required the lowest sound level to elicit an evoked response. Thresholds were determined audiovisually for each neuron prior to data collection and were defined as a clear response of at least 0.5 spikes per stimulus above the rate of spontaneous activity. Spontaneous and evoked activity were determined by windowing the recording window online into separate epochs before and during the stimulus presentation and measuring the spiking rates for each time period. We targeted fusiform cells whose responses consisted of simple spikes, moderate levels of spontaneous activity, and V-shaped frequency tuning curves (Rhode and Smith, 1986), which were determined audiovisually. Recordings from other types of neurons in DCN were excluded, including cartwheel cells that produced characteristic complex spikes (Portfors and Roberts, 2007) and vertical cells that exhibited high thresholds to broad band noise stimuli combined with narrow frequency tuning (Rhode, 1999). The recording protocol was conducted for presumptive fusiform cells before, during, and after the application of pharmacologic agents. The experimental protocol consisted of the recording of activity in the absence of sound (spontaneous spiking) prior to each test, followed by the generation of a peristimulus time histogram (PSTH) of responses to 100 presentations of the CF pure tones presented 10 dB above threshold (evoked spiking), and a rate-level test that varied the intensity of the CF tone (10 presentations) from 10 dB below threshold to 40 dB above threshold.

While searching for neurons, a retention current of -10 nA was continually applied to prevent leakage from the drug barrels. After the isolation of a single unit response and the recording of baseline tests, agents were ejected with currents ranging from $+45$ to $+75$ nA for a variable period of time up to 20 minutes. Retention and ejection currents for each drug solution were controlled by a micro-iontophoresis unit (model 6400, Dagan Corporation). Maximal drug effects were signaled by changes in the neuron's spiking pattern greater than 10% of baseline that remained stable for two consecutive minutes. If this threshold was not reached, tests in the drug condition were terminated after 20 minutes of application. After data were collected for the drug condition, the ejection current was terminated and the retention current reapplied to allow the response to recover. In cases where the response was lost prior to recovery, 30 minutes elapsed before the electrode was moved to search for another neuron. To ensure that changes in responses were not due to the application current alone, we applied vehicle solutions for pharmacologic agents at the maximum ejection current used ($+75$ nA), as is routine in our lab (Gittelman et al., 2013, Mayko et al., 2012), and observed no effects on spiking rate as determined by a paired student's t-test ($p > 0.5$ in all cases).

2.5 Recording site deposits and 5-HT fiber staining

Recording sites in nine experiments were labeled by ejecting biotinylated dextran amine (BDA) from the recording pipette via current applied for five minutes (0.5 μ A, 50% duty cycle). To recover the tracer deposits, animals were deeply anesthetized with isoflurane and perfused with 10% phosphate-buffered formalin. The brains were cryoprotected overnight in a 20% sucrose solution and sectioned coronally at 30 μ m using a freezing microtome. Alexa Fluor 488 conjugated streptavidin (1:250, Life Technologies) was used to visualize BDA. In five experiments, we labeled 5-HT fibers in the same tissue that contained BDA deposits. These sections were incubated overnight at room temperature in a primary antibody solution of goat anti-serotonin polyclonal antibody (1:1500, Abcam), 3% normal donkey serum (Millipore), and 0.4% Triton X-100 (Sigma) in phosphate buffer (PB). Sections were then rinsed in PB and incubated for two hours at room temperature in a secondary antibody solution using a Donkey anti-goat Alexa Fluor 568 to visualize 5-HT fiber staining. Following washes in PB, tissue sections were mounted on Superfrost Plus slides (Fisher Scientific), dehydrated and cleared, and coverslipped with DPX (Electron Microscopy Sciences). Images were taken with a confocal microscope (TCS SP8, Leica).

2.6 Data analysis

Recorded waveforms were analyzed using custom software written in Python (SSHF). Spiking events were detected by a threshold feature that reliably separated single unit activity with consistent waveforms from the noise floor, with at least a 4:1 signal to noise ratio. Spiking rate, first spike latency, as well as the standard deviation of first spike latency (jitter) were calculated from PSTH tests. Prior to statistical analyses, tests were performed to determine whether each data set fit a normal distribution (D'Agostino and Stephens, 1986). Statistics were applied using either SSHF or graphing software used to generate data plots (Prism, Graph Pad). The paired Student's t test (two-tailed) was used to determine whether spiking in the control condition differed from the 5-HT condition for each neuron. In each case, differences in response magnitude that exceeded 10% corresponded to a significant

change (t-test, $p < 0.05$) in spiking, whereas differences below a 10% change were non-significant ($p > 0.05$). To simplify comparisons of responses from neurons with vastly different rates, we normalized spiking rates by dividing the mean spiking rate in the 5-HT condition by that in the control value. Thus, normalized values greater than 1.1 represented an increase in spiking ($> 10\%$ change), normalized values less than 0.9 represented a spiking decrease, and values between 0.9 and 1.1 represented no change. For cases in which data were not normally distributed, the Wilcoxon matched-pairs signed rank test was used to assess statistical significance. The Pearson correlation coefficient was used to quantify relationships between normalized values of measured parameters (Sheskin, 2011) and the F-test was used to determine whether the variance of first spike latencies was different between control and 5-HT conditions (Snedecor and Cochran, 1983). The critical significance level for each test was set at $\alpha = 0.05$ and values in the text represent the sample mean and standard deviation (SD).

3. Results

We recorded spontaneous and tone-evoked activity from 43 neurons (CFs: 7–50 kHz) to investigate how 5-HT affects basic auditory response properties in the DCN. Recordings were conducted before, during, and when possible, following the application of either 5-HT ($n = 23$), the selective 5-HT₂ receptor agonist DOI ($n = 12$), or the selective 5-HT₂ receptor antagonist Ketanserin ($n = 8$). We targeted fusiform cells, which represent the principal outputs neurons of the DCN (Cant and Benson, 2003; Roberts and Portfors, 2007), and observed spiking patterns that resembled those previously reported including pauser, build-up, and primary-like responses (Young and Brownell, 1976; Rhode et al., 1983). Tracer deposits in nine animals confirmed that recordings sites guided by stereotaxic coordinates were in layer II of the DCN (Fig. 1A), which primarily contains fusiform cells (Ryugo and Willard, 1985). Because the innervation pattern of 5-HT in the cochlear nucleus of mice has not been previously reported, we labeled 5-HT fibers in the same tissue that contained BDA deposits (Fig. 1A) in five animals. We found that 5-HT fibers densely innervated the DCN, with relatively sparse fiber labeling in the ventral cochlear nucleus (Fig. 1B), similar to other species (rat: Klepper and Herbert, 1991; bat: Hurley and Thompson, 2001).

3.1 5-HT has heterogeneous effects on evoked and spontaneous spiking rates

The application of 5-HT had mixed effects on spiking rate. The example in Figure 2A shows a neuron that had a significant increase in spiking rate from a mean of 69.8 spikes/s (SD 25.8) in the control condition to a mean of 95.5 spikes/s (SD 29.9) during 5-HT application (t test, $p < 0.0001$). The spiking rate returned to 77.3 spikes/s (SD 22.5) during recovery. The example in Figure 2B shows a neuron that had a significant decrease in spiking rate from a mean of 27.1 spikes/s (SD 8.5) in the control condition to a mean of 16.3 spikes/s (SD 10.1) during 5-HT application (t test, $p < 0.0001$). The response recovered to the level of the control condition during recovery (26.2 spikes/s (SD 9.2)). The example in Figure 2C had no change ($p > 0.05$) in spiking rate, despite up to 20 minutes of continuous 5-HT delivery. Overall, 78% of neurons exhibited significant changes in evoked spiking during 5-HT application (Fig. 2D) with 56% increasing and 22% decreasing.

To further understand the effects of 5-HT in the DCN we compared evoked and spontaneous spiking rates. For example, if 5-HT changes spike rate in the same direction for evoked and spontaneous rates across experimental conditions, this would suggest that 5-HT alters the excitability of neurons in a general fashion, possibly as a type of gain control. Conversely, differential effects on evoked versus spontaneous rates or on responses that exhibited increases versus decreases in spiking may suggest a more complex contribution of 5-HT to DCN function. The majority of neurons that had significant increases in evoked spiking during 5-HT application (11/13) also had increases greater than 10% in spontaneous spiking rate (Fig. 2E). The responses of the remaining two neurons had no change in spontaneous rate despite an increase in evoked spiking with 5-HT. Four of the five neurons that had decreased evoked spiking during 5-HT application also had a decrease in spontaneous spiking, while the remaining neuron had no change in rate. Of the five neurons that exhibited no change in evoked spiking with 5-HT, four had no change in spontaneous rate, and one had a decrease. Overall, 5-HT had similar effects on evoked and spontaneous spiking rates for the majority (19/23) of neurons. There was a strong correlation between changes in evoked and spontaneous activity with 5-HT (Pearson $r = 0.65$, $p < 0.0001$) for the sample of neurons ($n = 23$; Fig. 2E) suggesting that 5-HT may be modulating the general excitability of many DCN neurons.

3.2 5-HT effects on evoked first spike latency and jitter reflect changes in spiking rate

The evoked first spike latency (FSL) and corresponding jitter were obtained for the sample of 23 DCN recordings and, similar to spiking rate, 5-HT had heterogeneous effects on spike timing measures. Figure 3A (left panels) shows a neuron whose FSL significantly decreased from a mean of 19.3 ms in the control condition (top left) to a mean of 5.9 ms with 5-HT (bottom left; t test, $p < 0.0001$). The jitter of the evoked response also significantly decreased from 4.3 ms in the control condition to 3.1 ms with 5-HT (F-test, $p = 0.0004$). For a subset of neurons, 5-HT application resulted in increased FSL and jitter. The mean FSL of the second neuron shown in Figure 3A (right panels) significantly increased from 13.1 ms in the control condition (top right) to 18.2 ms with 5-HT (bottom right; t test, $p < 0.0001$) and the jitter significantly increased from 7.2 ms to 11.6 ms with 5-HT (bottom right; F-test, $p = 0.0001$).

Overall, there was a wide range of mean FSLs in the control condition (3.3 to 23.2 ms). A 5-HT-mediated decrease in FSL was recorded for eight neurons (t test, $p < 0.05$), while six neurons exhibited an increase, and the remaining nine had no change (Fig. 3B top). Similar results were observed with respect to the mean response jitter (0.7 to 22.8 ms). Seven of 23 neurons had a decrease in jitter during 5-HT application, six had an increase, and ten had no significant change (Fig. 3B bottom). To determine whether the direction and magnitude of changes in response jitter could be predicted by corresponding changes in latency, the normalized values for each parameter were examined (Fig. 3C). The significant correlation between FSL and jitter (Pearson $r = 0.45$, $p = 0.047$) indicates that a relationship between the two exists in many DCN neurons, although there are examples where no relationship is present and cases where a change in FSL in one direction was accompanied by a change in jitter in the opposite direction (Fig. 3C).

Evoked response strength signaled by increases or decreases in spiking rate could have an impact on spike timing. To examine this possibility, we compared normalized changes in FSL and jitter to changes in evoked spiking rate (Fig. 3D). There was a strong negative correlation between FSL and spiking rate (Pearson $r = -0.69$, $p = 0.0005$), and a weaker, but significant, negative correlation between jitter and spiking rate ($r = -0.47$, $p = 0.032$). These results suggest that in cases where 5-HT increases responses strength, the precision of spike timing increases and, conversely, a decrease in response magnitude results in a smearing of spike timing properties for many DCN neurons.

3.3 5-HT shifts decreases response thresholds of many DCN neurons

If the 5-HT system functions as a gain control for excitability of neurons in the DCN, the threshold for sound levels needed to evoke responses would be expected to decrease or increase depending on respective increases or decreases in spiking rate. For example, an overall depolarization of the cell membrane mediated by 5-HT might enable a neuron to reach spiking threshold at lower sound levels, whereas a hyperpolarization may cause a shift to higher thresholds. The example in figure 4A shows a 20 kHz CF tone response at threshold (18 dB SPL) that increased in magnitude from 38 total spikes (left, 10 presentations) to 75 spikes with the addition of 5-HT (middle), and recovered to 47 spikes after 5-HT was removed (right). To determine whether this increase in spiking was accompanied by a shift in threshold, the sound level of the CF tone was systematically varied from 15 to 50 dB SPL in control, 5-HT, and recovery conditions (Fig. 4B). For this example, the spiking rate to each sound level increased with 5-HT application and the threshold shifted from 18 dB SPL in the control condition to 14 dB SPL in the 5-HT condition. In addition, there were no differences in spiking rate between control and recovery conditions for each presented sound level.

Response rates at different sound levels, like the example described above (Fig. 4A, B), were recorded for 14 DCN neurons. Nine of the 14 neurons had lower thresholds during 5-HT application that ranged from 2 to 6 dB, while the remaining five neurons had no observable change in threshold (Fig. 4C). As expected, nearly all neurons (8/9) that had significant increases in spiking rate also had lower threshold during 5-HT delivery, while one neuron that had no change in rate had small decrease in threshold (2 dB). However, in each of the three cases where the spiking rate significantly decreased in the 5-HT condition, there was no change rather than the predicted increase in threshold. The remaining two neurons in the sample had no change in both spiking rate and threshold. Overall, there was a large degree of variability in the thresholds of DCN neurons, which ranged from 3 to 44 dB SPL in the control condition (Fig. 4D). Although the shifts in threshold with 5-HT did not exceed 6 dB, the changes were statistically significant (Wilcoxon matched-pairs signed rank test, $p = 0.0039$).

3.4 The 5-HT_{2A} receptor subtype contributes to increased excitability of DCN neurons

Previous studies of DCN responses *in vitro* (Tang and Trussell, 2015) and IC responses *in vivo* (Hurley, 2006) demonstrated a clear role for the 5-HT_{2A} receptor (5HT_{2A}R) mediating increases in the excitability of auditory neurons. To determine whether this receptor subtype

contributes to 5-HT effects in the DCN *in vivo*, we applied the selective 5HT_{2A}R agonist DOI to 12 neurons.

The majority (9/12) of neurons had a significant increase in spiking with DOI (Fig. 5A), only one neuron had a decrease, and the remaining two neurons had no change in spiking rate (Fig. 5B). Overall, the mean spiking rate for the sample of neurons ($n = 12$) significantly increased from 44.8 spikes/s (SD 15.2) in the control condition to 55.1 spikes/s (SD 21.4) with DOI (t test, $p = 0.017$; Fig. 5C).

Although the mean FSL slightly decreased with DOI application (control: 10.3 ms, DOI: 9.8 ms), this change was not statistically significant (t test, $p = 0.253$; Fig. 5D). The mean response jitter for the sample of neurons exhibited no change from the control value of 4.1 ms to 4.3 ms with DOI (t test, $p = 0.650$). Unlike DOI effects on FSL, changes in the direction of jitter values were heterogeneous, with five neurons that exhibited a decrease, two that had an increase, and five that showed no change (Fig. 5D). Although DOI had opposing effects on FSL and jitter for two of the 12 neurons in the sample, there was a strong positive correlation overall between changes in these two parameters during drug application (Pearson $r = 0.80$, $p = 0.0017$; Fig. 5E).

We examined changes in response threshold with DOI application in eight neurons. Four of the eight neurons had thresholds that shifted lower with DOI, although these differences were not significant (Wilcoxon matched-pairs signed rank test, $p = 0.125$), and the remaining neurons had no change in threshold (Fig. 5F). There were no cases in which response thresholds were elevated with DOI, similar to the effects observed with 5-HT (Fig. 4C). There was a large range of minimum thresholds in the control condition from 18 to 55 dB and, similar to 5-HT application, shifts in threshold were observed with DOI for neurons that had relatively low and high thresholds (Fig. 5G).

The effects of 5-HT and DOI on DCN responses were very similar, suggesting that increases in excitability may be mediated in large part by the 5HT_{2A}R. If this were the case, blocking 5HT_{2A}Rs would likely result in a decrease in response magnitude for the majority of neurons. To test this possibility, we applied the selective 5HT_{2A}R antagonist KS to eight neurons. Overall, the effects of KS were opposite to those observed with DOI application; the spiking of the majority of neurons decreased (Fig. 6A). Six of the eight neurons had a significant decrease in spiking rate with KS, one had an increase, and the remaining neuron had no change (Fig. 6B). The mean spiking rate for the sample ($n = 8$) was 61.8 spikes/s (SD 34.4) in the control condition and decreased to 48.2 spikes/s (SD 30.5) with KS (t test, $p = 0.0544$; Fig. 6C).

4. Discussion

Serotonergic neurons of the raphe nuclei exhibit levels of activity that change in response to behavioral state (Trulson and Jacobs, 1979; Jacobs and Fornal, 1999) and these neurons provide a dense projection to the DCN (Klepper and Herbert, 1991; Hurley and Thompson, 2001) suggesting they may mediate modulation of auditory responses in the DCN. The results of the present study demonstrate that 5-HT fibers densely innervate each layer of the

mouse DCN and that 5-HT affects spiking properties of fusiform cells. Although there was diversity in the effects of exogenous 5-HT application, the majority of neurons we recorded in the DCN showed increases in spiking rate as well as decreases in first spike latencies, jitter, and sound evoked thresholds. A smaller number of neurons exhibited 5-HT-mediated changes in the opposite direction as those described above or experienced no change at all. In addition, we found that the 5-HT_{2A} receptor subtype plays a role in mediating increases in neural excitability in the DCN *in vivo*. These findings demonstrate that the serotonergic system modulates sound-evoked responses of neurons at early stages of auditory processing.

4.1 Distribution of 5-HT fibers in the cochlear nucleus

Previous studies have reported a higher density of 5-HT fibers in the DCN compared to the neighboring ventral cochlear nucleus in a variety of species including rat (Klepper and Herbert, 1991), guinea pig (Thompson et al., 1994; 1995), cat (Thompson et al., 1994; Thompson and Thompson, 2001), bat (Hurley and Thompson, 2001), opossum (Willard et al., 1984), and bush baby (Thompson et al., 1994). 5-HT fibers have also been shown to innervate all subdivisions of the DCN (Willard et al., 1984; Klepper and Herbert, 1991; Thompson et al., 1994; Hurley and Thompson, 2001). Our findings are in agreement with these previous studies and suggest that 5-HT projections to the DCN may allow for strong modulation of both auditory (Portfors and Roberts, 2007) and somatosensory (Shore, 2005) information, and this may have implications for the multimodal processing of self-generated sounds such as suppression of neural responses to self-vocalizations (Shore and Zhou, 2006).

4.2 Potential mechanisms of 5-HT-mediated effects on response properties

The current study is the first to examine the effects of 5-HT on DCN neurons in awake animals. Ebert and Ostwald (1992) recorded neuronal responses during 5-HT application in the cochlear nucleus of anesthetized rats and observed both decreases and increases in evoked spiking rate, as well as responses that did not change. Although the majority of changes in their study were decreases in spiking with drug delivery, only four of their 35 recorded neurons were reported to be in the DCN, providing an incomplete comparison with the present results in the mouse. A thorough characterization of 5-HT effects on DCN neurons *in vitro* revealed strong increases in neuronal excitability through 5-HT_{2A/2C} and 5-HT₇ receptors via augmentation of the *I_h* current of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Tang and Trussell, 2015). The majority of our recordings were consistent with this finding, as the application of 5-HT acted to significantly increase the rate of both spontaneous and evoked spiking in the same neurons, supporting a role for depolarizing current through HCN channels in these cases (Pal et al., 2003). Because the 5-HT_{2A}R has been implicated in increasing neuronal excitability in the DCN *in vitro* (Tang and Trussell, 2015) and the IC *in vivo* (Hurley, 2006), we compared the effects of 5-HT with those of the 5-HT_{2A/2C}R agonist DOI. Responses during DOI application were similar to those in which 5-HT increased neuronal excitability, and application of the selective 5-HT_{2A}R antagonist KS induced opposing effects, suggesting that increases in excitability following 5-HT application are likely mediated in large part by activation of the 5-HT_{2A}R. However, other receptor subtypes may also contribute to increased excitability in the DCN including the 5-HT_{2C}R, the 5-HT₇R (Tang and Trussell, 2015) and the 5-HT_{1B}R, which has

been implicated in increasing spiking rates of IC neurons through suppression of inhibitory inputs (Hurley et al., 2008; Ramsey et al., 2010). Future studies will be needed to further examine these other 5-HTR subtypes to determine their contributions to auditory processing in the DCN.

During 5-HT application, we observed that increases in spiking rate were accompanied by modest but consistent decreases in threshold, resulting in shifts in spiking rate versus sound level curves. For many auditory neurons, systematic changes in stimulus intensity result in predictable changes in spiking rate and spike timing. Specifically, increases in sound level often correspond to increases in rate accompanied by decreases in FSLs and jitter, while decreasing the sound level produces the opposite effects (Klug et al., 2000; Heil, 2004). These relationships are important because FSL has been implicated in the processing of a variety of auditory features including intensity (Klug et al., 2000; Galazyuk and Feng, 2001), duration (Faure et al., 2003), frequency tuning (Kitzes et al., 1978), and gap detection (Barsz et al., 1998). The high variability of FSLs in fusiform cells observed *in vivo* by us and others (Rhode et al., 1983) is likely due in large part to varying strengths of the A-type K⁺ current (Kanold and Manis, 1999). We found that both the mean and standard deviation (jitter) of FSLs decreased for some fusiform cells and increased for others in the presence of 5-HT. These changes in FSL typically correlated with changes in spiking rate, with lower FSLs and jitter associated with increased spiking and, conversely, higher FSLs and jitter were accompanied by decreased spiking. A similar relationship between changes in FSL and spiking rate was reported during 5-HT delivery in the IC *in vivo* (Hurley and Pollak, 2005b).

In cases where 5-HT shifted rate-level relationships, increases in neuronal excitability in the DCN may have driven changes in spike rate, threshold, and spike timing through a general mechanism that modulates resting membrane potential. For instance, HCN channels have been implicated in increasing the spiking rate during 5-HT application via enhanced activation near the resting membrane potential (Tang and Trussell, 2015). The HCN2 isoform that is expressed in DCN neurons (Koch et al., 2004) typically activates at hyperpolarized potentials (Engbers et al., 2011), however, 5-HT acts to increase the number of open HCN channels at the resting membrane potential (Tang and Trussell, 2015). This increase in HCN2-mediated inward current (I_h) near rest can contribute to lowering spiking thresholds. In addition, I_h current can act to lower first spike latency and jitter, thereby increasing temporal precision of auditory processing (Kim and Holt, 2013), which is known to be important for the reliable processing of natural sounds (Rieke et al., 1995; Rokem et al., 2006; Chase and Young, 2007). Thus, modulation of HCN channels by 5-HT can account for our main findings for the majority of DCN neurons that exhibited increases in excitability.

In addition to increases in neuronal excitability with 5-HT application, we also recorded opposing effects in some neurons. Decreases in spiking may be caused by activation of a different complement of 5-HTR subtypes than those of neurons that had increases in excitability. For instance, spiking rates of neurons in other auditory nuclei including the medial superior olive (Ko et al., 2016) and IC (Hurley, 2007) are suppressed via the 5-HT_{1A}R, and there is evidence that this receptor type is present in the DCN, albeit at low levels of expression (Wright et al., 1995). While activation of the 5-HT_{1A}R may contribute

to cases of decreased excitability, this potential role is questionable, as suppressive effects of 5-HT application were not observed *in vitro* despite a thorough sampling of neurons in the fusiform cell layer of the DCN (Tang and Trussell, 2015). An alternative explanation for decreased activity involves effects of 5-HT on synaptic inputs to fusiform cells. Decreases in fusiform cell spiking observed with 5-HT application may be caused by enhanced activity of inhibitory inputs from glycinergic vertical cells located in nearby deep layers of the DCN (Oertel and Young, 2004). Recent *in vitro* recordings support this possibility, as vertical cells exhibited increased excitability in the presence of exogenously applied 5-HT that induced suppression of fusiform cell activity via feedforward inhibition (Tang and Trussell, 2016). Also in support of this view, local blockade of glycine receptors in the DCN results in increased spiking rates of fusiform cells *in vivo* (Caspary et al., 1987). Thus, 5-HT-mediated increases in the activity of vertical cells may enhance inhibitory input to fusiform cells, acting to decrease postsynaptic spiking rates of some neurons.

Neurons that exhibited decreased spiking rates during 5-HT delivery typically had degraded spike timing. This relationship was opposite of the majority of neurons that had increased rates accompanied by increased sharpness of spike timing. Interestingly, the opposing effects of 5-HT on these subpopulations did not extend to evoked spiking thresholds. For instance, in each case where changes in spiking rate and rate-level curves were examined, neurons that had increased excitability also had decreased thresholds, while neurons with decreased excitability had no change in threshold. Although 5-HT modulation is capable of lowering thresholds of DCN neurons, this mechanism is not expected to cause increases in thresholds due to the kinetics of HCN channels (Bal and Oertel, 2000) and the results of HCN channel blockade in auditory neurons *in vivo* (Shaikh and Finlayson, 2003; Felix et al., 2011). Thus, changes in response properties associated with 5-HT application likely reflect differing contributions of intrinsic and synaptic mechanisms for DCN neuron subpopulations.

4.3 Functional implications

The DCN has an established role in sound localization (Young and Davis, 2002; Oertel and Young, 2004) and evidence is mounting for contributions to other aspects of auditory processing. For instance, the DCN may be involved in the emergence of sound identification pathways (Young and Davis, 2002). This notion is supported by the fact that fusiform cells respond selectively to some conspecific vocalizations over others and are in a position to transmit this information directly to the IC (Roberts and Portfors, 2015), where further processing occurs (Klug et al., 2002; Hurley and Pollak, 2005a; Portfors et al., 2009; Holmstrom et al., 2010). Behaviorally important interactions, such as courtship or stress caused by the presence of an intruder, lead to changes in 5-HT levels in the IC and this is thought to affect the reception of vocalizations (Hall et al., 2011; Keesom and Hurley, 2016). Based on the current findings in the DCN, the serotonergic system is capable of modulating the excitability of neurons at low levels of the ascending auditory pathway, potentially affecting the salience of vocalizations depending on arousal state, attention, and behavioral context.

The DCN has also been identified as a key locus in the emergence of auditory disorders that may arise, in part, by impaired function of the serotonergic system (Katzenell and Segal,

2001; McKendrick et al., 2011). For instance, tinnitus, which is characterized as the perception of phantom ringing or buzzing sounds (Henry et al., 2005; Roberts et al., 2010), has been linked to increased 5-HT levels in the auditory system (Simpson and Davies, 2000; Caperton and Thompson, 2011). The generation of tinnitus is also associated with trauma-induced increases in the activity of fusiform cells of the DCN (Brozoski et al., 2002; Finlayson and Kaltenbach, 2009), partially through HCN channels (Li et al., 2015). Thus, increased spiking activity of fusiform cells that involves the serotonergic system may contribute to the development of tinnitus (Tang and Trussell, 2015). Our finding that exogenously applied 5-HT increased spiking for the majority of fusiform cells *in vivo* is in line with this view, although tinnitus likely arises due to a combination of factors. For example, a trauma-related decrease in the strength of synaptic inhibition could lead to the hyperactivity of fusiform cells thought to underlie tinnitus (Wang et al., 2009; Middleton et al., 2011), as could an increase in activation of the cholinergic system (Jin et al., 2006; Godfrey et al., 2013). The present study provides a framework to further examine how 5-HT and other transmitters systems contribute to auditory processing in normal and impaired listening states.

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Research Highlights

- The dorsal cochlear nucleus (DCN) of the mouse contains dense innervation from serotonergic fibers.
- Application of serotonin has heterogeneous effects on sound-evoked response properties of DCN neurons, but primarily acts to increase excitability.
- Increased excitability of DCN neurons is largely mediated by the 5-HT₂ receptor.

Figure 1.

5-HT immunoreactive fibers innervate the mouse DCN. (A) Coronal section depicting the molecular layer (I), fusiform layer (II), and deep layers (III/IV) of the DCN (Ryugo and Willard, 1985). BDA labeling (green) of tissue near the electrode tip is shown, along with a filled neuron. The tracer deposit was made immediately following a fusiform cell recording (CF: 25 kHz). The tissue was also labeled to reveal 5-HT fibers throughout the DCN (magenta). The labeled neuron and surrounding 5-HT varicosities are magnified in the inset. (B) There was dense labeling of 5-HT fibers in the DCN, but relatively sparse labeling in the neighboring ventral cochlear nucleus (VCN). Scale bars: *A*, *B* 100 μ m; *A* inset 25 μ m. The inferior cerebellar peduncle (ICP) is shown for reference.

Figure 2.

5-HT has heterogeneous effects on evoked and spontaneous spiking of DCN neurons. (A) Representative example in which 5-HT application increased the spiking rate. The first three panels show PSTHs of the response before (left), during (middle), and following (right) the delivery of 5-HT. The bars below each PSTH mark the duration of the 25 kHz CF tone in the recording window. The last panel represents the spiking rate at time points over the course of the recording protocol, with the time course of 5-HT application depicted as a bar below the data points. Error bars represent the standard deviation of spiking to 100 presentations of the stimulus at each time point. (B, C) Example neurons that exhibited a decrease (B) and no change (C) in spiking during 5-HT application in response to respective 36 and 8 kHz CF tones. All tones had durations of 50 ms and were presented 10 dB above threshold. The colored symbols in the far right panels correspond to the middle PSTHs that represent the respective increase, decrease, and no change in response during 5-HT application. The corresponding colors in figures 3–5 also represent data from these example neurons. (D) Normalized change in evoked spiking for the sample of neurons was determined by dividing the mean spiking rate at the maximal 5-HT effect by the control rate prior to 5-HT application. Changes greater than 1.0 represent increases in spiking and changes less than 1.0 represent decreases. Changes greater than 0.1 in either direction were statistically significant (black bars; paired student's t test, $\alpha = 0.05$), while changes less than 0.1 (white bars) could not be attributed to an effect of 5-HT. The majority of neurons (13/23) responded to 5-HT application with a significant increase in evoked spiking rate. (E) Normalized change in spontaneous versus evoked spiking rate during 5-HT application. The significant positive correlation of normalized responses (Pearson $r = 0.65$, $p < 0.0001$) indicates that 5-HT affected spontaneous and evoked spiking similarly.

Figure 3.

5-HT effects on evoked first spike latency and jitter reflect changes in spiking rate. (A) Raster plots of example neurons showing respective decreases (left) and increases (right) in first spike latency (FSL) and jitter during 5-HT application. Only the first spike is shown for each trial. The bars below the data points represent the duration of respective 20 and 9 kHz CF tones in the recording window. All tones had durations of 50 ms and were presented 10 dB above threshold. (B) Mean FSL (top) and jitter (bottom) for responses before and during 5-HT application. (C) Scatter plot of normalized change in jitter versus FSL in response to 5-HT application. The positive correlation of normalized responses (Pearson $r = 0.45$, $p = 0.042$) indicates that 5-HT may affect FSL and jitter in a similar manner. (D) Scatter plot illustrating normalized changes in FSL (filled circles) and jitter (open circles) versus normalized changes in spiking rate during 5-HT application. Changes in spiking rate are negatively correlated with the magnitude of change for both FSL ($r = -0.69$, $p = 0.0005$) and jitter ($r = -0.47$, $p = 0.032$). Dashed lines in (B) represent the population mean values. Colored symbols represent data from corresponding example neurons shown in Figure 2.

Figure 4.

5-HT can shift evoked spiking thresholds. (A) An example 20 kHz CF tone response recorded at the minimum threshold (obtained before drug application) before (left), during (middle), and after (right) 5-HT application. The bars below each PSTH represent the duration of the tone in the recording window. (B) Spiking rate-sound level relationship of the neuron shown in (A) before, during, and after application of 5-HT. Error bars represent the standard deviation for responses to 10 CF tone presentations at each intensity. (C) Change in threshold during 5-HT application for the sample of recorded neurons in the DCN. (D) Threshold values for each evoked response before and during 5-HT application. Dashed lines in (D) represent the population mean values. Colored symbols represent data from corresponding example neurons shown in Figure 2.

Figure 5.

The selective 5-HT₂ receptor agonist DOI increases excitability in many DCN neurons. (A) Example of an increase in spiking during DOI application. The bars below each PSTH represent the duration of the 17 kHz CF tone in the recording window. (B) Normalized change in spiking response for the sample of recorded neurons during DOI application. The convention for determining significant (black bars) and nonsignificant (white bars) changes are the same as described in Figure 2. (C) Spiking rates of evoked responses before and during DOI application for each recorded neuron. (D) First spike latency and jitter values for evoked responses before and during DOI application. (E) The strong correlation between normalized changes in jitter versus latency ($r = 0.80$, $p = 0.002$) indicates that DOI acts to reduce the magnitude of each property in a similar manner. (F) Change in threshold during DOI application for the sample of recorded neurons. (G) Threshold values for each evoked response before and during DOI application. Dashed lines in (C), (D), and (G) represent the population mean values. Colored symbols represent data from the corresponding example neuron shown in (A).

Figure 6.

The selective 5-HT₂ receptor antagonist Ketanserin (KS) decreases excitability in DCN neurons. (A) Representative example in which KS application decreased spiking rate. The first three panels show PSTHs of the response before (left), during (middle), and following (right) the delivery of KS. The bars below each PSTH mark the duration of the 10 kHz CF tone in the recording window. The last panel represents the spiking rate at time points over the course of the recording protocol, with the time course of KS application depicted with a bar below the data points. Error bars represent the standard deviation of spiking to 100 presentations of the stimulus at each time point. (B) Normalized change in spiking response for the sample of recorded neurons during KS application. The convention for determining significant (black bars) and nonsignificant (white bars) changes are the same as described for Figure 2. (C) Spiking rate values for each evoked response before and during KS application. Dashed lines in (C) represent the population mean values. Colored symbols represent data from the corresponding example neuron shown in (A).