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Research paper

Modulation of auditory brainstem responses by serotonin and specific serotonin receptors

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ABSTRACT

The neuromodulator serotonin is found throughout the auditory system from the cochlea to the cortex. Although effects of serotonin have been reported at the level of single neurons in many brainstem nuclei, how these effects correspond to more integrated measures of auditory processing has not been well-explored. In the present study, we aimed to characterize the effects of serotonin on far-field auditory brainstem responses (ABR) across a wide range of stimulus frequencies and intensities. Using a mouse model, we investigated the consequences of systemic serotonin depletion, as well as the selective stimulation and suppression of the 5-HT1 and 5-HT2 receptors, on ABR latency and amplitude. Stimuli included tone pips spanning four octaves presented over a forty dB range. Depletion of serotonin reduced the ABR latencies in Wave II and later waves, suggesting that serotonergic effects occur as early as the cochlear nucleus. Further, agonists and antagonists of specific serotonergic receptors had different profiles of effects on ABR latencies and amplitudes across waves and frequencies, suggestive of distinct effects of these agents on auditory processing. Finally, most serotonergic effects were more pronounced at lower ABR frequencies, suggesting larger or more directional modulation of low-frequency processing. This is the first study to describe the effects of serotonin on ABR responses across a wide range of stimulus frequencies and amplitudes, and it presents an important step in understanding how serotonergic modulation of auditory brainstem processing may contribute to modulation of auditory perception.

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1. Introduction

Evidence accumulated over the past twenty years indicates that serotonin plays an important role in modulating auditory responses with potentially significant functional consequences. Serotonin has been linked to clinical auditory disorders such as tinnitus (Noreña et al., 1999; Simpson and Davies, 2000; Caperton and Thompson, 2011) and hyperacusis (Marriage and Barnes, 1995; Attri and Nagarkar, 2010), as well as the auditory manifestations associated with non-auditory-specific disorders including migraine (Goadsby, 1998; Hamel, 2007; Panconesi, 2008; Sand et al., 2008), depression (Gopal et al., 2000; Chen et al., 2002; Kampf-Sherf et al., 2004; Gopal et al., 2005), schizophrenia (Bleich et al., 1988; Breier, 1995; Park et al., 2010), and post-traumatic stress disorder (Southwick et al., 1999; van der Kolk, 2001; Lee et al., 2005), among others. Associations between serotonergic activity and auditory responsivity have been supported in part by auditory evoked potential (AEP) measures assessing cortical function. Here, changes in endogenous serotonin levels have been linked to changes in peak component amplitudes (Ehlers et al., 1991; Manjarrez et al., 2005) and latencies (Concu et al., 1978) as well as dynamic variation in responses to stimuli across time (Johnson et al., 1998; Stevens et al., 2006) and stimulus level (Hegerl and Juckel, 1993; Juckel et al.,

Abbreviations: 5-HT, 5-hydoxytryptamine, serotonin; 8-OH-DPAT, 8-hydroxy-DPAT hydrobromide, 5-HT1A receptor agonist; ABR, auditory brainstem response; AEP, auditory evoked potential; ANOVA, analysis of variance; dB, decibel; dB SL, decibel referenced to sensation level; dB SPL, decibel referenced to sound pressure level; IC, inferior colliculus; IP, intraperitoneal; SEM, standard error of the mean; CP93129, CP93129 dihydrochloride, 5-HT1B receptor agonist; DOI, (±)-DOI hydrochloride, 5-HT2A/C receptor agonist; Ketanserin, ketanserin tartrate, 5-HT2A/C receptor antagonist; NAS-181, (2R)-2-[[[3-(4-Morpholinylmethyl)-2H-1-benzopyran-8-yl] oxy]methyl] morpholinedimethanesulfonate, 5-HT1B receptor antagonist; pCPA, 4-chloro-DL-phenylalanine methyl ester hydrochloride, endogenous serotonin depletion agent; S-WAY, (S)-WAY 100135 dihydrochloride, 5-HT1A receptor antagonist

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1997; Juckel et al., 1999; Gallinat et al., 2000; Bruder et al., 2001; Hegerl et al., 2001; Jaworska et al., 2013). These studies generally support the theory that reduced serotonergic function is associated with increased activity and responsivity in the auditory cortex. Effects of serotonin lower within the auditory pathway have also been demonstrated in numerous extra- and intracellular studies (Ebert and Ostwald, 1992; Mizutani et al., 2006; Wang and Robertson, 1997; Fitzgerald and Sanes, 1999; Faingold, 1999; Hurley and Pollak, 1999; Hall and Hurley, 2007; Miko and Sanes, 2009), indicating the likelihood that serotonergic effects begin well before the level of the cortex. Auditory brainstem responses (ABRs) can serve as a versatile tool allowing comparison of serotonergic effects across levels of analysis. Since ABRs represent the summed activity across large populations of auditory neurons, they can illustrate whether effects observed at the level of single neurons are prominent enough to influence the amplitude or synchrony of population activity. ABRs can also be used to compare the influence of serotonergic manipulation at different sites along the auditory neuraxis, a type of information often used to identify potential sites of origin for auditory phenomena (Starr and Achor, 1975; Stockard and Rossiter, 1977). Finally, ABRs can be compared between human subjects and animal models of specific auditory disorders, facilitating testing of hypotheses on the mechanistic role of serotonin in disorders like tinnitus or hyperacusis (Tziridis et al., 2015; Heeringa and van Dijk, 2014). Despite these advantages, the effects of serotonin, and particularly specific receptor pathways, on ABR remain largely unexplored.

Serotonergic fibers and varicosities are abundant in the cochlea and each level of the auditory brainstem (Steinbusch, 1981; Willard et al., 1984; Fitzpatrick et al., 1989; Klepper and Herbert, 1991; Harvey et al., 1993; Gil-Loyzaga et al., 1997; Hurley and Thompson, 2001; Thompson and Hurley, 2004; Papesh and Hurley, 2012). Though fewer in number, some physiological investigations of serotonergic effects in the brainstem support a suppressive or partially suppressive role such that application of serotonin leads to reduced spontaneous and driven firing in the cochlear nucleus (Ebert and Ostwald, 1992), the trapezoid body (Mizutani et al., 2006), the superior olivary complex (Fitzgerald and Sanes, 1999), and the inferior colliculus (IC) (Hurley and Pollak, 2001; -Wang et al., 2008). In the IC, serotonin affects first-spike latencies and interspike intervals (Hurley and Pollak, 2005; Hurley, 2006) as well as changes in frequency tuning most often resulting in reduced response areas following serotonin appliction (Hurley and Pollak, 2001; 2005; Hurley, 2006; Hall and Hurley, 2007). ABRs provide a useful tool by which to study the aggregate effects of serotonergic manipulations across a large population of cells from the auditory nerve to the IC. Thus, a main goal of the present study was to use ABRs to characterize the effects of endogenous serotonin depletion in the mouse model across a wide range of stimulus frequencies and intensities, with the prediction that low levels of endogenous serotonin should elicit larger response amplitudes and shorter response latencies compared to baseline measures. Since the densities of serotonergic fibers are reported to be greater in low-frequency regions of several brainstem nuclei (Klepper and Herbert, 1991; Hurley and Thompson, 2001; Hurley et al., 2002; Papesh and Hurley, 2012), a further prediction is that depletion of serotonin should have larger effects on low-frequency than on high-frequency responses.

Although the effects of serotonin in the auditory brainstem are often suppressive, multiple studies reveal subsets of cells showing the opposite trend of increased responsivity with serotonin application. The diversity in serotonergic effects is largely mediated by the type and distribution of serotonergic receptors present on pre- and post-synaptic cells. Of the seven serotonin receptor families, the 5-HT1 and 2 families have been well-documented throughout the auditory system, including the periphery. Predominant effects of 5-HT1A and 5-HT2A/C receptor stimulation in the IC include reduced spiking and increased firing latencies in most cells tested in the central nucleus of the IC (Hurley, 2006, 2007), and increased frequency and amplitude of GABAergic postsynaptic currents (Wang et al., 2008). In contrast, stimulation of the 5-HT1B receptor is reported to most often elicit excitatory effects such as increases in bandwidth of frequency response maps, higher firing rates, and lower thresholds of auditory neurons (Hurley, 2006; Hurley et al., 2008; Ramsey et al., 2010). Thus, a second goal of the present work is to begin an exploration of the role of multiple specific serotonergic receptors, the 5-HT1A, 1B, and 2A/C, on the modulation of ABRs. Although this is complicated by the fact that even the same type of serotonin receptor may have different effects in different brainstem nuclei, a general prediction was that manipulating different types of serotonin receptor will create different suites of effects on the ABR.

2. Methods

2.1. Subjects

Subjects in the current study were male CBA/J (Jackson Laboratories) mice. This inbred strain is frequently chosen for auditory studies due to its good hearing sensitivity maintained across the lifespan (Mikaelian and Ruben, 1965; Willott et al., 1991). A schematic of subject groups and experimental procedures is shown in Fig. 1. A total of 56 subjects were tested at an average age of 2.3 months (standard deviation of 0.6) at baseline testing. Twenty-four of these subjects underwent depletion of endogenous serotonin. Following serotonin depletion, these 24 subjects were further divided into three groups of eight, each of which received an agonist of the 5-HT1A, 1B, or 2A/C receptor. An additional 24 subjects were placed into three groups of eight with each group receiving an antagonist of either the 5-HT1A, 1B, or 2A/C receptor. Finally, the last eight subjects received saline injections but no serotonergic manipulations. This group served as a control to ensure that changes recorded after serotonin depletion were not due to the stress of injections or handling of animals. All protocols were approved by the Bloomington Institutional Animal Care and Use Committee and were carried out in accordance with EU Directive 2010/63/EU.

2.2. Serotonergic pharmaceutical agents

Depletion of endogenous serotonin was achieved by intraperitoneal injection of 4-Chloro-DL-phenylalanine methyl ester hydrochloride (pCPA; Sigma Aldrich, Cat#C3635), which inhibits the function of tryptophan hydroxylase (Koe and Weissman, 1966). Repeated application of pCPA leads to progressive decreases in endogenous serotonin levels as serotonin stores are released and metabolized, but not replenished (Dailly et al., 2006). Subjects received a dose of 150 mg/kg pCPA diluted in physiological saline and administered intraperatoneally (i.p.) once every 24 h for six days, a regimen of pCPA injections that reduces endogenous serotonin levels by approximately 80–90% in mice (Dailly et al., 2006) while minimizing impact on nonserotonergic systems (Bauer et al., 2002). Because pCPA is reported to reduce appetite (Bubenik and Pang, 1993), subjects' weight was monitored daily to ensure that any changes in appetite resulting from pCPA did not cause undue weight loss.

To explore the role of specific serotonergic receptors on ABR measures, selective agonists and antagonists of the 5-HT1A, 1B, and 2 receptors were administered to designated subject groups (Fig. 1). Agonists were administered to subjects only after serotonin

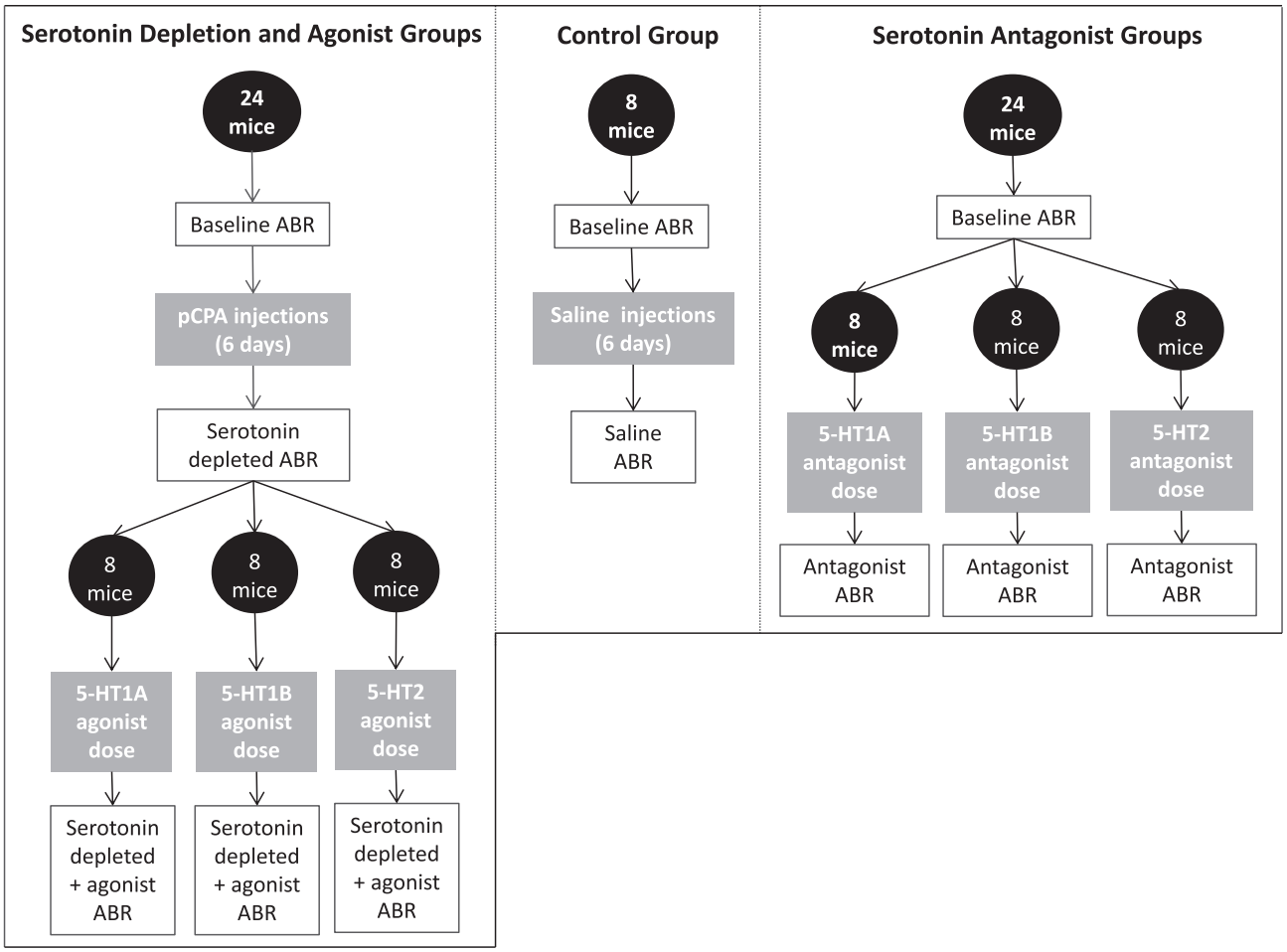

Fig. 1. Schematic of subject groups and timeline of experimental procedures. Subject groups are shown in black circles, ABR measurements are shown in white boxes, and pharmaceutical doses are shown in gray boxes.

depletion while antagonists were administered to unmanipulated subjects (e.g. those with normal endogenous serotonin levels). The intent of this approach was to provide a crosscheck of the effects of certain receptors on ABR measures such that responses were examined when only one receptor type was stimulated (the serotonin-depletion + agonist groups) and also when that same receptor type was blocked while all other receptors were functioning normally (antagonist groups). To activate the 5-HT1A receptor agonist, 8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; Tocris Bioscience, Cat#0529) was administered. This chemical has as high affinity for the 5-HT1A subtype (Middlemiss and Fozard, 1983) as well as a moderate affinity for the 5-HT7 receptor (Wood et al., 2000). Additional agonists included CP93129 dihydrochloride (CP93129, Sigma–Aldrich, Cat#PZ0102) which is a potent and selective agonist of the 5-HT1B receptor, and (±)-DOI hydrochloride (DOI; Sigma Aldrich, Cat#D101) which is a potent agonist of the 5-HT2A and 2C receptors (Hoyer et al., 2002). Serotonin receptor antagonists included (S)-WAY 100135 dihydrochloride (S-WAY; Tocris Bioscience, Cat#1253) with high affinity for the 5-HT1A receptor, (2R)-2-[[[3-(4-Morpholinylmethyl)-2H-1-benzopyran-8-yl]oxy]methyl] morpholinedimethanesulfonate (NAS-181; Tocris Bioscience, Cat#1314) which is selective for the 5-HT1B receptor, and ketanserin tartrate (Tocris Bioscience, Cat#0908) (Hoyer et al., 2002). Though ketanserin has some affinity for both 2A and 2C receptors, its affinity for the 5-HT2A receptor is between 10 and 30 times greater than for the 5-HT2C receptor (Vollenweider et al., 1999). 8-OH-DPAT and NAS-181 were administered subcutaneously at a dosage of 0.5 mg/kg (Revelis et al., 1998) and 10 mg/kg (Stenfors et al., 2001), respectively. All other agonists and antagonists were administered intraperitoneally at the following dosages: 1 mg/kg for both CP93129 and DOI, 10 mg/kg of S-WAY, and 1.5 mg/kg ketanserin (Ninan and Kulkarni, 1999). Lastly, a saline solution was administered to a control group in order to control for the effects of repeated handling and injections of serotonin-depleted subjects. The quantity of injected saline was equal to the amount of serotonergic drug administered to subjects receiving pCPA injections, and was administered on the same timeline.

2.3. Electrode implantation

Measurement of ABRs required placement of subdermal silver wires which served as electrodes at the vertex of the head, nape of the neck, and on the bulla located just behind each ear. Mice were briefly anesthetized with isoflurane fumes, and then injected intraperitoneally with 120 mg/kg ketamine and 5 mg/kg xylazine. Surgical anesthesia was achieved as assessed by lack of response to tail and toe pinch. Following induction of anesthesia, ophthalmic ointment was placed over the eyes of the mouse to prevent drying. Depilatory cream was used to clear each electrode site of hair. Each site was then immediately rinsed and cleaned with three applications of betadine and 70% ethanol. Electrodes were constructed of sterilized silver wire loops (26 G). The electrodes were inserted

subcutaneously through a hypodermic needle (18 G) passed through a fold of skin held by forceps. Once inserted, the hypodermic needle was removed, leaving the silver wire electrodes in place. The ends of the electrodes were twisted together and clipped off approximately 3 mm from the subject's scalp. Subjects were then treated with topical antibiotic and allowed to regain consciousness and mobility prior to being returned to animal quarters. Subjects were monitored daily following surgery to ensure good recovery and analgesics were given if indicated. No ABR measurements were made until at least one week following electrode implantation to allow for adequate healing time.

2.4. Auditory brainstem response (ABR) recordings

Prior to beginning ABR measurements, subjects were anesthetized via brief exposure to isofluorane before receiving intraperitoneal (IP) injections of a mixture of ketamine (120 mg/kg) and xylazine (5 mg/kg). Anesthetic level was confirmed via toe and tail pinch. Subjects were then transported to a custom-built recording apparatus within a sound-attenuating booth. Subject head placement was maintained using a bite bar. Rectal temperature was maintained at 36.6 ± 0.2 °C using a heating pad connected to a temperature control module (FHC; Bowdoinham, ME). Anesthetic level was checked every fifteen minutes throughout the recording session via tail pinch, and additional anesthetics consisting of 1/5 of the initial dose were given as needed to maintain a standard anesthetic plane.

ABR stimuli consisted of tone bursts centered at frequencies of 4, 8, 16, and 32 kHz with an 8 m s duration including linear 0.5 ms rise/fall times. Stimuli were generated at a sampling rate of 333 kHz, passed through a Microstar Data Acquisition Processor (model #5216a) to an anti-aliasing filter (model FT6-2), and a programmable attenuator (TDT PA5; Tucker–Davis Technologies, Alachua, FL) before being amplified (Samson Servo-170; Samson Technologies, Hauppauge, NY) and delivered to the speaker. Stimuli were presented in free-field via a Panasonic EAS10TH100B speaker placed at 0° azimuth and 30° above the subject at a distance of eight inches between the center of the speaker and the center of the subject's head. The rate of stimulus presentation was 20 Hz. A minimum of 1024 stimulus repetitions were included in each run, with many more presentations as levels approached hearing thresholds. The majority of runs were recorded twice in order to ensure good reliability of traces. The order of presentation across frequencies was counterbalanced within each group such that half the group heard frequencies presented from low to high, and the other half of the group was presented the frequencies starting with the highest and moving to the lowest. The total duration of each ABR recording session was approximately 1 h from presentation of the first stimulus to the last. The inverting electrode was located at vertex, the non-inverting electrode just ventrolateral to the right pinna, and the ground electrode just ventrolateral to the left pinna. Response signals were sent to a pre-amplifier (Grass P5 series; Warwick, RI) powered by a regulated power supply (Grass RPS 107; Warwick, RI), bandpass filtered between 30 and 3000 Hz with a 60 Hz notch filter engaged.

For experimental measures, each tone pip was presented across a 40 dB range in 10 dB intervals starting at the highest level and descending to the lowest. Presentation levels varied across stimulus frequency due to the limitations of the mouse hearing sensitivity (Henry, 1979) and system constraints. The presentation levels of each frequency in both dB SPL and dB sensation level (SL) are shown in Table 1. Hence, 4, 8, 16, and 32 kHz stimuli were presented at levels between 70 and 100 dB SPL, 52 and 82 dB SPL, 48 and 78 dB SPL, and 41 and 71 dB SPL, respectively. In addition to these presentation levels, ABR thresholds were also estimated for each subject at each frequency with threshold defined as the lowest presentation level which elicited identifiable ABR wave morphology as assessed by an experienced, but not blinded, evaluator. Determination of thresholds was conducted by first presenting tone pips at a clearly suprathreshold level. The presentation level was then reduced in 10 dB steps until a response was no longer visually discernable. The presentation level was then increased by 10 dB, recordings were made, and the presentation level was then sequentially stepped down in 5 dB steps until threshold was identified. As the presentation level decreased, a minimum of 2048 sweeps were obtained for each response in order to ensure that noise was averaged out and the best possible responses were acquired. All responses to threshold and near-threshold level stimuli were repeated to ensure that true responses were obtained. Threshold was defined as the lowest level at which wave I of the ABR was discernable to an experienced electrophysiologist with knowledge of each subject's experimental condition. Wave I was selected for threshold estimates because it is generally has the largest amplitude in mouse and thus most consistently referenced for mouse ABR thresholds (Henry, 1979). A 5 dB step size allowed for unambiguous distinction of present versus absent responses, however, smaller shifts in threshold may have occurred which were not captured in this protocol. Stimulus levels were calibrated using a ¼" microphone having a flat frequency response through 120 kHz (ACO Pacific, Model 7017) placed in the location of the subject's head during ABR recordings against a pistonphone producing 94 and 104 dB (ACO Pacific, Model 511E). Stimulus presentation and response measures were obtained using custom software (Batlab; Dr. Donald Gans).

Table 1
Free-field presentation levels in dB SPL and dB SL for each test frequency.

4 kHz		8 kHz		16 kHz		32 kHz	
Average threshold: 55 dB SPL		Average threshold: 32 dB SPL		Average threshold: 22.5 dB SPL		Average threshold: 31 dB SPL	
dB SPL	dB SL	dB SPL	dB SL	dB SPL	dB SL	dB SPL	dB SL
100	45	82	50	77.5	55	71	40
90	35	72	40	67.5	45	61	30
80	25	62	30	57.5	35	51	20
70	15	52	20	47.5	25	41	10

Auditory thresholds for each test frequency were estimated to be the lowest level at which wave I morphology was clearly discernable. Auditory thresholds obtained in the current study corresponded well to previously published work using tone-pip ABRs in CBA/J mice (Henry, 1979). Waveform morphology varies with changes in stimulus frequency and intensity such that not all waves may be observable at every level for each test frequency, particularly wave V (Henry, 1979). For this reason, sample sizes for a given wave may vary across stimulus frequencies (e.g. wave V was more often observable in response to 8 kHz stimuli compared to 32 kHz stimuli).

2.5. Procedure

Subject groups and experimental procedures are outlined in Fig. 1. Immediately following completion of baseline ABR measures, subjects undergoing serotonin depletion (24 in total) received their first injection of pCPA while still anesthetized. Subjects were then allowed to regain consciousness and were not returned to animal quarters until they had regained mobility. For the next five days, each of these subjects was weighed daily and examined for both behavioral changes and proper healing and maintenance of

electrodes and surgical sites. Each was then briefly anesthetized with isofluorane fumes prior to receiving a dose of pCPA. Thus, each subject in the serotonin-depletion group received a total of six applications of pCPA over the course of six days beginning just after baseline ABR measures. On the seventh day, subjects again underwent ABR testing to assess the effects of serotonin depletion. Immediately following serotonin-depleted ABR measures, each subject received a dose of one of three serotonergic agonists described above. ABR measures were repeated one hour later to assess the effects of stimulating only one receptor type while other serotonin receptors were inactive due to serotonin depletion. This timeframe was selected based upon previous reports indicating that the effects of most systemically administered serotonergic agonists and antagonists are maximal beginning approximately one hour following administration (Stevens et al., 2006). Subjects remained anesthetized from the beginning of serotonin-depleted ABR recordings through the administration of serotonergic agonists and subsequent recordings.

Twenty-four additional subjects were tested to assess the effects of blocking one specific serotonin receptor type using a selective antagonist while all other receptor subtypes were operating normally with normal levels of endogenous serotonin. These subjects underwent baseline ABR testing as described above and then immediately received an injection of one of the three serotonin receptor antagonists while still under anesthesia. As with those subjects receiving serotonergic agonists following serotonin depletion, a period of one hour elapsed between antagonist administration and beginning of ABR recordings to assess the effects of the antagonist. Each subject received only one dose of a single antagonist, with eight total subjects receiving each specific receptor antagonist.

2.6. Measurement and statistical analysis of auditory brainstem responses

Response waveforms were converted to ASCII file format and then read into Matlab (Version 7.0, MathWorks, Natick, MA) where they were offline bandpass filtered from 30 to 1500 Hz. Filtered waveforms were then imported into Excel (Microsoft Corporation, USA) where peak analysis was completed by an experienced, but not blinded, evaluator who determined waveform peaks based upon the highest amplitudes occurring within appropriate latency regions of the ABR waveform (Hall, 2007). Peak amplitudes and latencies were measured from up to 2048 stimulus presentations per run, with many more presentations at lower sensation levels. Runs were recorded twice in order to ensure good reliability of traces. The latencies of peaks were selected based upon the highest amplitude locations within the appropriate latency boundaries for each component. The amplitude of each peak component was calculated by adding the positive peak amplitude value with the absolute value of the following trough.

Statistical analysis was completed using SPSS software (IBM, USA). In order to determine the effects of serotonin depletion on ABR amplitudes and latencies, peak measures were analyzed using repeated-measures ANOVA including the within-subjects variables 'Condition' (two levels: baseline and serotonin depletion, baseline and agonist, or serotonin depleted and antagonist) and 'Presentation Level' (4 levels). ANOVAs were conducted on the latencies and amplitudes of ABR peaks obtained at each test frequency (4, 8, 16, and 32 kHz). Separate ANOVAs were run for each ABR wave since the sample size of each waveform varied depending upon the stimulus frequency and the particular ABR wave. The effect of serotonin depletion on amplitude-intensity and latency-intensity functions were assessed both via analysis of the interactions between 'Condition' and 'Presentation Level' as well as by calculating the slope of the latency/intensity and amplitude/intensity function for each individual for each ABR wave, condition, and stimulus frequency. For each stimulus frequency, latency/intensity slopes for each individual were assessed via repeated-measures ANOVA with the within-subject factor Condition (two levels: baseline and pCPA). This analysis was repeated for amplitude/intensity measures. For all analyses, Greenhouse-Geisser corrections were applied when any factor indicated violation of the assumption of sphericity based upon Mauchly's test of sphericity. When the need for Greenhouse-Geisser corrections was indicated, degrees of freedom were altered respectively, appearing as decimal points in Tables 2–4. As expected, decreases in stimulus intensity led to significantly smaller amplitudes and longer latencies of all waves in response to all test frequencies. Thus, further analysis focused primarily on main effects and interactions with 'Condition' since this was the primary experimental measure of interest.

3. Results

3.1. Effects of serotonin depletion

Compared to baseline ABR measures, serotonin depletion led to significantly reduced latencies of most ABR peaks in response to 8 and 16 kHz stimuli. This effect is shown in Fig. 2 which displays ABR recording from a representative individual in response to the four stimulus frequencies presented at a similar sensation level. Notice that in response to 8 and 16 kHz, waves II through V occurred earlier in time in the pCPA condition (broken gray line) compared to the baseline condition (solid black line). The group effects of serotonin depletion on ABR peak latencies are further demonstrated in Fig. 3 where ABR peaks are plotted as a function of the change in latency between baseline and serotonin-depleted conditions in response to each of the four stimulus frequencies. On this plot, that positive latency difference values indicate decreases in latency with serotonin depletion relative to baseline while negative values indicate increases in latency. Though the average latency changes for peaks in response to 4 (panel A) and 32 kHz (panel D) stimuli hover around zero, average latency differences in waves II through V in response to 8 and 16 kHz (panels B and C, respectively) presentations show consistent latency decreases in serotonin-depleted conditions relative to baseline. Statistical analysis revealed that the change in latency was significant for waves II through V and waves III through V in response to 8 and 16 kHz stimuli, respectively (Table 2, left panel). In contrast to the effects of serotonin depletion of peak latencies, peak amplitudes were generally unaffected by serotonin depletion with the one exception of a small but significant decrease in wave I amplitude in response to 4 kHz following serotonin depletion.

It is possible that the daily handling of subjects in the serotonin-depletion group influenced test results. To control for this possibility, a separate group of eight subjects received injections of saline on the same time scale and in the same amount as subjects receiving injections of pCPA. The results of repeated-measures ANOVA on this control group are shown in the right panel of Table 2. Notice that neither the amplitude nor latency of any ABR wave was significantly affected in response to any stimulus frequency following saline injection. This finding provides evidence that the effects of serotonin depletion reported above are truly a reflection of experimental manipulations rather than stress or fatigue associated with daily handling.

3.2. Serotonergic effects on wave V amplitude-intensity and latency-intensity functions

Various studies have indicated that decreased serotonergic

Table 2
Results of repeated-measures ANOVA comparing baseline and serotonin-depletion conditions (left panel) and baseline and saline treatments (right panel) across four presentation levels. Separate ANOVAs were conducted for the amplitudes and latencies of each wave at each of the four stimulus frequencies. Significant results ($p \leq 0.05$) are in bold text.

		Serotonin depletion (pCPA) compared to baseline					Saline compared to baseline				
		Wave I	Wave II	Wave III	Wave IV	Wave V		Wave I	Wave II	Wave III	Wave IV	Wave V
4 kHz	Latency:	$n = 23$	$n = 22$	$n = 22$	$n = 22$	$n = 16$	Latency:	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 6$
	Condition	$F_{(1,22)} = 0.195$ $p = 0.663$	$F_{(1,21)} = 1.994$ $p = 0.173$	$F_{(1,21)} = 0.021$ $p = 0.886$	$F_{(1,21)} = 0.192$ $p = 0.666$	$F_{(1,15)} = 3.691$ $p = 0.074$	Condition	$F_{(1,7)} = 0.256$ $p = 0.628$	$F_{(1,7)} = 0.081$ $p = 0.785$	$F_{(1,7)} = 0.765$ $p = 0.411$	$F_{(1,7)} = 0.220$ $p = 0.653$	$F_{(1,5)} = 0.094$ $p = 0.771$
	Cond*Lvl	$F_{(2.4, 53.0)} = 0.107$ $p = 0.928$	$F_{(2.03, 42.6)} = 1.292$ $p = 0.286$	$F_{(1.68, 35.3)} = 0.783$ $p = 0.444$	$F_{(2.17, 45.6)} = 0.626$ $p = 0.552$	**$F_{(1.95, 29.3)} = 3.701$ $p = 0.038$**	Cond*Lvl	$F_{(1.57, 11.1)} = 2.707$ $p = 0.071$	$F_{(3,21)} = 2.615$ $p = 0.078$	$F_{(3,21)} = 1.752$ $p = 0.187$	$F_{(3,21)} = 0.480$ $p = 0.700$	$F_{(3,15)} = 2.670$ $p = 0.085$
	Amplitude:						Amplitude:					
	Condition	**$F_{(1,22)} = 5.163$ $p = 0.033$**	$F_{(1,21)} = 1.762$ $p = 0.199$	$F_{(1,21)} = 0.226$ $p = 0.639$	$F_{(1,22)} = 2.751$ $p = 0.112$	$F_{(1,15)} = 1.395$ $p = 0.254$	Condition	$F_{(1,7)} = 0.087$ $p = 0.777$	$F_{(1,7)} = 0.024$ $p = 0.882$	$F_{(1,7)} = 1.499$ $p = 0.260$	$F_{(1,7)} = 0.278$ $p = 0.614$	$F_{(1,5)} = 0.265$ $p = 0.628$
	Cond*Lvl	$F_{(1.65, 36.2)} = 1.105$ $p = 0.332$	$F_{(1.81, 38.1)} = 2.166$ $p = 0.133$	**$F_{(3,63)} = 4.429$ $p = 0.007$**	$F_{(1.53, 32.1)} = 1.425$ $p = 0.253$	$F_{(1.75, 29.8)} = 1.638$ $p = 0.213$	Cond*Lvl	$F_{(1.48, 10.3)} = 3.319$ $p = 0.087$	$F_{(3,21)} = 1.383$ $p = 0.275$	$F_{(3,21)} = 1.549$ $p = 0.231$	$F_{(3,21)} = 0.315$ $p = 0.814$	$F_{(1.82, 9.09)} = 2.143$ $p = 0.174$
8 kHz	Latency:	$n = 23$	$n = 23$	$n = 22$	$n = 22$	$n = 21$	Latency:	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 8$
	Condition	$F_{(1,22)} = 0.532$ $p = 0.474$	**$F_{(1,22)} = 4.952$ $p = 0.037$**	**$F_{(1,21)} = 8.506$ $p = 0.008$**	**$F_{(1,21)} = 6.181$ $p = 0.021$**	**$F_{(1,20)} = 5.34$ $p = 0.032$**	Condition	$F_{(1,7)} = 0.771$ $p = 0.409$	$F_{(1,7)} = 0.491$ $p = 0.506$	$F_{(1,7)} = 0.001$ $p = 0.970$	$F_{(1,7)} = 0.363$ $p = 0.566$	$F_{(1,7)} = 0.089$ $p = 0.774$
	Cond*Lvl	$F_{(3, 66)} = 1.098$ $p = 0.356$	$F_{(3, 66)} = 0.294$ $p = 0.830$	$F_{(2.06, 43.3)} = 2.170$ $p = 0.125$	$F_{(3, 63)} = 1.569$ $p = 0.206$	$F_{(2.33, 46.6)} = 1.935$ $p = 0.149$	Cond*Lvl	$F_{(3,21)} = 1.793$ $p = 0.179$	$F_{(1.53, 10.70)} = 1.365$ $p = 0.281$	$F_{(3,21)} = 1.678$ $p = 0.202$	$F_{(2.04, 14.27)} = 0.504$ $p = 0.618$	$F_{(1.19, 8.34)} = 3.07$ $p = 0.051$
	Amplitude:						Amplitude:					
	Condition	$F_{(1,22)} = 0.015$ $p = 0.902$	$F_{(1,22)} = 0.435$ $p = 0.517$	$F_{(1,21)} = 0.112$ $p = 0.741$	$F_{(1,21)} = 2.607$ $p = 0.121$	$F_{(1,20)} = 0.924$ $p = 0.347$	Condition	$F_{(1,7)} = 0.293$ $p = 0.605$	$F_{(1,7)} = 1.449$ $p = 0.268$	$F_{(1,7)} = 0.756$ $p = 0.413$	$F_{(1,7)} = 0.003$ $p = 0.956$	$F_{(1,5)} = 0.700$ $p = 0.430$
	Cond*Lvl	$F_{(1.81, 39.8)} = 0.218$ $p = 0.783$	$F_{(1.56, 34.4)} = 0.833$ $p = 0.417$	$F_{(1.78, 39.2)} = 0.448$ $p = 0.620$	$F_{(2.13, 44.7)} = 1.947$ $p = 0.152$	**$F_{(3, 63)} = 3.392$ $p = 0.023$**	Cond*Lvl	$F_{(3,21)} = 0.900$ $p = 0.458$	$F_{(3,21)} = 1.624$ $p = 0.214$	$F_{(3,21)} = 0.581$ $p = 0.634$	$F_{(3,21)} = 0.415$ $p = 0.744$	$F_{(3,21)} = 2.128$ $p = 0.127$
16 kHz	Latency:	$n = 24$	$n = 23$	$n = 24$	$n = 23$	$n = 22$	Latency:	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 8$
	Condition	$F_{(1,23)} = 0.152$ $p = 0.700$	$F_{(1,22)} = 3.105$ $p = 0.092$	**$F_{(1,23)} = 7.677$ $p = 0.011$**	**$F_{(1,22)} = 12.328$ $p = 0.002$**	**$F_{(1,21)} = 5.934$ $p = 0.024$**	Condition	$F_{(1,7)} = 0.357$ $p = 0.569$	$F_{(1,7)} = 0.086$ $p = 0.778$	$F_{(1,7)} < 0.001$ $p = 1.000$	$F_{(1,7)} = 0.104$ $p = 0.757$	$F_{(1,7)} = 0.213$ $p = 0.659$
	Cond*Lvl	$F_{(1.34, 30.8)} = 0.733$ $p = 0.437$	$F_{(1.92, 42.2)} = 0.296$ $p = 0.736$	$F_{(3, 63)} = 0.882$ $p = 0.455$	$F_{(1.48, 32.6)} = 0.462$ $p = 0.576$	$F_{(3,63)} = 1.785$ $p = 0.170$	Cond*Lvl	$F_{(3,21)} = 0.466$ $p = 0.709$	$F_{(3,21)} = 0.272$ $p = 0.845$	$F_{(3,21)} = 1.555$ $p = 0.230$	$F_{(3,21)} = 2.017$ $p = 0.185$	$F_{(3,21)} = 0.218$ $p = 0.883$
	Amplitude:						Amplitude:					
	Condition	$F_{(1,23)} = 0.089$ $p = 0.768$	$F_{(1,22)} = 1.155$ $p = 0.294$	$F_{(1,23)} = 0.087$ $p = 0.771$	$F_{(1,22)} = 3.301$ $p = 0.084$	$F_{(1,21)} = 0.74$ $p = 0.399$	Condition	$F_{(1,7)} = 0.0207$ $p = 0.890$	$F_{(1,7)} = 2.333$ $p = 0.170$	$F_{(1,7)} = 0.040$ $p = 0.848$	$F_{(1,7)} = 0.321$ $p = 0.588$	$F_{(1,7)} = 5.321$ $p = 0.054$
	Cond*Lvl	$F_{(1.69, 38.8)} = 0.147$ $p = 0.829$	$F_{(2.06, 45.2)} = 1.034$ $p = 0.365$	$F_{(1.93, 42.4)} = 1.058$ $p = 0.354$	$F_{(1.73, 36.3)} = 0.635$ $p = 0.513$	$F_{(2.08, 45.8)} = 0.190$ $p = 0.836$	Cond*Lvl	$F_{(1.72, 12)} = 1.005$ $p = 0.383$	$F_{(3,21)} = 0.688$ $p = 0.569$	$F_{(1.56, 10.9)} = 1.518$ $p = 0.239$	$F_{(3,21)} = 0.432$ $p = 0.732$	$F_{(3,21)} = 0.907$ $p = 0.455$
32 kHz	Latency:	$n = 22$	$n = 22$	$n = 22$	$n = 21$	$n = 15$	Latency:	$n = 7$	$n = 7$	$n = 7$	$n = 7$	$n = 6$
	Condition	$F_{(1,21)} = 2.863$ $p = 0.105$	$F_{(1,21)} = 1.994$ $p = 0.173$	$F_{(1,21)} = 0.976$ $p = 0.335$	$F_{(1,20)} = 2.216$ $p = 0.152$	$F_{(1,14)} < 0.001$ $p = 0.985$	Condition	$F_{(1,6)} = 1.478$ $p = 0.270$	$F_{(1,6)} = 0.508$ $p = 0.503$	$F_{(1,6)} = 2.470$ $p = 0.167$	$F_{(1,6)} = 1.615$ $p = 0.251$	$F_{(1,5)} = 1.662$ $p = 0.254$
	Cond*Lvl	$F_{(2.03, 42.6)} = 1.172$ $p = 0.32$	$F_{(1.41, 29.6)} = 2.659$ $p = 0.102$	$F_{(1.22, 25.6)} = 1.990$ $p = 0.169$	$F_{(3, 60)} = 0.536$ $p = 0.659$	$F_{(3, 42)} = 0.129$ $p = 0.942$	Cond*Lvl	$F_{(1.35, 8.07)} = 0.857$ $p = 0.481$	$F_{(3,18)} = 2.265$ $p = 0.116$	$F_{(3,18)} = 0.433$ $p = 0.732$	$F_{(1.34, 8.02)} = 0.148$ $p = 0.781$	$F_{(3,15)} = 1.12$ $p = 0.372$
	Amplitude:						Amplitude:					
	Condition	$F_{(1,21)} = 1.186$ $p = 0.289$	$F_{(1,21)} = 2.006$ $p = 0.171$	$F_{(1,21)} = 0.105$ $p = 0.749$	$F_{(1,20)} = 2.55$ $p = 0.125$	$F_{(1,14)} = 0.501$ $p = 0.489$	Condition	$F_{(1,6)} = 0.412$ $p = 0.545$	$F_{(1,6)} = 0.047$ $p = 0.836$	$F_{(1,6)} = 2.834$ $p = 0.143$	$F_{(1,6)} = 0.020$ $p = 0.893$	$F_{(1,5)} = 0.961$ $p = 0.372$
	Cond*Lvl	$F_{(1.59, 33.4)} = 0.710$ $p = 0.468$	$F_{(3, 63)} = 1.589$ $p = 0.201$	$F_{(1.71, 35.9)} = 1.600$ $p = 0.218$	$F_{(1.75, 36.8)} = 0.013$ $p = 0.979$	$F_{(3, 48)} = 0.436$ $p = 0.728$	Cond*Lvl	$F_{(1.35, 8.07)} = 0.907$ $p = 0.401$	$F_{(3,18)} = 2.137$ $p = 0.131$	$F_{(3, 18)} = 1.664$ $p = 0.210$	$F_{(3, 18)} = 2.665$ $p = 0.079$	$F_{(3,15)} = 3.085$ $p = 0.059$

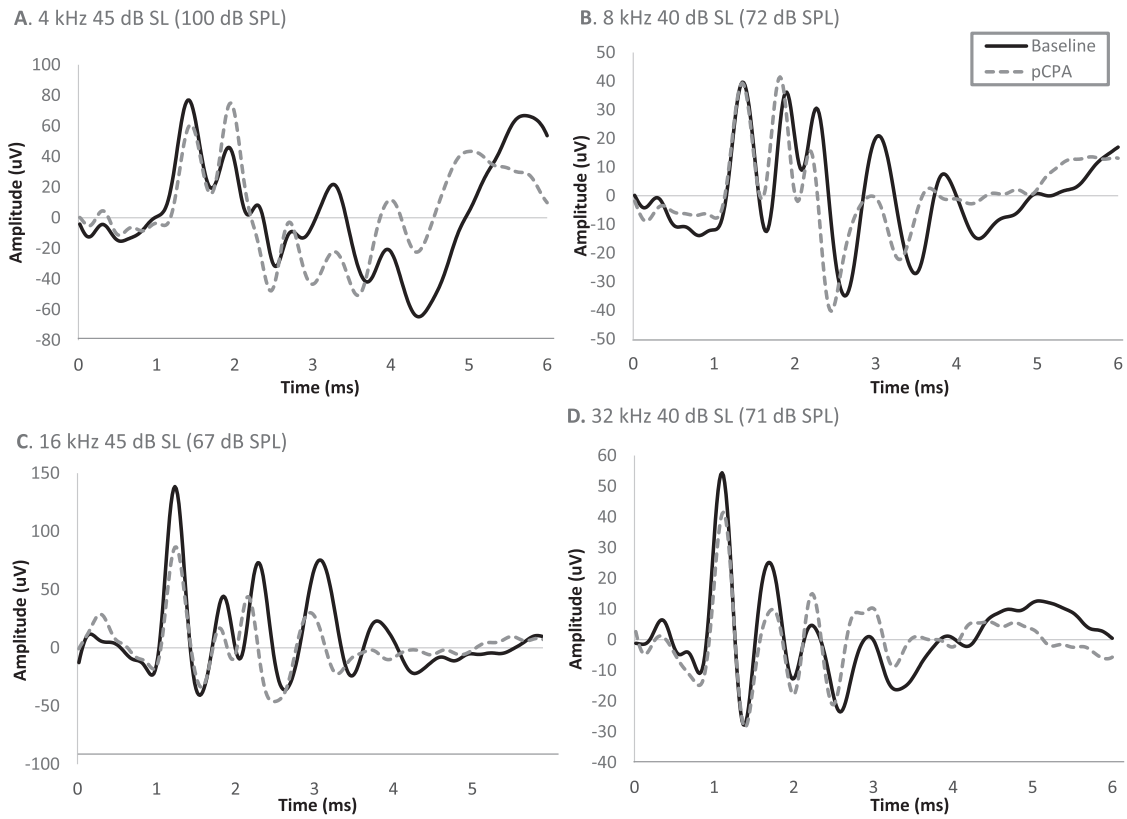

Fig. 2. ABR measures obtained during baseline (solid black) and after serotonin depletion with pCPA (broken gray) for a representative subject. Panels A through D show waveforms in response to stimulus frequencies of 4, 8, 16, and 32 kHz, respectively, presented at similar sensation levels. Notice different amplitude scales for each panel reflecting differences in response amplitudes across stimulus frequencies and presentation levels.

neurotransmission is associated with increases in the slope of the amplitude-intensity functions of the cortical N1/P2 response (Hegerl et al., 2001), although the robustness of this phenomenon in human patients is debated (O'Neill et al., 2008). One previous study assessing auditory brainstem effects in depressed patients also reported steeper amplitude intensity functions for wave V of the ABR (Gopal et al., 2004). To assess the potential effects of serotonin depletion on wave V of the ABR across stimulus intensity, we analyzed both the interaction between 'Condition' and 'Presentation Level', and also calculated the slope of each individual's latency/intensity and amplitude/intensity functions for wave V in both baseline and serotonin-depleted conditions. These individual slope measures were then submitted to repeated-measures ANOVA with 'Condition' as the within-subjects factor with two levels (baseline and serotonin-depleted). Both of these metrics revealed a significant effect of serotonin depletion on the latency-intensity function of wave V in response to 4 kHz stimuli only. The interaction between 'Condition' and 'Presentation Level' was significant at the level of $p = 0.038$ ($F_{(1.95,\ 29.266)} = 3.701$; Table 2) and the main effect of individual slope comparisons between baseline and serotonin-depleted conditions was significant at the level of $p = 0.003$ ($F_{(1,\ 20)} = 11.732$). The group average effect of serotonin depletion on wave V latencies in response to 4 kHz stimuli across a 40 dB range of intensities is shown in Fig. 4. Compared to baseline measures, the slope of the latency-intensity function is shallower following serotonin depletion. This effect is driven by shorter latencies in response to low level stimuli in the serotonin-depleted condition with more similar latencies occurring in response to higher level stimuli. No significant changes were found with regard to the amplitude-intensity functions of wave V at any stimulus frequency or on the latency-intensity functions of wave V in response to stimulus frequencies other than 4 kHz.

3.3. *Effects of serotonergic agonists*

In order to assess the effects of stimulating only select serotonergic receptors, three groups of subjects were given injections of agonists selective for the 5-HT1A, 1B, and 2 receptors immediately following ABR assessments of serotonin depletion. This experimental design allowed us to isolate the influence of specific serotonergic receptors by comparing conditions in which virtually no receptors were activated (serotonin depletion conditions) to those conditions in which only select serotonergic receptors were activated (agonist conditions). Repeated-measures ANOVAs were conducted on the within-subjects variables 'Condition' (two levels including 'serotonin-depleted' and 'agonist') and 'Presentation Level' (4 levels), with separate ANOVAs conducted for latency and amplitude measures. Outcomes from these analyses are presented in Table 3.

3.3.1. *8-OH-DPAT (5-HT1A) agonist*

Compared to the serotonin-depleted condition, application of the 5-HT1A agonist 8-OH-DPAT led to significant changes in both the amplitude and latency of many peak ABR components across a wide range of stimulus frequencies (Table 3). These changes were particularly pervasive in responses to the lowest frequency stimulus, 4 kHz stimuli, at which 8-OH-DPAT significantly reduced the latencies of waves II through V and decreased the amplitudes of waves II and IV. These effects on the latency and amplitude of responses at 4 kHz are shown in Fig. 5, panels A and B, respectively.

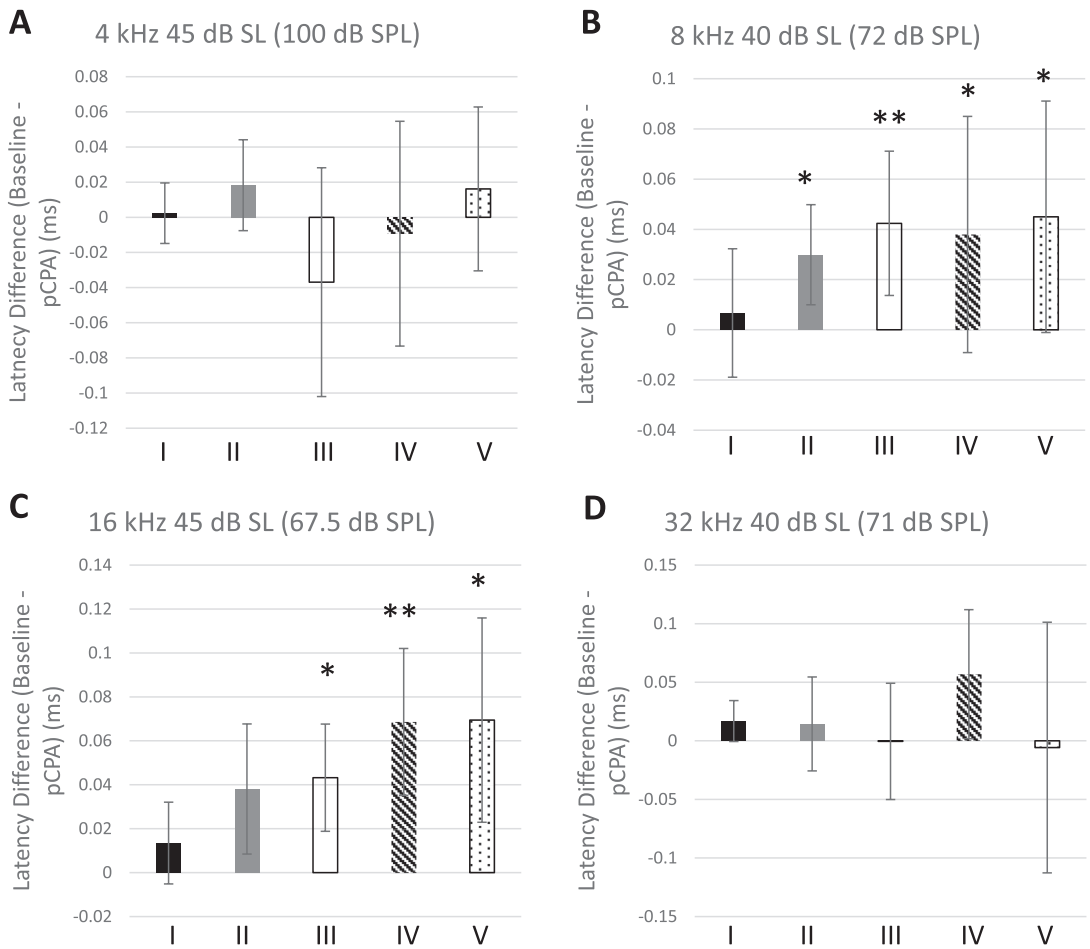

Fig. 3. Average individual change in latency following serotonin depletion. For each individual, latencies measured after pCPA administration were subtracted from baseline latency measures. Positive values indicate shorter latencies following serotonin depletion, and negative values represent longer latencies relative to baseline measures. Error bars represent ±1 SEM. * indicates significance at the level of $p < 0.05$. ** indicates significance at the level of $p < 0.01$.

Latency and amplitude changes in response to other stimulus frequencies were more sporadic, but displayed similar patterns particularly with respect to amplitude changes. In response to 8 kHz stimuli, all waves had generally smaller amplitudes with significant decreases identified at waves I, III, and IV. Significant effects in response to 16 kHz stimuli included smaller amplitudes of waves IV and V during 5-HT1A activation compared to serotonin depletion, with significant latency decreases and amplitude increases of wave II in response to 32 kHz stimuli (Table 3).

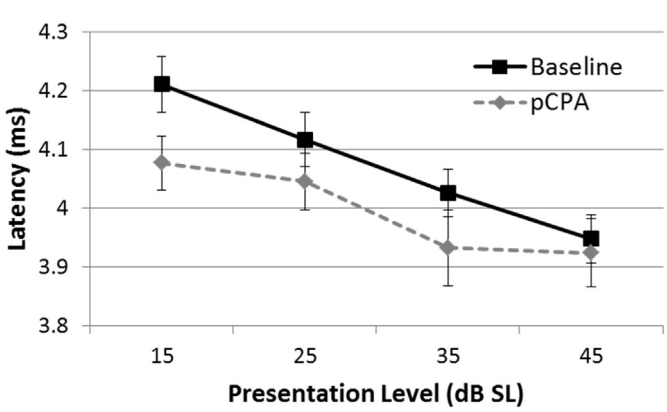

Fig. 4. Changes in the slope of latency/intensity function of wave V in response to 4 kHz stimuli presented over a range of 40 dB measured during baseline (solid black line) and following serotonin depletion with pCPA (broken gray line). Error bars indicate ±1 SEM.

3.3.2. 5-HT1B agonist (CP93129) effects

Application of the 5-HT1B agonist led to few significant changes in ABR responses compared to serotonin-depleted recordings. Analysis of responses to 32 kHz stimuli indicated a significant main effect of 'Condition' on the latencies of waves IV and V (Table 3) such that application of CP93129 led to shorter latencies compared with the pCPA condition. However, due to the small amplitude of wave V responses to 32 kHz stimuli (see Fig. 2), the sample sizes upon which these analyses were conducted are quite small (*n* of 5 and 4 for waves IV and V, respectively) such that any interpretations must be drawn with caution.

3.3.3. 5-HT2 agonist (DOI) effects

Similar to results from the 5-HT1A receptor agonist, application of a 5-HT2 agonist in serotonin-depleted subjects led to significant decreases in the latencies of waves II, IV, and V in response to 4 kHz stimuli as evidenced by a significant main effect of 'Condition' at this frequency (Table 3). Waves I and III also demonstrated similar trends toward significant latency decreases. Fig. 6, panel A, plots the average latency measured during serotonin-depleted conditions

Table 3
Results of repeated-measures ANOVA comparing selective serotonin receptor agonists to serotonin-depletion conditions. Separate ANOVAs were conducted for the amplitudes and latencies of each wave at each of the four stimulus frequencies. Significant results ($p \leq 0.05$) are in bold text.

	Serotonin depletion (pCPA) compared to 5-HT1A agonist (8-OH-DPAT)					Serotonin depletion (pCPA) compared to 5-HT1B agonist (CP93129)					Serotonin depletion (pCPA) compared to 5-HT2 agonist (DOI)				
	Wave I	Wave II	Wave III	Wave IV	Wave V	Wave I	Wave II	Wave III	Wave IV	Wave V	Wave I	Wave II	Wave III	Wave IV	Wave V
	F	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
4 kHz	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 6	*n* = 5	*n* = 4	*n* = 6	*n* = 6	*n* = 3	*n* = 8	*n* = 8	*n* = 7	*n* = 8	*n* = 7
Latency	$F_{(1,7)} = 3.91$	$\mathbf{F_{(1,7)} = 15.840}$	$\mathbf{F_{(1,7)} = 52.320}$	$\mathbf{F_{(1,6)} = 22.639}$	$\mathbf{F_{(1,5)} = 8.653}$	$F_{(1,4)} = 4.704$	$F_{(1,3)} = 2.853$	$F_{(1,5)} = 0.108$	$F_{(1,5)} = 0.137$	$F_{(1,2)} = 0.142$	$F_{(1,7)} = 4.034$	$\mathbf{F_{(1,7)} = 6.644}$	$F_{(1,6)} = 3.324$	$\mathbf{F_{(1,7)} = 6.260}$	$\mathbf{F_{(1,6)} = 14.587}$
	$p = 0.090$	$\mathbf{p = 0.005}$	$\mathbf{p < 0.001}$	$\mathbf{p = 0.003}$	$\mathbf{p = 0.032}$	$p = 0.096$	$p = 0.190$	$p = 0.756$	$p = 0.726$	$p = 0.743$	$p = 0.085$	$\mathbf{p = 0.037}$	$p = 0.118$	$\mathbf{p = 0.041}$	$\mathbf{p = 0.009}$
Amplitude	$F_{(1,7)} = 0.174$	$\mathbf{F_{(1,7)} = 22.381}$	$F_{(1,7)} = 0.470$	$\mathbf{F_{(1,6)} = 7.010}$	$F_{(1,5)} = 4.849$	$F_{(1,4)} = 1.204$	$F_{(1,3)} = 6.329$	$F_{(1,5)} = 1.346$	$F_{(1,5)} = 0.221$	$F_{(1,2)} = 1.475$	$F_{(1,7)} = 1.953$	$\mathbf{F_{(1,7)} = 6.584}$	$F_{(1,6)} = 1.258$	$F_{(1,7)} = 0.947$	$F_{(1,6)} = 0.074$
	$p = 0.690$	$\mathbf{p = 0.002}$	$p = 0.515$	$\mathbf{p = 0.038}$	$p = 0.079$	$p = 0.334$	$p = 0.086$	$p = 0.298$	$p = 0.658$	$p = 0.311$	$p = 0.205$	$\mathbf{p = 0.037}$	$p = 0.305$	$p = 0.363$	$p = 0.793$
8 kHz	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 7	*n* = 7	*n* = 7	*n* = 7	*n* = 6	*n* = 8	*n* = 8	*n* = 7	*n* = 8	*n* = 7
Latency	$F_{(1,7)} = 0.452$	$F_{(1,7)} = 1.264$	$\mathbf{F_{(1,7)} = 6.060}$	$F_{(1,7)} = 3.832$	$F_{(1,6)} = 0.863$	$F_{(1,6)} = 2.090$	$F_{(1,6)} = 0.025$	$F_{(1,6)} = 0.010$	$F_{(1,6)} = 1.231$	$F_{(1,5)} = 5.001$	$F_{(1,7)} = 0.630$	$F_{(1,7)} = 4.543$	$F_{(1,6)} = 3.092$	$F_{(1,7)} = 1.690$	$F_{(1,6)} = 0.792$
	$p = 0.523$	$p = 0.298$	$\mathbf{p = 0.043}$	$p = 0.091$	$p = 0.389$	$p = 0.198$	$p = 0.879$	$p = 0.925$	$p = 0.310$	$p = 0.076$	$p = 0.0453$	$p = 0.071$	$p = 0.129$	$p = 0.235$	$p = 0.408$
Amplitude	$\mathbf{F_{(1,7)} = 23.825}$	$\mathbf{F_{(1,7)} = 6.820}$	$F_{(1,7)} = 0.786$	$\mathbf{F_{(1,7)} = 17.939}$	$F_{(1,6)} = 2.772$	$F(1,6) = 0.244$	$F_{(1,6)} = 1.409$	$F_{(1,6)} < 0.001$	$F_{(1,6)} = 0.519$	$F_{(1,5)} = 4.406$	$F_{(1,7)} = 1.483$	$F_{(1,7)} = 4.146$	$F_{(1,6)} = 0.307$	$F_{(1,7)} = 0.329$	$F_{(1,6)} = 0.610$
	$\mathbf{p = 0.002}$	$\mathbf{p = 0.350}$	$p = 0.405$	$\mathbf{p = 0.004}$	$p = 0.147$	$p = 0.639$	$p = 0.280$	$p = 0.987$	$p = 0.498$	$p = 0.090$	$p = 0.263$	$p = 0.081$	$p = 0.597$	$p = 0.584$	$p = 0.460$
16 kHz	*n* = 7	*n* = 7	*n* = 7	*n* = 7	*n* = 7	*n* = 6	*n* = 7	*n* = 7	*n* = 6	*n* = 6	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7
Latency	$F_{(1,6)} < 0.000$	$F_{(1,6)} = 0.153$	$F_{(1,6)} = 0.965$	$F_{(1,6)} = 0.653$	$F_{(1,6)} = 1.462$	$F_{(1,5)} = 2.195$	$F_{(1,6)} = 5.165$	$F_{(1,6)} = 0.195$	$F_{(1,5)} = 0.133$	$F_{(1,5)} = 0.200$	$F_{(1,7)} = 1.455$	$F_{(1,7)} = 0.943$	$F_{(1,7)} = 0.775$	$F_{(1,7)} = 0.407$	$F_{(1,6)} = 1.063$
	$p = 1.000$	$p = 0.709$	$p = 0.364$	$p = 0.450$	$p = 0.272$	$p = 0.199$	$p = 0.063$	$p = 0.674$	$p = 0.730$	$p = 0.673$	$p = 0.267$	$p = 0.364$	$p = 0.408$	$p = 0.544$	$p = 0.342$
Amplitude	$F_{(1,6)} = 0.321$	$F_{(1,6)} = 3.715$	$F_{(1,6)} = 0.913$	$\mathbf{F_{(1,6)} = 15.701}$	$\mathbf{F_{(1,6)} = 9.985}$	$F(1,5) = 0.076$	$F_{(1,6)} = 0.892$	$F_{(1,6)} = 1.569$	$F_{(1,5)} = 0.924$	$F_{(1,5)} = 6.329$	$F_{(1,7)} = 0.015$	$F_{(1,7)} = 0.419$	$F_{(1,7)} = 1.783$	$F_{(1,7)} = 0.556$	$F_{(1,6)} = 1.545$
	$p = 0.592$	$p = 0.102$	$p = 0.913$	$\mathbf{p = 0.007}$	$\mathbf{p = 0.020}$	$p = 0.794$	$p = 0.381$	$p = 0.257$	$p = 0.381$	$p = 0.053$	$p = 0.905$	$p = 0.538$	$p = 0.224$	$p = 0.480$	$p = 0.254$
32 kHz	*n* = 4	*n* = 5	*n* = 5	*n* = 5	*n* = 4	*n* = 5	*n* = 5	*n* = 5	*n* = 5	*n* = 4	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 2
Latency	$F_{(1,3)} = 0.820$	$\mathbf{F_{(1,4)} = 28.560}$	$F_{(1,4)} = 0.267$	$F_{(1,4)} = 0.925$	$F_{(1,3)} = 0.137$	$F_{(1,4)} = 0.112$	$F_{(1,4)} = 0.611$	$F_{(1,4)} = 0.598$	$\mathbf{F_{(1,4)} = 67.529}$	$\mathbf{F_{(1,3)} = 12.835}$	$F_{(1,7)} = 3.619$	$F_{(1,7)} = 3.098$	$F_{(1,7)} = 0.231$	$F_{(1,6)} = 0.008$	$F_{(1,1)} = 4.098$
	$p = 0.432$	$\mathbf{p = 0.006}$	$p = 0.633$	$p = 0.925$	$p = 0.736$	$p = 0.755$	$p = 0.478$	$p = 0.482$	$\mathbf{p = 0.001}$	$\mathbf{p = 0.037}$	$p = 0.099$	$p = 0.122$	$p = 0.645$	$p = 0.933$	$p = 0.292$
Amplitude	$F_{(1,3)} = 6.736$	$\mathbf{F_{(1,4)} = 11.665}$	$F_{(1,4)} = 0.021$	$F_{(1,4)} = 0.427$	$F_{(1,3)} = 6.377$	$F_{(1,4)} = 0.375$	$F_{(1,4)} = 0.527$	$F_{(1,4)} = 0.786$	$F_{(1,4)} = 0.501$	$F_{(1,3)} = 0.180$	$F_{(1,7)} = 0.191$	$F_{(1,7)} = 1.373$	$F_{(1,7)} = 0.317$	$F_{(1,6)} = 2.245$	$F_{(1,1)} = 5.030$
	$p = 0.060$	$\mathbf{p = 0.027}$	$p = 0.892$	$p = 0.549$	$p = 0.086$	$p = 0.573$	$p = 0.508$	$p = 0.425$	$p = 0.518$	$p = 0.700$	$p = 0.677$	$p = 0.280$	$p = 0.591$	$p = 0.185$	$p = 0.088$

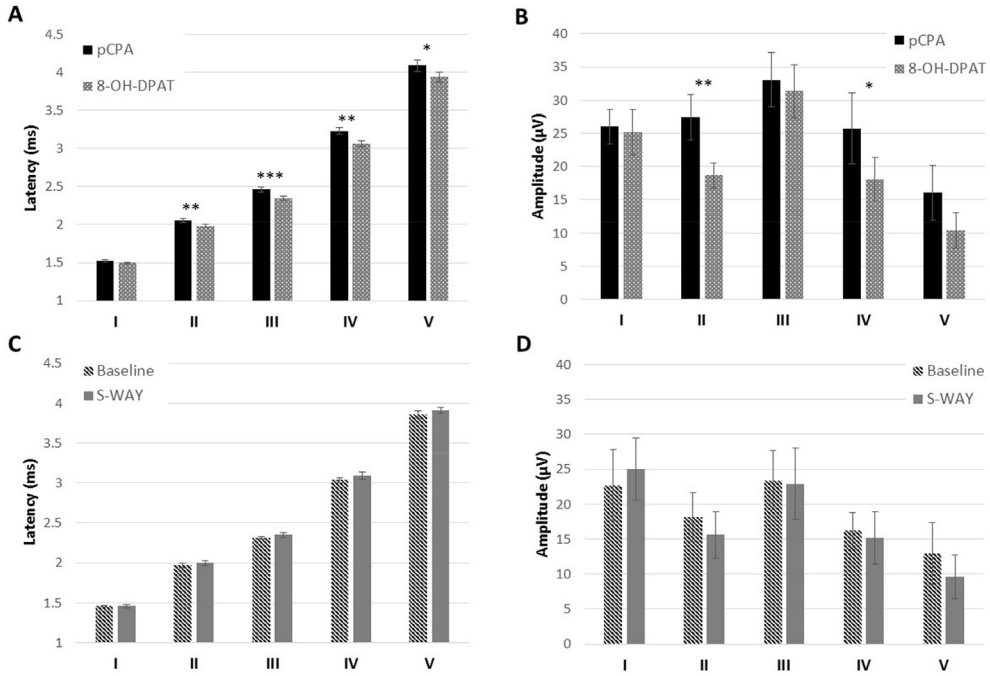

Fig. 5. Group average 5-HT1A receptor effects on ABR peaks I – V in response to 4 kHz stimuli averaged across all presentation levels. Panels A and B represent the average latency and amplitude measures, respectively, obtained during serotonin depletion with pCPA and following application of the 5-HT1A agonist 8-OH-DPAT. Panels C and D represent the average latency and amplitude measures, respectively, measured during baseline and following application of the 5-HT1A antagonist S-WAY. * indicates $p < 0.05$. ** indicates $p < 0.01$. *** indicates $p < 0.001$. Error bars = ±1 SEM.

and following DOI application in response to 4 kHz stimuli presented at 45 dB SL. From this figure, it is apparent that the latencies of waves II, IV, and V were consistently decreased relative to serotonin-depleted conditions across subjects. The amplitude of wave II in response to 4 kHz was also significantly decreased by the application of DOI (Table 3). Responses to stimulation at other test frequencies (8, 16, and 32 kHz) demonstrated no significant main effects of DOI application on either peak amplitudes or latencies.

3.4. Effects of serotonergic antagonists

To complement the agonist portion of the study in which only one specific serotonergic receptor was stimulated in the absence of serotonin to stimulate other receptors, the effects of deactivating these specific receptors were investigated in a group of previously untreated mice with normal levels of serotonergic activity. Hence, this paradigm allows for the assessment of responses when only one serotonergic receptor type is deactivated and all others are functioning normally, as well as when that serotonergic same receptor type is the only activated receptor. Serotonergic antagonists were administered immediately following baseline ABR measures with additional ABR measures occurring one hour after. The effects of antagonist application were assessed using repeated-measures ANOVAs on the within-subjects variables “Condition” (two levels including “baseline” and “agonist”) and “Presentation Level” (four levels), with separate ANOVAs conducted for latency and amplitude measures. Outcomes from these analyses are presented in Table 4.

3.4.1. 5-HT1A (S-WAY) antagonist

Compared to baseline measures, application of S-WAY had minimal effects on ABR. Average response latencies and amplitudes are shown in Fig. 5, panels C and D, respectively, in response to 4 kHz stimulus. This figure demonstrates the general lack of effects of S-WAY application on either response latencies or amplitudes at this frequency. Statistical analysis revealed no significant main effect of ‘Condition’ on either the amplitude of latency of any wave at any test frequency (Table 4).

3.4.2. 5-HT1B (NAS-181) antagonist

Application of the 5-HT1B antagonist, NAS-181, revealed a decrease in the latency of nearly all ABR peaks in response to 4 kHz stimuli. This effect is demonstrated in Fig. 6, panel B, which shows the average measured during baseline testing and following NAS-181 application. Statistical analysis indicates that the decrease in latency relative to baseline for responses to 4 kHz stimuli was significant for all waves with the exception of wave II which showed a clear trend toward significance (Table 4). No significant main effects of ‘Condition’ were found on the amplitudes of any peaks in response to 4 kHz. Effects of NAS-181 on responses to stimuli other than 4 kHz were minimal.

3.4.3. 5-HT2A (ketanserin)

Blocking the action of the 5-HT2A receptor with ketanserin revealed strong effects on response amplitudes to low and mid frequency stimuli, with no significant effects on responses to 32 kHz stimuli or response latencies at any frequency (Table 4). All significant amplitude effects indicated that application of ketanserin lead to significant amplitude decreases relative to baseline. An example of this effect is shown in Fig. 6, panel C, which displays the average amplitudes measured during baseline and following ketanserin application in response to 8 kHz stimuli presented at 40 dB SL. At this frequency, significant amplitude shifts were found for waves II, IV, and V (Table 4). Similar effects were found for waves I and II in response to 4 kHz stimuli and waves II and IV in response to 16 kHz stimuli (Table 4).

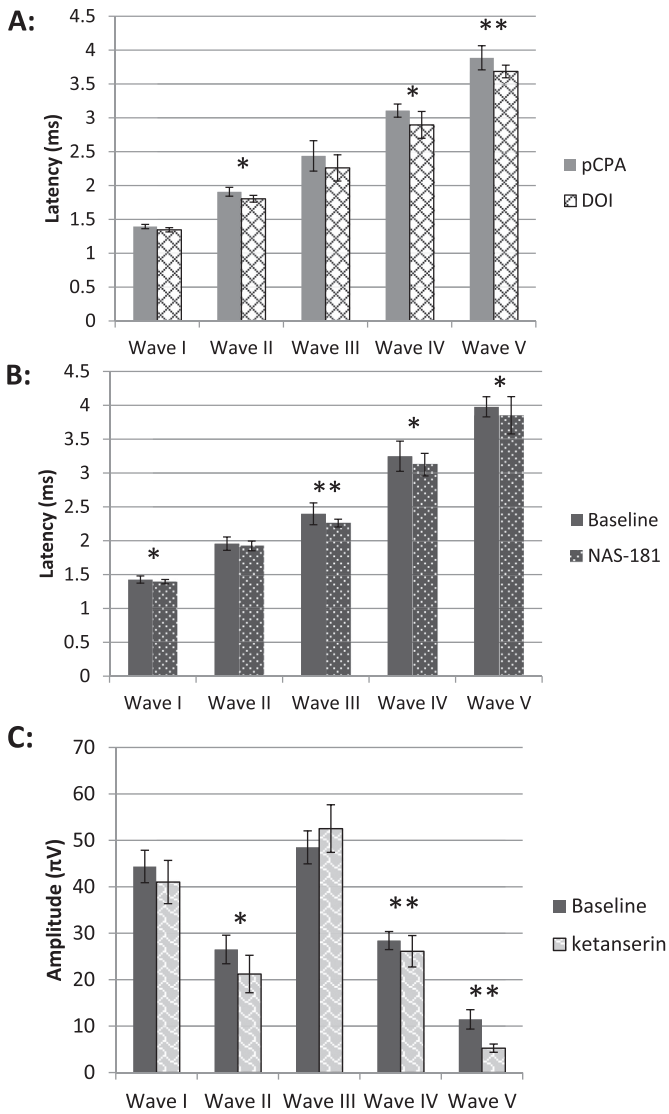

Fig. 6. Effects of serotonin receptor activation with DOI and deactivation using NAS-181 and Ketanserin on ABR components. Panel A demonstrates the group average latency in response to 4 kHz stimuli presented at 45 dB SL during serotonin depletion (pCPA) and after application of DOI. Panel B shows the group average latency before and after application of NAS-181 in response to the same stimulus as panel A. In Panel C, the group average amplitude is shown in response to 8 kHz stimuli presented at 40 dB SL before and after Ketanserin application. Error bars = SEM. * indicates $p < 0.05$. ** indicates $p < 0.01$.

4. Discussion

This is the first study to explore the effects of serotonin depletion and serotonin receptor modulation using the ABR across a wide range of stimulus frequencies and intensities. The data presented here strongly support a modulatory role of serotonin on auditory brainstem processing. With regard to our initial hypotheses, our results indicate that 1) depletion of serotonin results in reduced ABR latencies, 2) serotonergic effects are dependent upon the unique characteristics of specific serotonergic receptor agonists and antagonists, and 3) the effects of manipulating serotonin and its receptors are more pronounced for lower- and mid-frequency responses. The diverse manner in which such effects are exerted suggests a sophisticated role of serotonin in regulating the balance of excitation and inhibition within the auditory pathway beginning as early as the auditory nerve.

4.1. Serotonin depletion: latency and amplitude effects

Based upon data from cellular studies in the auditory brainstem demonstrating an often suppressive role for serotonin, we hypothesized that reduced endogenous serotonin levels would result in increased ABR amplitudes and decreased latencies, beginning as early as wave I. Depletion of endogenous serotonin led to significant decreases in the latencies of most waves in response to 8 and 16 kHz relative to latencies in the same individual mice before depletion. The fact that these changes begin with wave II of the ABR suggests that they begin in cochlear nucleus, although this interpretation must be regarded with some caution. The spiral ganglion and the cochlea receive innervation from serotonergic fibers, and there is also evidence of serotonergic turnover in the cochlea consistent with a functional role for serotonin (Gil-Loyzaga et al., 1997). Further, cultured spiral ganglion neurons are strongly responsive to serotonin receptor manipulation (Yu et al., 2013). Thus, a lack of effect of serotonin depletion on the earliest ABR wave may suggest that effects of serotonin are not sufficiently directional or prevalent to influence the ABR at the periphery. This interpretation is partially supported by the results of a previous study assessing the effects of pCPA in prosimian primates using ABR (Revelis et al., 1998). This study revealed no significant changes in early ABR components, with effects occurring only for wave V when measured using similar pCPA dosages and stimulus presentation rates as the present study. However, it should be noted that the effects they reported at wave V were in the opposite direction, reflecting *increased* wave V latencies after pCPA. This discrepancy between the results of the two studies may reflect differences in the effects of serotonin depletion across stimulus frequencies. The present study employed tone pips of relatively narrow frequency content presented at low to moderate levels compared to the considerably higher-level broadband clicks employed in the primate study (120 dB SPL). These two types of stimuli may be dominated by different cochlear regions (Eggermont and Don, 1980; Abdala and Folsom, 1995), raising the possibility that spectral or intensity differences in the stimuli would result in different effects of serotonin depletion. It is also possible that the differences between the studies may stem from differences between subject species.

In spite of significant reductions in latency, no significant amplitude effects were found following serotonin depletion. Reduced ABR latencies are most often associated with increased response amplitudes, such as occurs with increases in stimulus intensity (Picton et al., 1981). ABR amplitudes are primarily dependent upon the number of cells firing synchronously to the onset of an auditory stimulus with additional contributions of cranial anatomy and electrode placement (Picton et al., 1981). Significant latency decreases without significant amplitude effects following serotonin depletion could thus indicate a shift in the population response toward shorter response latencies without affecting either the number or synchrony of responsive cells. It is also possible that a lack of amplitude effects following serotonin depletion is related to the inherently greater variability of ABR amplitude compared to latencies which is reflected in the significantly larger relative standard deviations for ABR amplitudes compared to latencies (Musiek et al., 1984 & 1986; Yang et al., 1993). However, this possibility seems unlikely given that manipulation of specific serotonergic receptors were found to elicit significant amplitude effects. Overall, the pattern of reduced peak response latencies with minimal changes in amplitude in the absence of serotonin is somewhat consistent with cellular studies in various brainstem nuclei with regard to the intricate pattern of serotonergic effects in the brainstem. Rather than producing purely suppressive or excitatory effects, serotonin depletion in the current

Table 4
Results of repeated-measures ANOVA comparing selective serotonin receptor antagonists to baseline conditions. Separate ANOVAs were conducted for the amplitudes and latencies of each wave at each of the four stimulus frequencies. Significant results ($p \leq 0.05$) are in bold text.

	Baseline compared to 5-HT1A antagonist (S-WAY)					Baseline compared to 5-HT1B antagonist (NAS-181)					Baseline compared to 5-HT2 antagonist (ketanserin)				
	Wave I	Wave II	Wave III	Wave IV	Wave V	Wave I	Wave II	Wave III	Wave IV	Wave V	Wave I	Wave II	Wave III	Wave IV	Wave V
	F	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
4 kHz	*n* = 6	*n* = 7	*n* = 6	*n* = 7	*n* = 6	*n* = 7	*n* = 7	*n* = 7	*n* = 7	*n* = 2	*n* = 6	*n* = 7	*n* = 6	*n* = 7	*n* = 6
Latency	$F_{(1,5)} = 0.040$	$F_{(1,6)} = 3.166$	$F_{(1,5)} = 4.127$	$F_{(1,6)} = 2.907$	$F_{(1,5)} = 3.270$	$\mathbf{F_{(1,6)} = 6.035}$	$F_{(1,6)} = 4.794$	$\mathbf{F_{(1,6)} = 15.534}$	$\mathbf{F_{(1,6)} = 6.926}$	$\mathbf{F(1,1) = 29.766}$	$F_{(1,5)} = 0.016$	$F_{(1,6)} = 0.028$	$F_{(1,5)} = 3.204$	$F_{(1,6)} = 5.534$	$F_{(1,5)} = 0.095$
	p = 0.849	p = 0.125	p = 0.098	p = 0.139	p = 0.130	**p = 0.049**	p = 0.071	**p = 0.008**	**p = 0.039**	**p = 0.042**	p = 0.902	p = 0.873	p = 0.117	p = 0.051	p = 0.766
Amplitude	$F_{(1,5)} = 0.419$	$F_{(1,6)} = 1.320$	$F_{(1,5)} = 0.026$	$F_{(1,6)} = 0.219$	$F_{(1,5)} = 0.979$	$F_{(1,6)} = 0.585$	$F_{(1,6)} = 1.267$	$F_{(1,6)} = 3.745$	$F_{(1,6)} = 1.127$	$F_{(1,1)} = 13.884$	$\mathbf{F_{(1,5)} = 7.395}$	$\mathbf{F_{(1,6)} = 33.731}$	$F_{(1,5)} = 2.320$	$F_{(1,6)} = 0.333$	$F_{(1,5)} = 1.795$
	p = 0.546	p = 0.294	p = 0.877	p = 0.656	p = 0.368	p = 0.473	p = 0.303	p = 0.101	p = 0.329	p = 0.167	**p = 0.030**	**p = 0.001**	p = 0.172	p = 0.582	p = 0.222
8 kHz	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 7	*n* = 6	*n* = 7	*n* = 7	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7
Latency	$F_{(1,7)} = 3.671$	$F_{(1,7)} = 0.063$	$F_{(1,7)} = 2.408$	$F_{(1,7)} = 3.425$	$F_{(1,7)} = 0.287$	$F_{(1,6)} = 1.328$	$F_{(1,6)} = 0.222$	$F_{(1,5)} = 0.017$	$F_{(1,6)} = 0.048$	$F_{(1,6)} = 2.290$	$F_{(1,7)} = 1.423$	$F_{(1,7)} = 1.925$	$F_{(1,7)} = 0.045$	$F_{(1,7)} = 0.358$	$F_{(1,6)} = 0.880$
	p = 0.097	p = 0.809	p = 0.172	p = 0.107	p = 0.608	p = 0.293	p = 0.654	p = 0.901	p = 0.833	p = 0.181	p = 0.272	p = 0.208	p = 0.838	p = 0.568	p = 0.384
Amplitude	$F_{(1,7)} = 1.316$	$F_{(1,7)} = 0.200$	$F_{(1,7)} = 2.089$	$F_{(1,7)} = 0.548$	$F_{(1,7)} = 0.231$	$F_{(1,6)} = 0.129$	$F_{(1,6)} = 1.755$	$F_{(1,5)} = 0.040$	$F_{(1,6)} = 1.608$	$F_{(1,6)} = 0.015$	$F_{(1,7)} = 1.308$	$\mathbf{F_{(1,7)} = 6.276}$	$F_{(1,7)} = 0.011$	$\mathbf{F_{(1,7)} = 14.792}$	$\mathbf{F_{(1,6)} = 19.111}$
	p = 0.289	p = 0.668	p = 0.192	p = 0.483	p = 0.645	p = 0.731	p = 0.233	p = 0.849	p = 0.252	p = 0.907	p = 0.290	**p = 0.041**	p = 0.921	**p = 0.006**	**p = 0.005**
16 kHz	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 8	*n* = 8	*n* = 8	*n* = 8	*n* = 8
Latency	$F_{(1,7)} = 0.070$	$F_{(1,7)} = 0.106$	$F_{(1,7)} = 0.410$	$F_{(1,7)} = 0.064$	$F_{(1,6)} = 0.261$	$F_{(1,7)} < 0.001$	$F_{(1,7)} = 0.895$	$F_{(1,7)} = 1.330$	$F_{(1,7)} = 0.022$	$F_{(1,6)} = 3.712$	$F_{(1,7)} = 1.957$	$F_{(1,7)} = 1.566$	$F_{(1,7)} = 0.122$	$F_{(1,7)} = 0.978$	$F_{(1,7)} = 0.872$
	p = 0.799	p = 0.754	p = 0.542	p = 0.807	p = 0.628	p = 0.983	p = 0.482	p = 0.287	p = 0.886	p = 0.102	p = 0.205	p = 0.251	p = 0.737	p = 0.356	p = 0.381
Amplitude	$F_{(1,7)} = 0.039$	$F_{(1,7)} = 0.222$	$F_{(1,7)} = 0.123$	$F_{(1,6)} = 0.010$	$F_{(1,6)} = 1.206$	$F_{(1,7)} = 0.884$	$F_{(1,7)} = 0.827$	$F_{(1,7)} = 0.586$	$F_{(1,7)} = 0.797$	$F_{(1,6)} = 2.135$	$F_{(1,7)} = 0.239$	$\mathbf{F_{(1,7)} = 7.427}$	$F_{(1,7)} = 1.760$	$\mathbf{F_{(1,7)} = 5.806}$	$F_{(1,7)} = 2.310$
	p = 0.849	p = 0.222	p = 0.736	p = 0.923	p = 0.314	p = 0.378	p = 0.393	p = 0.469	p = 0.402	p = 0.194	p = 0.640	**p = 0.030**	p = 0.226	**p = 0.047**	p = 0.172
32 kHz	*n* = 8	*n* = 8	*n* = 8	*n* = 7	*n* = 5	*n* = 5	*n* = 5	*n* = 5	*n* = 5	*n* = 2	*n* = 8	*n* = 8	*n* = 7	*n* = 8	*n* = 5
Latency	$F_{(1,7)} = 1.976$	$F_{(1,7)} = 1.828$	$F_{(1,7)} = 0.359$	$F_{(1,6)} = 0.109$	$F_{(1,4)} = 0.717$	$F_{(1,4)} = 0.093$	$F_{(1,4)} = 5.286$	$F_{(1,4)} = 1.144$	$F_{(1,4)} = 0.001$	$F_{(1,1)} = 0.001$	$F_{(1,7)} = 1.648$	$F_{(1,7)} = 0.803$	$F_{(1,6)} = 1.447$	$F_{(1,7)} = 0.077$	$F_{(1,4)} = 0.118$
	p = 0.203	p = 0.218	p = 0.568	p = 0.753	p = 0.445	p = 0.775	p = 0.083	p = 0.345	p = 0.973	p = 0.980	p = 0.240	p = 0.400	p = 0.274	p = 0.789	p = 0.749
Amplitude	$F_{(1,7)} = 1.705$	$F_{(1,7)} = 2.018$	$F_{(1,7)} = 1.039$	$F_{(1,6)} = 0.911$	$F_{(1,4)} = 0.705$	$F_{(1,4)} = 0.356$	$\mathbf{F_{(1,4)} = 8.694}$	$F_{(1,4)} = 5.161$	$F_{(1,4)} = 2.117$	$F_{(1,1)} = 2.932$	$F_{(1,7)} = 1.244$	$F_{(1,7)} = 2.453$	$F_{(1,6)} = 0.584$	$F_{(1,7)} = 0.071$	$F_{(1,4)} = 0.051$
	p = 0.233	p = 0.198	p = 0.342	p = 0.372	p = 0.448	p = 0.583	**p = 0.042**	p = 0.086	p = 0.219	p = 0.336	p = 0.302	p = 0.161	p = 0.474	p = 0.798	p = 0.832

study may reveal reduced suppression of short latency neurons with concomitant increased suppression of longer latency neurons.

In addition to general changes in ABR amplitudes and latencies, we investigated the possibility that low serotonin levels would increase the slope of ABR amplitude/intensity and latency/intensity functions. Though it is currently the subject of debate, multiple studies in both animals and humans have reported steeper slopes of amplitude/intensity functions of the cortical N1/P2 complex in individuals with low serotonergic function (Juckel et al., 1997 & 1999; Manjarrez et al., 2005; Chen et al., 2005; Gopal et al., 2005; Juckel et al., 2008; Wutzler et al., 2008; Simmons et al., 2011; Schaaf et al., 2012). In addition to these cortical studies, one study reported similar findings of wave V of the ABR in a population of depressed female patients tested both on and off selective serotonin reuptake inhibitors (SSRIs), a class of medications which increases the availability of extracellular serotonin (Gopal et al., 2005). Compared to ABR responses obtained when patients were non-medicated, SSRI use led to significantly shallower wave V amplitude/intensity functions. In the current study, the effects of intensity level on ABR wave V were analyzed both via assessing the interaction between 'Condition' and 'Presentation, and by analyzing the difference in slope between baseline and serotonin depleted conditions for each individual for each wave and stimulus frequency. Overall, our results indicate no significant changes in the slope of the amplitude-intensity function of wave V at any test frequency, but a significant *reduction* in the latency-intensity function for wave V at 4 kHz such that responses to lower level stimuli elicited shorter wave V latencies in the serotonin-depleted condition while higher level stimuli elicited similar latencies compared to baseline measures (Fig. 4). Discrepancies between our present results and those of cortical studies may be due to a number of experimental factors, including the time course of serotonergic manipulations, which have been proposed as an important variable in such effects (Lee et al., 2011; Simmons et al., 2011). It is also possible that our results indicate an important contrast in serotonergic processing in the auditory brainstem compared to the auditory cortex.

4.2. Serotonin receptor types

A second major hypothesis supported by our data was that agonists and antagonists for different serotonin receptor types would have distinct suites of effects on ABR latencies and amplitudes. This hypothesis was based on the diversity of effects of serotonin and selective serotonin receptor agonists or antagonists in multiple auditory brainstem nuclei, but the characteristic effects of agonists and antagonists we observed could arise through multiple potential pathways. These pathways include direct effects on auditory neurons expressing serotonin receptors, combined effects on auditory neurons at different stages of auditory processing, or even through effects in non-auditory brain regions projecting to auditory nuclei.

For example, the 5-HT1A receptor is expressed prominently in auditory brainstem nuclei as well as in many other brain regions (Hoyer et al., 1986; Pompeiano et al., 1992; Thompson et al., 1994). Activation of this receptor in serotonin-depleted animals caused an interesting combination of effects: a concurrent decrease in the amplitudes and latencies of multiple waves. This is different from what might be expected from a uniform decrease or increase in auditory activity, but could potentially result from effects on different subsets of auditory neurons expressing the 5-HT1A receptor. Within the IC, the selective activation of the 5-HT1A receptor strongly decreases the responses of longer-latency neurons to tones at the characteristic frequency, but has less of an effect on neurons with the shortest response latencies (Hurley, 2007). Furthermore, activation of the 5-HT receptor is more likely to suppress secondary spikes than initial spikes. In combination, these effects could advance ABR peaks to earlier latencies while decreasing amplitude. In an alternative to this scenario, the 5-HT1A receptor is expressed by both excitatory and inhibitory neurons within the IC (Peruzzi and Dut, 2004). Suppressive effects of the 5-HT1A receptor on inhibitory interneurons could cause a mix of inhibitory and disinhibitory effects apparent at the level of the ABR. Finally, the 5-HT1A receptor is an inhibitory autoreceptor within serotonergic nuclei (Hjorth, 1993). Activation of this receptor type in the Raphénuclei, and subsequent decrease of serotonin release in the auditory system, could therefore also have contributed to the combination of effects we observed.

Similar considerations apply to other types of serotonin receptors. 5-HT1B receptors are located presynaptically where they regulate the release of either excitatory or inhibitory neurotransmitters into the synaptic cleft (Sari, 2004; Xiao et al., 2008). These effects may vary among auditory nuclei; in the IC, the effect of activating this receptor is consistent with disinhibition, while at the Calyx of Held in the medial nucleus of the trapezoid body, 5-HT1B receptor stimulation reduces excitatory post-synaptic potentials (Mizutani et al., 2006). Like the 5-HT1A receptor, the 5-HT1B receptor is also an inhibitory autoreceptor in some brain regions, so could potentially act to decrease serotonin release at the level of serotonergic terminals (Engel et al., 1986). In a similar vein, pharmacological agents targeting receptors in the serotonin 2 receptor family excite neurons at multiple levels of the auditory brainstem (Hurley, 2006; Tang and Trussell, 2015), but also have inhibitory effects (Hurley, 2006). Thus, the effects of serotonin receptor manipulation that we observed are likely to represent a broad-scale integration of effects along the auditory neuraxis, and even between auditory and non-auditory systems. Despite this impressive breadth of potential mechanisms, the fact that manipulation of different receptor types produced different outcomes suggests the potential for somewhat targeted effects on auditory function. For example, activation of the 5-HT1A receptor and block of 5-HT2 receptors were two manipulations in our study that produced significant effects on ABR amplitude. This suggests that the 5-HT1A receptor could be interesting to explore in the context of auditory disorders potentially involving gain, such as hyperacusis or tinnitus (Marriage and Barnes, 1995; Noreña et al., 1999; Simpson and Davies, 2000).

4.3. Frequency

A final hypothesis regarding the effects of serotonin depletion on ABRs was that effects would be greater in response to low frequency stimuli compared to higher frequency stimuli. This speculation was based upon immunohistochemical studies indicating that serotonergic innervation to lower-frequency response regions of auditory brainstem nuclei is denser than innervation to higher-frequency response regions (Klepper and Herbert, 1991; Hurley et al., 2002; Papesh and Hurley, 2012). Present results provide limited confirmation of this hypothesis in that serotonin depletion was most likely to significantly affect responses to low- and mid-frequency stimuli while no effects on amplitude, latency, or intensity functions were found for responses to the highest frequency stimuli. This trend generally continued when assessing the effects of serotonin receptor activation and blockade in that the majority of significant effects were found in response to low- and mid-frequency stimuli (Tables 2 and 3).

This finding is especially notable, because serotonergic effects on the responses of single neurons with high characteristic frequencies has been documented in several studies (Hurley and Pollak, 1999, 2001). Our results suggest that the largest or most

directional effects of serotonin nevertheless occur for low-frequency stimuli. Since topographic patterns of serotonergic innervation are conserved across a range of mammalian species, particularly at the midbrain level (Thompson et al., 1994; Hurley and Thompson, 2001), our results further suggest that low-frequency effects could likewise be a conserved feature of serotonergic modulation of brainstem auditory processing.

4.4. Perceptual and behavioral implications

The present study clearly demonstrated that changes in endogenous serotonin levels significantly alter temporal aspects of the ABR, and thus that serotonin could have considerable consequences for auditory processing at very early stages within the auditory pathway. Although changes in ABR latencies following reduced serotonin levels were relatively small, these changes were measurable using a far-field measure such as the ABR, which integrates the electrical activity of large populations of auditory neurons. This finding suggests that either small changes in latency or spike train timing occur in large numbers of neurons, or that larger changes occur in a smaller number of neurons. Although it is not possible to distinguish among these possibilities with the ABR measurement, each of these would be likely to have functionally relevant effects, even presuming that individual neurons show very small shifts in latency. This is because the precise timing of excitatory and inhibitory inputs to auditory brainstem neurons are critical to determining selectivity of response to important acoustic features of vocalizations, such as direction and velocity of frequency changes (Andoni et al., 2007; Kao et al., 1997). Hence, even small changes in neural response timing may alter the brain's ability to detect important distinguishing characteristics of rapidly fluctuating sounds such as speech. Such difficulty is likely to be compounded in noisy listening environments. Indeed, poor neural synchrony in the brainstem is associated with poor speech understanding in noise (Wible et al., 2005; Song et al., 2011, 2012). This may partially account for why patients with disorders associated with poor serotonergic function report difficulty understanding speech in noise (Gopal et al., 2004, 2005), and why auditory perceptions are altered by drugs affecting the serotonergic system (Parrott, 2002; Meltzer et al., 2006; Geyer and Vollenweider, 2008). We further note that ABR latency changes on a highly comparable timescale have been previously reported as consequences of pCPA administration (Revelis et al., 1998). Although we observed limited changes in the amplitudes of the ABR following manipulation of serotonin or serotonin receptors, changes in auditory gain have also been frequently-reported consequences associated with altered serotonergic function (Norra et al., 2003; Hensch et al., 2006; Simmons et al., 2011). Because serotonergic innervation of the auditory system as well as serotonin receptor expression are altered following noise exposure, cochlear ablation, or age-related hearing loss (Holt et al., 2005; Tadros et al., 2007; Papesh and Hurley, 2012; Smith et al., 2014), these findings are in concert with the notion that serotonin is involved in auditory-specific disorders such as tinnitus and hyperacusis (Marriage and Barnes, 1995; Noreña et al., 1999; Simpson and Davies, 2000; Attri and Nagarkar, 2010; Caperton and Thompson, 2011).

5. Summary

The serotonergic system is a diffuse neuromodulatory network through which diverse brain regions are simultaneously influenced by the release of serotonin from the Raphé nuclei in response to both external stimuli and internal states (Hurley and Sullivan, 2012). The present work provides one of the first extensive examinations of the role of serotonin and specific serotonin receptors on far-field evoked responses in the auditory brainstem. Our results indicate that reductions in endogenous serotonin levels decrease peak ABR latencies beginning at wave II and persisting through wave V. This decrease in peak latencies is consistent with the hypothesis that serotonin plays a predominantly suppressive role in auditory processing such that low serotonin levels lead to increased auditory responsiveness. We clearly showed different serotonin receptors affect different aspects of ABR responses. Further, the effects of serotonin depletion on ABR latencies demonstrated frequency-specificity with more pervasive effects occurring in response to low and mid frequency stimuli compared to higher frequency stimuli. Taken as a whole, our findings suggest that serotonin plays a powerful role in coordinating auditory activity with behavioral states from an early level of auditory processing.

Authorship roles

Dr. Papesh was responsible for project design, implementation, analysis, and initial manuscript preparation. Dr. Hurley provided funding and oversight of project design as well as assisting in the editing and revision of the manuscript.

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