Published in final edited form as:

Hear Res. 2016 February ; 332: 217–222. doi:10.1016/j.heares.2015.09.013.

Audiograms, gap detection thresholds, and frequency difference limens in cannabinoid receptor 1 knockout mice

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Abstract

The cannabinoid receptor 1 (CB1R) is found at several stages in the auditory pathway, but its role in hearing is unknown. Hearing abilities were measured in CB1R knockout mice and compared to those of wild-type mice. Operant conditioning and the psychophysical Method of Constant Stimuli were used to measure audiograms, gap detection thresholds, and frequency difference limens in trained mice using the same methods and stimuli as in previous experiments. CB1R knockout mice showed deficits at frequencies above 8 kHz in their audiograms relative to wild-type mice. CB1R knockouts showed enhancements for detecting gaps in low-pass noisebursts relative to wild-type mice, but were similar for other noise conditions. Finally, the two groups of mice did not differ in their frequency discrimination abilities as measured by the frequency difference limens task. These experiments suggest that the CB1R is involved in auditory processing and lay the groundwork for future physiological experiments.

Keywords

CB1R KO; CBA/CaJ; audiogram; difference limens; gap detection; mouse

1. Introduction

The endocannabinoid signaling pathway plays an important role in regulating neuronal activity. The primary receptor for endocannabinoids in the nervous system is the CB1 receptor (CB1R), which has widespread expression throughout the brain (Mechoulam and Parker, 2013; Ohnu-Shosaku et al., 2012; Pettit et al., 1998), presumably reflecting its importance in many aspects of neuronal processing. Behavioral evidence has been found for

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its role in various systems, including motor, memory, anxiety, and appetite (reviewed in Chaperon and Thiebot, 1999). At the cellular level, CB1Rs are responsible for depolarization-induced suppression of inhibition and excitation (DSI and DSE, Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001), and also play a role in long-term depression (LTD, Safo and Regehr, 2005; Sjostrom et al., 2003; Tzounopoulos et al., 2007). However, it is not clear how such cellular effects contribute to behavior.

One approach to linking behavior with cellular function is to investigate how CB1Rs influence perception. CB1Rs are expressed at several stages in the auditory pathway, including the medial nucleus of the trapezoid body (MNTB, Kushmerick et al., 2004), the ventral cochlear nucleus (VCN, Zheng et al., 2007), and the dorsal cochlear nucleus (DCN, Zhao et al., 2009). Studies in zebra finches (*Taeniopygia guttata*) have revealed the importance of CB1R expression for the production of stereotyped birdsong through synaptic plasticity (Soderstrom and Tian, 2004) and have shown high levels of CB1R expression in brain regions involved in song learning and vocal production (Soderstrom and Johnson, 2000). CB1Rs have been implicated in tinnitus (Zheng et al., 2007; Zheng et al., 2015). However, the function of CB1Rs in hearing is largely unknown.

To address this, we compared the hearing abilities of CB1R knockout (KO) mice to mice without any known hearing impairment (wild-type CBA/CaJ mice). Three experiments were performed with these KO mice: audiograms, gap detection thresholds, and frequency difference limens (FDLs). We first measured audiograms, which are a measure of general hearing ability across different frequencies. We used the same methods and stimuli as, Radziwon et al. (2009) to obtain comparable audiograms for the CB1R KO mice, and we found that the KO mice had poorer hearing at mid-range and high frequencies than the wild-type CBA/CaJ mice. We next measured gap detection thresholds to assess the sensitivity of the auditory system for fine temporal features of sounds. Radziwon et al. (2009) obtained gap detection thresholds for wild-type CBA/CaJ mice using the same methods used here. We found that the KO mice were *more* sensitive, i.e., lower thresholds, to small gaps than wild-type mice. Finally, we measured FDLs, which are a finer test of frequency sensitivity than detection thresholds, using the same methods and stimuli as Radziwon and Dent (2014) used in wild-type CBA/CaJ mice. We found no differences between the two populations, suggesting the detection impairments at mid-frequencies do not extend to discriminating between those same frequencies. These experiments provide an important starting point for understanding how CB1Rs contribute to hearing, and also provide direction for future cellular studies.

2. Methods

2.1 Subjects

Ten adult, CB1R KO mice (*Mus musculus*) were used as subjects in these experiments. The knockouts were originally a gift from Andreas Zimmer, in a C57/B6 background. These mice were backcrossed into the CBA/CaJ line for at least ten generations before establishing a breeding line of homozygous KOs, which have been maintained for several generations. Six subjects (all M) were used to obtain audiograms. Four subjects (2 M, 2 F) were used to obtain thresholds for gap detection, and three of those gap detection subjects (2 M, 1 F) also

completed the frequency difference limens experiment. Mice began training at approximately two months of age and the experiments lasted approximately 12 months. The mice were individually housed and kept on a reverse day/night cycle (lights off at 6 am and on at 6 pm). The mice were tested during the dark portion of their cycle. All of the mice were water restricted and maintained at 85% of their free-drinking weight during the course of the experiment. Food was available ad libitum, except during testing sessions. All procedures were approved by the University at Buffalo, SUNY's Institutional Animal Care and Use Committee.

2.2 Apparatus

The mice were tested in a wire cage (23×39×15.5 cm, see Figure 1) located in a sound attenuated chamber (53.5×54.5×57 cm) lined with 4 cm thick Sonex sound attenuating foam (Illbruck, Inc., Minneapolis, MN). The chamber contained an overhead web camera (Logitech QuickCam Pro, Model 4000) and a small 25 watt white-light bulb to monitor the animal during test sessions. Sounds were played from an electrostatic speaker (Tucker-Davis Technologies (TDT), Gainesville, FL, Model ES1). The cage also contained two nose-poke holes surrounded by infrared sensors (Med Associates Model ENV-254), and a response dipper (Med Associates Model ENV-302M-UP).

The experiments were conducted with Dell Optiplex 580 computers running Matlab R2007b software, which operated Tucker-Davis Technologies (TDT, Gainesville, FL) modules and Rpvds software. Stimuli were sent through an RX6 processor, an RP2 signal processor, a PA5 attenuator, an ED1 speaker driver, and then finally to the speaker. Inputs to and outputs from the testing cages were controlled by the RX6 processor. The response dipper and infrared sensors were powered by Elenco Precision (Models XP-603 and XP-650 respectively) power supplies. A Tektronix (model TDS 2014) oscilloscope was attached to the PA5 attenuator to ensure that the stimuli were being sent to the speaker.

2.3 Stimuli

The stimuli were created and edited in Adobe Audition. Sound pressure levels for frequencies up to 12 kHz were calibrated using a sound level meter (Larson Davis system 824) with a condenser microphone (PRM902) placed at the position where the animal's head would be during testing. Sound pressure levels for higher frequencies were calibrated using an ultrasound recording system (Avisoft, Model USG 116–200) and Raven Pro (v 2.3, Cornell University) software, with the microphone placed at the same location. Calibrations were conducted weekly, and did not vary by more than 2 dB at any frequency tested throughout the course of the experiments.

Audiogram stimuli were the same as those used in Radziwon et al. (2009). Mice were tested on eight pure tones (1, 2, 4, 8, 12, 16, 24, and 42 kHz) with a 2 s duration and cosine rise/fall times of 20 ms. In the gap detection task, the repeated background was a noise burst with an 800 ms duration, and cosine rise/fall times of 100 ms. Stimuli were broadband or band passed, and only one stimulus type was presented per session. The four band-limited stimuli that were used consisted of (1) noise high passed at 18 kHz, or noise low passed at (2) 18 kHz, (3) 12 kHz, or (4) 8 kHz. The broadband stimuli contained frequencies up to 100 kHz.

All stimuli were presented at a sensation level (SL) of 30 dB (calculated by measuring detection thresholds for the broadband noise stimuli) and roved by ± 3 dB from presentation to presentation to eliminate any possible level cue for the mice. For each condition, target stimuli with gaps were created from the background stimuli. That is, for the target stimuli, silence was inserted into the center of the stimulus while the rest of the stimulus remained identical to the background stimulus. All stimuli remained 800 ms in duration, and the gaps ranged from 1–100 ms, depending on the animal's performance.

In the FDL experiment, stimuli were 500 ms pure tones with cosine rise/fall times of 20 ms. The four background reference frequencies tested were 12, 16, 24, and 42 kHz. The tones were presented at 10 dB SL (calculated from the audiogram experiment above) and roved by ± 3 dB from presentation to presentation to eliminate any possible level cue for the mice. The target tones were higher in frequency than the reference stimulus, with step sizes varying by subject and frequency (usually 2–5% of the baseline frequency), although this was adjusted dependent upon each mouse's performance.

2.4 Audiogram procedures

In the audiogram experiment, the mice were trained using a go/no-go operant conditioning procedure to respond to pure tones in a detection task. The mouse began a trial by nose poking through an observation nose-poke hole two times, which initiated a variable waiting interval ranging from 1 to 4 s. After this waiting interval, a test stimulus was played in the go trials. If the mouse detected this stimulus, it was trained to nose poke to the report nose-poke hole within 2 s of the onset of the target. A *hit* was recorded if the mouse correctly responded to the stimulus within the allotted time, and the animal received 0.01 ml of Ensure® as a reinforcement. A *miss* was recorded if the mouse either failed to nose poke through the report hole or if it did not respond within 2 s. If the mouse responded to the report nose-poke hole during the waiting interval, the trial was aborted and the mouse received a 2 s timeout, during which no test stimulus would play. Approximately 30% of all trials were catch trials. These constituted the no-go part of the procedure and no target stimulus was presented during these trials. These trials were required to measure the false alarm rate and calculate the animal's response bias. If the subject nose poked to the report hole during a catch trial, a *false alarm* was recorded. However, if the subject continued to nose poke to the observation nose-poke hole, a *correct rejection* was recorded. In either case, no reinforcement was given and the next trial began immediately. Sessions were excluded from analysis if the percentage of false alarms was greater than 30%.

Testing began when the animals were approximately 5 months old, after which they ran two 30-min sessions per day, 5–6 days per week. Only one frequency was tested per session. Typically, the mice ran between 50 and 100 trials within each session. All mice were tested on all eight frequencies in a random order and a different random order was used for each mouse. We presented the pure tone stimuli according to the Method of Constant Stimuli (MOCS). Within a session, one frequency was presented in 10-trial blocks. Within each 10-trial block, each test tone was presented randomly at seven different pre-determined levels using a step size of 5 or 10 dB (3 catch trials were also included in each block). The levels were chosen so that only the quietest one or two stimuli could not be heard by the mouse,

whereas the loudest tones were well above threshold. Starting stimulus intensities for each frequency were based on previous audiograms from our laboratory (Radziwon et al., 2009). Chance performance was represented by the animal's *false alarm* rate. Sessions were excluded from analysis if the false alarm rate was greater than 30%. Approximately 5% of all sessions were eliminated due to a high false alarm rate.

2.5 Gap detection and FDL procedures

The mice were trained using a go/no-go operant conditioning procedure to discriminate between a repeating background and a target stimulus. The mouse began a trial by nose poking the observation hole, which initiated a variable waiting interval ranging from 1 to 4 s. During this time, the repeating background played at a rate of once every 1 s. Only one repeating background was presented per session. For the gap detection experiment, the repeating background was one of the un-gapped noisebursts (broadband or band passed). For the FDL experiment, it was one of the reference pure tones (12, 16, 24, or 42 kHz). After the waiting interval, the mouse poked the observation hole again. In the go condition, the target stimulus would then alternate twice with the background. If the mouse discriminated this change between background and target, it was required to nose poke the report hole within 2 s of the onset of the target. A *hit* was then recorded if the mouse correctly responded to the target within the allotted time, and the animal received 0.01 mL of Ensure® as a reinforcement at the dipper. A *miss* was recorded if the mouse either failed to poke the report hole or if it did not respond within 2 s. If the mouse poked the report hole during the waiting interval, the trial was aborted and the mouse received a 3 s timeout during which no test stimulus would play.

Approximately 30% of all trials were *catch* trials. These constituted the no-go part of the procedure and no target stimulus was presented during these trials. Instead, the repeating background simply continued to play. These trials were required to measure the false alarm rate and calculate the animal's response bias. If the subject nose poked the report hole during a *catch* trial, a *false alarm* was recorded. However, if the subject continued to poke the observation hole, a *correct rejection* was recorded. In either case, no reinforcement was given and the next trial began immediately. Chance performance was represented by the animal's *false alarm* rate. Sessions were excluded from analysis if the percentage of false alarms was greater than 20%. Approximately 1% of all sessions were thrown out due to a high false alarm rate. Testing began when the animals were approximately seven months old and, during the data collection period, they ran two 30–40 minute sessions per day, 5 days per week. Typically, the mice ran between 30 and 60 trials within each session. All mice were tested on stimulus types and a different random order was used for each mouse.

The stimuli were presented according to the psychophysical Method of Constant Stimuli (MOCS). For the gap detection experiment, within a session, one noise burst bandwidth was tested. Within each 10-trial block in a session, seven predetermined gap target stimuli were presented randomly using a step size of 1–10 ms (3 *catch* trials were also included in each block). The stimuli were chosen so that only the smallest one or two gaps could not be heard by the mice, while the largest gaps were well above threshold. Gap detection thresholds

were first measured for broadband noise bursts, and then for four 30 dB SL band passed noise bursts (high passed at 18 kHz and low passed at 18, 12, and 8 kHz) in a random order.

In the FDL experiment, all of the mice were tested on all four reference tones in a random order and a different random order was used for each mouse. The tone stimuli were presented according to the psychophysical MOCS. Within each 10-trial block, seven predetermined targets were presented randomly (3 *catch* trials were also included in each block). The target frequencies were chosen so that only the smallest one or two frequency differences were not discriminated by the mice, whereas the largest frequency differences were well above threshold.

2.6 Data analysis

Signal detection analysis was performed to factor out the animals' motivational biases since bias is independent of sensitivity (e.g., Steckler, 2001). At least 200 trials were obtained for each condition from each mouse. Thresholds were calculated using the last 200 trials as long as performance was similar from session to session. If performance varied, testing was continued until performance stabilized and the last 200 trials were used to obtain thresholds. Mean *hit* and *false alarm* rates were used to calculate thresholds using signal detection theory with a threshold criterion of $d' = 1.5$. Although any threshold criterion is chosen arbitrarily, we set our threshold criterion at 1.5 for two main reasons. For one, this d' value is comparable to the values used by other animal researchers (e.g., Klink et al., 2006; Radziwon et al., 2009, 2014; Wagner et al., 2003). Also, a d' of 1.5 is a relatively conservative criterion (compared to lower d' values such as 1.0). It corresponds to low false alarm rates which ensure that the animal is responding primarily to the target stimuli and not responding randomly (Klump and Maier, 1989).

Statistical analysis was performed using IBM SPSS Statistics (v. 21). Means are reported for wild-type mice with standard errors of the mean from Radziwon et al. (2009, audiograms and gap detection) and Radziwon and Dent (2014, FDLs). For the audiogram experiment, a two-way mixed model ANOVA with frequency as a repeated measure and mouse type (wild-type CBA/CaJ or KO) as a between-subjects factor was used. A Holm-Sidak post-hoc test was conducted to interpret the significant interaction.

For the gap detection experiment, a two-way mixed model ANOVA with sound type (broadband, noise high passed at 18 kHz, noise low passed at 18 kHz, noise low passed at 12 kHz, and noise low passed at 8 kHz) as a repeated measure and mouse type (wild-type CBA/CaJ or KO) as a between-subjects factor was used. Mauchly's test of sphericity was conducted, and when significant, a Greenhouse-Geisser correction was applied. A Holm-Sidak post-hoc test was conducted to interpret the significant interaction. For the FDLs, a two-way mixed model ANOVA with frequency (12, 16, 24, and 42 kHz) as a repeated measure and mouse type (wild-type CBA/CaJ or KO) as a between-subjects factor.

3. RESULTS

There were no noticeable differences in training or testing time between the two groups of mice. There were also no differences in overall performance across the two groups; the

number of sessions thrown out due to high false alarm rates for the KOs did not differ from the wild-type CBA/CaJ mice in Radziwon et al. (2009) or Radziwon and Dent (2014).

3.1 Audiograms

Figure 2 shows audiograms for individual KOs, the mean KO results, and the mean audiograms for wild-type CBA/CaJ mice from Radziwon et al. (2009). Thresholds for the KOs were significantly higher than the wild-type CBA/CaJs (Fig. 2). The two-way ANOVA revealed significant differences across frequency ($F_{(7,62)} = 11.78, p < 0.001$) and subject type ($F_{(1,9)} = 177.44, p < 0.001$), and a significant interaction between frequency and subject type ($F_{(7,7)} = 11.78, p < 0.001$). The post-hoc test further showed that the two groups were significantly different from each other at the higher frequencies: 8, 12, 16, 24, and 42 kHz ($p < 0.05$). From 8–42 kHz, thresholds for the KOs were higher than those for the wild-type CBA/CaJ mice.

3.2 Gap Detection

Figure 3 shows gap detection thresholds for broadband and band passed noise for each individual KO mouse, mean KO results, and the mean thresholds for wild-type CBA/CaJ mice from Radziwon et al. (2009). An ANOVA revealed a significant effect of stimulus ($F_{(1,2, 6,1)} = 17.39, p < 0.01$), a non-significant effect of subject type ($F_{(1, 5)} = 5.50, p > 0.05$), and a significant interaction between stimulus and subject type ($F_{(1,2, 6,1)} = 5.92, p < 0.05$). Post-hoc analyses revealed that the thresholds from the KOs were similar to those obtained from the wild-type CBA/CaJ mice for the broadband noise, noise high passed at 18 kHz, and noise low passed at 18 kHz and 12 kHz. When noise was low passed at 8 kHz, however, the KOs had lower (better) thresholds than the wild-type CBA/CaJ mice ($p < 0.05$).

3.3 FDLs

Figure 4 shows FDLs for 12, 16, 24, and 42 kHz for each individual KO mouse, mean KO results, and the mean FDLs obtained from CBA/CaJ mice from Radziwon and Dent (2014). The KOs had FDLs similar to CBA/CaJ mice. Overall, FDLs increased with increasing frequency for both subject types, although there was some variability across frequency for the KOs. The ANOVA revealed a main effect of stimulus frequency only ($F_{(3, 12)} = 19.10, p < 0.001$). There was no effect of subject type ($F_{(1, 4)} = 0.66, p = 0.46$), and the interaction between stimulus frequency and subject type was also not significant ($F_{(3, 12)} = 3.36, p = 0.055$). The knockouts performed the same as the normal-hearing CBA/CaJ mice in the frequency discrimination experiment.

4. Discussion

We used operant conditioning techniques and the psychophysical MOCS to test basic hearing abilities of CB1R KO mice. By employing the same stimuli and procedures that were used by Radziwon et al. (2009) and Radziwon and Dent (2014) to behaviorally test hearing abilities of wild-type CBA/CaJ mice, we were able to make direct comparisons in order to isolate the role that CB1Rs have in auditory processing. Overall, it appears that deleting the CB1R gene does not have a negative impact on learning the task as the training

time for KOs was similar to that of the wild-type CBA/CaJ mice. It does, however, affect basic hearing abilities of mice, as detection thresholds for tone frequencies above 8 kHz were significantly higher in CB1R KO mice relative to wild-type CBA/CaJ mice.

There were also differences in gap detection thresholds for certain frequencies of noise stimuli between the two populations, although here the direction of the difference was quite surprising. The CB1R KO mice had gap detection thresholds for broadband noise that were similar to thresholds obtained in normal-hearing CBA/CaJ mice (Radziwon et al., 2009). Gap detection thresholds for band passed noise were also similar between the two populations when the noise was high passed at 18 kHz or low passed at 18 kHz. However, when noise was low-passed at 8 kHz, the KO mice had lower thresholds than those measured in normal CBA/CaJ mice by Radziwon et al. (2009). Radziwon and colleagues noted in their experiment with wild-type CBA/CaJ mice that thresholds could not be obtained for two of their five subjects for the low-pass 8 kHz noise. We had no difficulty obtaining gap detection thresholds for any of our KO subjects, and the thresholds were as low as they were for the other stimulus conditions.

It is surprising that mice with a deleted CB1R gene would perform better than wild-type mice with normal hearing. It appears that for the KOs but not the wild-types, frequencies above 8 kHz are not necessary in the detection of gaps. The CB1R KO mice do not seem to rely on high frequencies contained in the noise bursts to complete the gap detection task, whereas wild-type CBA/CaJ mice do. The audiogram experiment showed that the CB1R KO mice lack the peak auditory sensitivity at 24 kHz that is exhibited by wild-type CBA/CaJ mice, and that their hearing is significantly worse than wild-types at frequencies above 8 kHz. It is possible that since the CB1R KO mice have a deficit in their audiogram from 8–24 kHz, they attend to lower frequencies better than CBA/CaJ mice. Ison et al. (2005) found that gap detection performance increased as noise bands included frequencies of peak auditory sensitivity (i.e. 16 kHz) and concluded that mice have sensitive temporal acuity because they integrate information across frequency channels. The CB1R KO mice might not be integrating across as many frequency channels since they do not rely on higher frequencies in the gap detection task. Since gap detection is best when noise bands include frequencies of peak auditory sensitivity, it is possible that gap detection performance does not decrease in CB1R KO mice when noise is low passed at 12 kHz because the KOs lack the peak auditory sensitivity that CBA/CaJ mice have for frequencies between 16 and 24 kHz. Another possibility is that neurons involved in temporal processing in KOs are altered. CB1Rs are expressed in AVCN, MNTB, and the octopus cell area (Kushmerick et al., 2004; Zheng et al., 2007). These areas all appear to be important in temporal processing. Enhanced gap detection performance at low frequencies could result from differences in processing or development of these areas in the CB1R KO mice. In the DCN, CB1Rs have a role in short- and long-term changes in synaptic strength (Tzounopoulos et al., 2007), although it has not been thought that the DCN plays a role in temporal processing. It is clear that further work will be required to understand the cellular basis for these observed changes.

The FDLs obtained from the CB1R KO mice were similar to those obtained by Radziwon and Dent (2014) in wild-type CBA/CaJ mice. Additionally, the FDLs are similar to those obtained in wild mice (Heffner et al., 1971). It appears that lacking CB1R does not impact

the ability of mice to discern frequencies across a wide range of frequencies, despite having an impairment in hearing those stimuli. Although the KOs exhibit deficits in their audiogram, for frequencies above 8 kHz, they are still able to distinguish changes in frequencies as well as the wild-type CBA/CaJ mice in the same frequency range.

The experiments reported here represent an early attempt at understanding the role of CB1Rs in auditory processing and are one starting point for establishing how cellular processes associated with CB1R signalling influence neuronal function. Overall, the results point towards differences in frequency processing as being important. One possible future avenue would be to establish where in the auditory pathway a difference emerges between normal CBA/CaJ mice and CB1R KOs. It may be constructive to compare frequency sensitivity among auditory nerve fibers, for instance. Gap sensitivity may involve parts of the auditory pathway that encode precise timing, and the MNTB, which expresses CB1Rs (Kushmerick et al., 2004) is also a possible candidate. However, it is not clear how those receptors are endogenously activated. In addition, it will be useful to see which locations in the auditory pathway show strong CB1R labelling in addition to the MNTB and DCN (Tzounopoulos et al., 2007), which may suggest additional behavioral tasks related to those areas.

Acknowledgements

This project was supported by National Institute on Deafness and Other Communication Disorders grants R01-DC012302 to MLD and R01-DC008125 to MXF. We would like to thank the numerous undergraduates in the Dent Lab for assistance with data collection and Dr. L. Pliss for breeding the CB1R KO allele into the CBA/CaJ background.

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HIGHLIGHTS

- Audiograms, gap detection thresholds, and frequency difference limens were measured in CB1R knockout mice and compared to wild-type mice
- Knockouts had deficient hearing as measured by the audiogram at frequencies above 8 kHz
- Gap detection thresholds for knockouts were better than those for wild-type mice on low passed noise but otherwise similar to the wild-type mice
- Frequency difference limens did not differ between the two populations

Fig. 1. Schematic of experimental setup. Mice began a trial by nose poking to the observation hole. When they detected or discriminated between stimuli presented from the speaker, they poked to the report hole. If correct, the mice received Ensure from the dipper.

Fig. 2.

Audiograms for each individual KO mouse (open symbols), mean audiograms with standard errors for those KOs (filled hexagons), and mean results from wild-type CBA/CaJ mice from Radziwon et al. (2009, filled triangles). * = $p < 0.05$ between groups.

Fig. 3. Gap detection thresholds for broadband and band passed noise (HP18 = high-passed at 18 kHz, BB = broadband, LP18 = low passed at 18 kHz, LP12 = low passed at 12 kHz, and LP8 = low passed at 8 kHz) for each individual KO mouse (open symbols), the mean plus standard error for all KOs (filled circles), and the mean thresholds with standard errors for wild-type CBA/CaJ mice from Radziwon et al. (2009, filled triangles). * = $p < 0.05$ between groups.

Fig. 4.

FDLs across frequencies for each individual KO mouse (open symbols), mean thresholds with standard errors for the KOs (filled circles), and the mean thresholds with standard errors for wild-type CBA/CaJ mice from Radziwon and Dent (2014) (filled symbols). The groups were not statistically different from each other.