Mice with behavioral evidence of tinnitus exhibit dorsal cochlear nucleus hyperactivity because of decreased GABAergic inhibition

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Tinnitus has been associated with increased spontaneous and evoked activity, increased neural synchrony, and reorganization of tonotopic maps of auditory nuclei. However, the neurotransmitter systems mediating these changes are poorly understood. Here, we developed an in vitro assay that allows us to evaluate the roles of excitation and inhibition in determining the neural correlates of tinnitus. To measure the magnitude and spatial spread of evoked circuit activity, we used flavoprotein autofluorescence (FA) imaging, a metabolic indicator of neuronal activity. We measured FA responses after electrical stimulation of glutamatergic axons in slices containing the dorsal cochlear nucleus, an auditory brainstem nucleus hypothesized to be crucial in the triggering and modulation of tinnitus. FA imaging in dorsal cochlear nucleus brain slices from mice with behavioral evidence of tinnitus (tinnitus mice) revealed enhanced evoked FA response at the site of stimulation and enhanced spatial propagation of FA response to surrounding sites. Blockers of GABAergic inhibition enhanced FA response to a greater extent in control mice than in tinnitus mice. Blockers of excitation decreased FA response to a similar extent in tinnitus and control mice. These findings indicate that auditory circuits in mice with behavioral evidence of tinnitus respond to stimuli in a more robust and spatially distributed manner because of a decrease in GABAergic inhibition.

excitability | neurotransmitters | GABA inhibition | in vitro imaging

Tinnitus, the persistent perception of a subjective sound in the absence of an acoustic stimulus (ringing of the ears), is often a debilitating condition that reduces the quality of life for many of those chronically affected. Estimates of the number of people experiencing tinnitus range from 8% to 20% of the general population (1). Despite the prevalence and growing incidence of tinnitus, the mechanisms underlying the induction and maintenance of tinnitus remain poorly understood.

Animal models of tinnitus have contributed significantly to the understanding of the pathophysiology of tinnitus (2–8). An emerging pattern associated with tinnitus pathology indicates that intense noise exposure leads to cochlear damage and hearing loss, which often is not clinically detected. Decreased cochlear input leads to hyperactive, more responsive central auditory circuits, which is evidenced by functional MRI (fMRI) studies in patients with tinnitus and in vivo recordings in animal models of tinnitus (9–13). Increased spontaneous firing rates, increased evoked responses, and reorganization of tonotopic maps are consistent with decreased inhibition (disinhibition) (1, 14). However, direct evidence that disinhibition mediates these changes is still lacking. In addition, the alternative hypothesis that predicts increased excitation as a potential mechanism in mediating these changes has not been tested (15). Addressing these unexamined questions could lead to pharmacological approaches for treating tinnitus patients.

We used flavoprotein autofluorescence (FA) imaging in brain slices prepared from control mice and mice with behavioral evidence of tinnitus. FA signals emanate from mitochondrial flavoproteins because of changes in oxidation state caused by neuronal activity (16–20). FA imaging exploits intrinsic changes in the optical characteristics of neural tissues and circumvents many problems associated with traditional imaging techniques. The most common approaches, such as voltage-sensitive dye and calcium imaging, involve bathing tissue in a potentially cytotoxic fluorophore before measuring optical signals (21, 22). These approaches require long incubation times and carry the complication of heterogeneous and inconsistent dye uptake as well as drop-off of signal over time. FA signals are intrinsic signals and provide stable amplitudes for several hours (16). Moreover, penetration of dyes can be limiting in tissue from adult animals. Finally, FA imaging has advantages over other imaging techniques that exploit intrinsic signals. PET and MRI studies have shown elevated blood flow and increased sound-evoked blood oxygenation level-dependent (BOLD) responses in central auditory structures of individuals experiencing tinnitus (9, 10, 23). Although PET and MRI rely on intrinsic changes, they are not suitable for mechanistic studies, unlike FA imaging, which allows us to address mechanistic details of tinnitus induction and expression.

Evidence suggests that the dorsal cochlear nucleus (DCN) is an important brain center involved in the triggering and modulation of tinnitus (24). Although other central auditory centers have been implicated in tinnitus (14, 25–27), the DCN deserves special emphasis, because as a primary acoustic nucleus, it occupies a pivotal position in the hierarchy of functional processes leading to the emergence of tinnitus percepts. In this study, we developed an in vitro paradigm for FA imaging of electrically evoked activity in DCN circuits in brainstem slices. We used this experimental paradigm in conjunction with a mouse model of tinnitus to evaluate the differences between tinnitus and control mice in the amplitude and spatial distribution of FA signals in the DCN. We then extended this analysis by pharmacologically dissecting the contribution of excitation and inhibition in the generation of enhanced and more spatially spread FA response.

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Results

We measured evoked FA responses from coronal DCN slices prepared from control and tinnitus mice (6–13 wk old). No obvious anatomical abnormalities were observed in the DCN of tinnitus mice. We evoked FA responses by electrical stimulation of the molecular layer of the DCN (Fig. 1*A*). In control mice, repetitive electrical stimulation (100 Hz for 1 s) produced a localized increase in green autofluorescence at the stimulated site (Fig. 1*B*). The peak increase was observed about 1 s after the initiation of the stimulation (Fig. 1 *B* and *C*). Application of tetrodotoxin (TTX) blocked the signal, indicating that action potential-driven neural activity generated the observed FA signal (Fig. 1*B*).

We used FA imaging to test whether DCN-evoked activity is increased in slices prepared from mice with behavioral evidence of tinnitus. Tinnitus is often induced after intense sound exposure, and therefore, we used a noise-induced animal model of tinnitus. We used a protocol [unilateral, 45-min exposure to 116-dB sound pressure level (SPL), 1-kHz band noise centered at 16 kHz] that does not lead to permanent hearing threshold shifts (8). To assess behavioral evidence of tinnitus, we used a reflex-based gap detection method (8). According to this paradigm, normal hearing animals exhibit an inhibited reflex to a startling sound (115 dB for 20 ms) when a silent gap (50 ms long) is embedded in a constant background sound (Fig. 2*A*). The background sound itself (70 dB) does not elicit a startle response. After noise exposure, animals that exhibit less inhibition of startle when the frequency of the background sound matches the frequency of their putative tinnitus are considered animals with behavioral evidence of tinnitus (Fig. 2*A*). The hypothesis is that the chronic tinnitus partially masks the silent gap perception, and therefore, mice show reduced ability to detect the silent gap and less inhibition of startle reflex (8). In the present study, gap detection performance was assessed before noise induction and 2–9 wk postnoise induction. Approximately

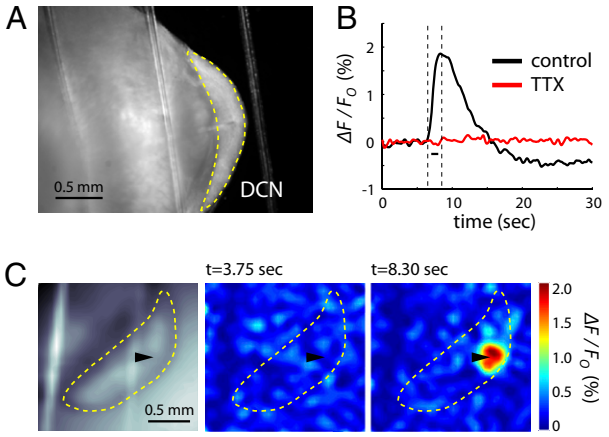

Fig. 1. Example of flavoprotein autofluorescence (FA) signal evoked by electrical stimulation of dorsal cochlear nucleus (DCN) brain slices prepared from control mice (6–13 wk old). (*A*) An image from a brainstem slice including the DCN. A stimulating electrode was placed in the molecular layer of the DCN to stimulate parallel fibers. (*B*) Electrical stimulation (100-Hz stimulation for 1 s is indicated by solid bar) resulted in evoked FA signal. FA signals represent percent increases relative to baseline (black). To confirm that these signals were dependent on neuronal action potentials, we applied TTX and observed that electrical stimulation no longer resulted in an increase of fluorescence above baseline (red). Vertical dashed lines illustrate the temporal window that we used to calculate average FA response for all successive analysis. This window begins 0.5 s before electrical stimulation and ends 0.5 s after the termination of electrical stimulation. (*C*) Single frames of the DCN optical field illustrate the spatial spread and magnitude of the FA response. A time point preceding stimulation and a time point during the peak of the response are shown. Arrowheads indicate the position of the stimulating electrode.

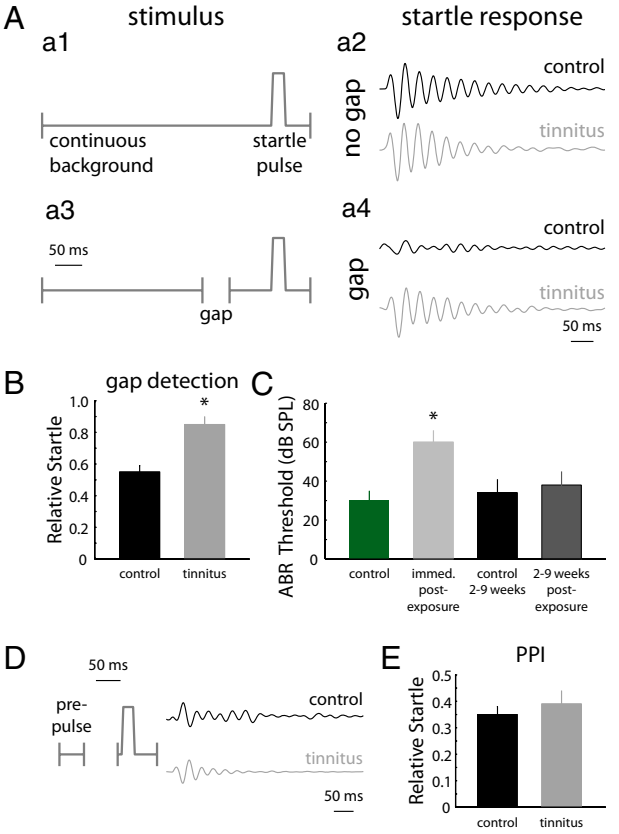

Fig. 2. Behavioral evidence of tinnitus. (*A*) Control and tinnitus mice show normal startle reflex (*a2*) in response to a startle stimulus (*a1*; 115 dB for 20-ms duration embedded in a 70-dB constant background sound). Startle responses represent the time course of the downward force that the mouse applies onto the platform after the startle pulse (*a2* and *a4*). Previous studies have shown that control animals detect the silent gap (*a3*) and that their startle reflex is inhibited robustly (*a4*, black trace). However, animals with behavioral evidence of tinnitus have difficulty detecting the gap when the frequency of the background sound is at or near the frequency of their putative tinnitus. Thus, their startle reflex is less inhibited (*a4*, gray trace). (*B*) Noise-exposed mice with behavioral evidence of tinnitus. Relative startle ratio is (response to gap + startle stimulus)/response to startle alone. Background noise was at 24 KHz. A response of one suggests no gap detection (no inhibition of the startle). Noise-induced mice revealed behavioral evidence of tinnitus 2–9 wk after exposure (control: relative startle = 0.55 ± 0.04, $n = 8$; tinnitus: relative startle = 0.85 ± 0.05, $n = 8$; $P < 0.05$). Only mice that showed increased relative startle ratios are included in this graph (about 50% of noise-induced mice). (*C*) ABR thresholds reveal only temporary threshold shifts for 24-KHz tones. Noise-exposed mice showed significant postexposure threshold elevation (control = 30 ± 5 dB; postexposure = 60 ± 6 dB; $P < 0.05$). This temporary elevation was recovered by the time that noise-exposed mice were behaviorally assessed (2–9 wk postexposure = 38 ± 7 dB). For the noise-induced mice in *C*, ABR measurements were obtained from the sound-exposed (ipsilateral) ears. (*D*) When a 50-ms pulse precedes the startle pulse by 130 ms (left side), the startle responses are similar in control (black) and noise-induced mice (gray). (*E*) Prepulse inhibition measured after a brief (50 ms) duration stimulus (10 KHz) was similar in controls and mice with behavioral evidence of tinnitus (control PPI = 0.35 ± 0.03; tinnitus PPI = 0.39 ± 0.05). Error bars indicate SEM.

50% of noise-exposed mice displayed decreased gap detection when the gap was embedded in 24-kHz bandpass background sound (Fig. 2*B*), suggesting that these mice developed a high frequency tinnitus 2–9 wk after sound exposure.

Deficits in gap detection performance of tinnitus mice were not caused by hearing loss, because auditory brainstem response (ABR) thresholds in control and tinnitus mice were identical (Fig. 2*C*). Moreover, deficits in gap detection performance of tinnitus mice were unlikely to be because of temporal processing deficits or

other general behavioral problems, because prepulse inhibition (PPI) in control and tinnitus mice was identical (Fig. 2*D*). PPI is the inverse of gap detection testing. In PPI, testing was done in a quiet background (rather than with a continuous 70 dB background), and a frequency band of 10 KHz was presented as a 50-ms duration prepulse stimulus before the startle stimulus. PPI was not different between control and tinnitus mice (Fig. 2*D*).

Next, we quantitatively compared evoked FA responses from control and tinnitus mice. Stimulation of the molecular layer of the DCN in slices from tinnitus mice revealed increased evoked FA signal at the site of stimulation (center signal) and enhanced spatial signal propagation to surrounding sites (surround signal) (Fig. 3 *A* and *B* and Movies S1 and S2). The average ratio of the amplitude of surround response over the amplitude of center response reveals a statistically higher ratio in tinnitus mice (Fig. 3 *C* and *D*). FA signal propagation to surround sites in tinnitus mice involved active processes (Fig. 3 *E* and *F*). To show active propagation, we made small radial cuts through the molecular layer and found that the signal stopped at the cuts (in three out of three experiments) (Fig. 3 *E* and *F*). However, when we moved the stimulation electrode to the other side of the cut, the signal propagated to that side (Fig. 3*F*). These results suggest that neural correlates of the hyperactivity of central auditory circuits, which have been reported in tinnitus patients and with in vivo recordings from animal models of tinnitus, can be identified in an in vitro slice preparation. This finding establishes an application of FA imaging that reveals tinnitus-related hyperactivity in vitro and allows for the pharmacological dissection of the underlying neurotransmitter systems that mediate tinnitus-associated hyperactivity of auditory circuits.

Input–output response functions revealed differential sensitivity of FA responses to weak and strong stimuli between control and tinnitus mice. To construct input–output response functions, we used an increasing number of pulses at a constant frequency (100 Hz) and measured the resultant FA responses. Averaged FA responses for control mice are shown in Fig. 4*A*. Increasing the number of pulses led to increased FA center and surround responses in control and tinnitus mice (Fig. 4*B*). Input–output relationships revealed that the slopes of the center response curves for weak stimuli (between 50 and 100 pulses) were similar in control and tinnitus mice (Fig. 4*C Left*). However, the slopes of the input–output functions of surround response curves for weak stimuli were significantly larger in tinnitus than in control mice (Fig. 4*C Left*). The slopes of the input–output functions for strong stimuli (200–400 pulses) were similar in control and tinnitus mice for either center or surround responses (Fig. 4*C Right*). These results indicate that the sensitivity of surround responses to weak stimuli is larger in tinnitus mice than in control slices.

Application of inhibitory and excitatory receptor antagonists revealed that increased FA signal propagation in tinnitus mice is mainly because of decreased GABAergic inhibition. For pharmacological analysis of FA responses, we used the 100-pulses stimulation protocol, because it provided robust but not saturated FA signals (Fig. 4*B*). Blockers of inhibition [glycinergic and GABAergic inhibition were blocked by strychnine and SR95531 (SR), respectively] did not differentially affect center FA responses in control and tinnitus mice. This result suggests equal contribution of inhibition in shaping center FA responses in control and tinnitus mice (Fig. 5 *A* and *C*). Blockers of excitation [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs) were blocked by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dionde (NBQX) and amino-5-phosphonovaleric acid (AP5), respectively] revealed a similar relative reduction of FA center response, suggesting equal contribution of excitation in shaping center responses in control and tinnitus mice (Fig. 5 *A* and *C*). When the same analysis was performed for surround responses, blockers of glycinergic inhibition did not reveal any significant difference in their relative effect (Fig. 5 *B* and *D*). In contrast, blockade of GABAergic inhibition revealed an enhanced effect in the surround response in control mice (Fig. 5*D*).

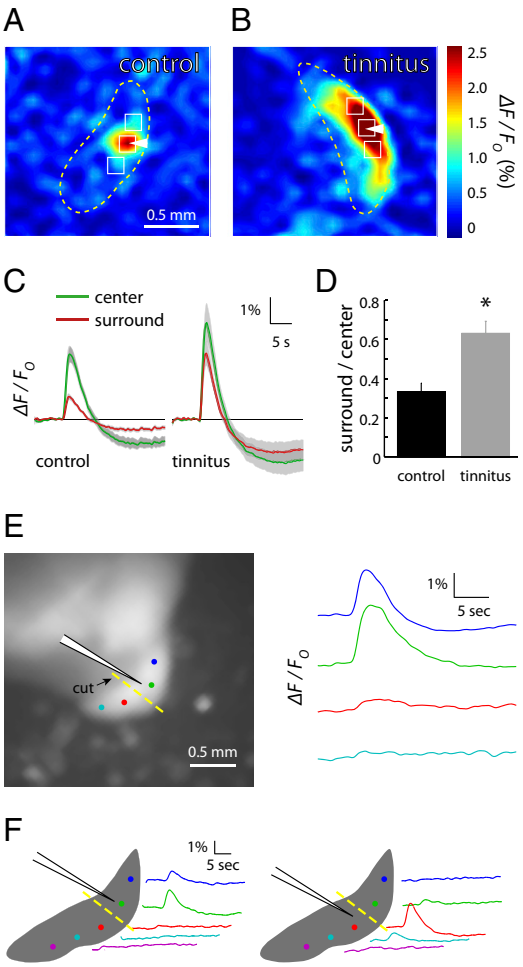

Fig. 3. FA responses to electrical stimulation are larger and more widespread in the DCN of tinnitus mice. (*A*) Single-image frame during electrical stimulation of a brain slice from a control mouse illustrating localized FA responses. All successive analysis was based on the average signals obtained from three regions of interest (ROIs; white squares)—the center ROI and the two surrounding ROIs. The site of the stimulation electrode defined the center pixel of the center ROI. Surround ROIs were positioned on either side of the center ROI along the fusiform cell layer. We measured a 200-μm distance from the center pixel of the center ROI. This measurement determined the center pixel of surrounding ROIs. (*B*) Single-image frame during electrical stimulation of a brain slice from a tinnitus mouse illustrating FA responses that spread to surrounding ROIs. (*C*) The population average of center responses is larger than the responses recorded at surround regions in control mice (*Left*; $n = 11$). The population average of responses in tinnitus mice reveals significantly smaller differences in responses from center and surrounding regions (*Right*; $n = 12$). The gray zone indicates SEM. (*D*) The ratio of the average surround response to the center response is significantly lower in tinnitus mice (control = 0.34 ± 0.04, $n = 11$; tinnitus = 0.63 ± 0.06, $n = 12$; $P < 0.01$). Surround signal for all graphs is the average of the signal obtained from both surrounding ROIs. (*E*) FA signal propagation to surrounding ROIs in tinnitus mice involves active processes. We used a sharp electrode to bisect the molecular layer near the middle of the DCN (*Left*, dashed yellow line). A stimulating electrode was placed on one side of the transection. Only sites located on the side of the transection where the stimulating electrode was positioned show FA responses. (*F*) Similar experiment performed in a different slice. Recordings on the side of the transection where the stimulating electrode is positioned reveal FA response, whereas sites on the other side do not. When the stimulating electrode is repositioned on the other side of the transection, FA response now appears on that side (*Right*). Error bars indicate SEM.

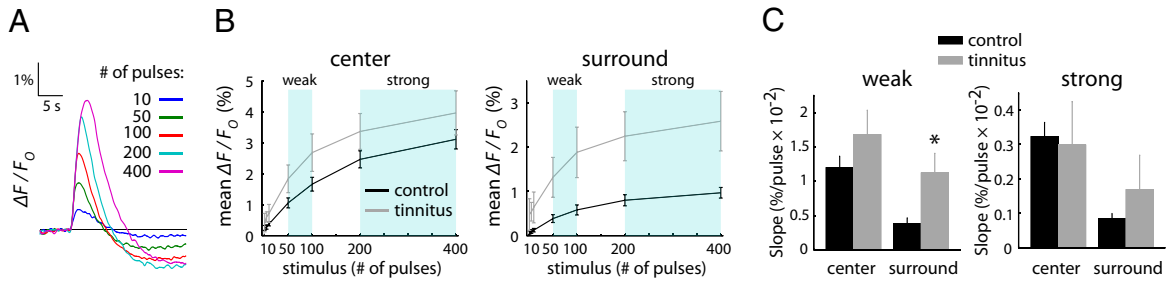

Fig. 4. Input–output functions reveal increased responsiveness of DCN FA responses for weak stimuli in tinnitus mice. (*A*) Population-averaged FA responses (from control mice, $n = 11$) to stimuli of different durations. All stimuli were composed of 100-Hz pulses. Responses were calculated as the average signal over the duration of the pulse train, including 0.5 s before and after the stimulus. (*B*) Input–output response functions for center and surrounding signals in control and tinnitus mice. Shaded areas indicate the regions for weak (50–100 pulses) and strong (200–400) stimuli. (*C*) Comparison of the average slope of input–output functions between control and tinnitus. The slope of the response curves was calculated as the difference in the signal amplitude between two points divided by the difference in the corresponding number of pulses delivered. The slopes of the center response curves for weak stimuli (*Left*) are similar in control ($n = 11$) and tinnitus ($n = 12$) slices. However, the average slope of the surround response curves for weak stimuli is significantly steeper in tinnitus mice (control = $0.40 \pm 0.08 \times 10^{-2}$% per pulse, $n = 11$; tinnitus = $1.16 \pm 0.29 \times 10^{-2}$% per pulse, $n = 12$; $P < 0.05$). The average slopes of center and surrounding response curves for strong stimuli (*Right*) are similar in tinnitus and control DCN slices. Error bars indicate SEM.

This result suggests that GABAergic inhibition limits the propagation of the FA response in surround sites to a larger extent in control than in tinnitus mice. This occlusion of the effect of GABA receptor (GABAR) antagonists on surround responses in tinnitus mice indicates that DCN tinnitus circuits are already disinhibited. This occlusion was not caused by potentially saturated surround FA responses (100 Hz and 100 stimuli), because weaker stimuli (100 Hz and 50 stimuli) revealed similar results in tinnitus mice (normalized $\Delta F/F_0$ after SR = 1.2 ± 0.33, $n = 12$, $P = 0.54$). Taken together, these results indicate that DCN circuits in tinnitus mice show evidence of decreased GABAergic inhibition. Although this finding is consistent with previous studies that have suggested reduced inhibition in DCN circuits in tinnitus animal models (11, 12, 28), our results are unexpected, because previous studies have proposed a reduced glycinergic inhibition as a mediator of tinnitus-induced disinhibition in the DCN (11, 12, 28).

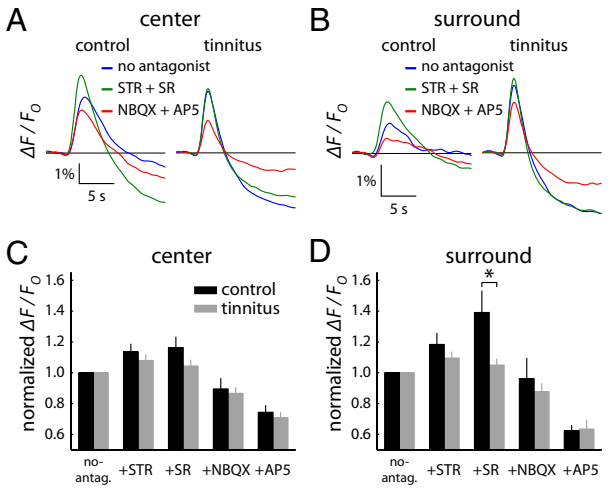

Fig. 5. Decreased GABAergic inhibition in tinnitus mice. (*A*) Individual traces for center signals from control (*Left*) and tinnitus mice (*Right*) before and after successive application of strychnine (STR; glycine receptor antagonist = 0.5 μM), SR-95531 (SR; GABA receptor antagonist = 20 μM), NBQX (AMPA receptor antagonist = 10 μM), and AP5 (NMDA receptor antagonist = 100 μM). (*B*) Individual traces for surround signals from control (*Left*) and tinnitus mice (*Right*) before and after successive application of STR, SR, NBQX, and AP5. (*C*) Average values of the relative center response amplitudes (normalized to no antagonist values) after sequential application of blockers of STR, SR, NBQX, and AP5 reveal no differences between control and tinnitus mice. (*D*) Average values of the relative surround response amplitudes (normalized to no antagonist values) after sequential application of blockers of STR, SR, NBQX, and AP5 reveal differences in GABAergic inhibition between control and tinnitus mice. There is a significant difference in the relative increase in signal amplitude after SR application (control: normalized $\Delta F/F_0$ after SR = 1.37 ± 0.13, $n = 11$; tinnitus: normalized $\Delta F/F_0$ after SR = 1.03 ± 0.04, $n = 12$; $P < 0.05$). STR, NBQX, and AP5 responses for control and tinnitus mice showed no difference on the relative change. For all experiments, drug application was sequential: STR was added first in the bath, then SR95531, NBQX, and finally, AP5. Error bars indicate SEM.

Discussion

Here, we developed an in vitro paradigm for imaging nucleus-level activity of auditory brainstem circuits in tinnitus mice. FA imaging revealed that DCN responses are increased in amplitude and their ability to spatially spread, a finding that is consistent with previously identified neural correlates of tinnitus. Moreover, we show pharmacological dissection of the contribution of excitation and inhibition in the generation of enhanced and more spatially spread FA response. Decreased GABAergic inhibition mediates, at least in part, these changes.

FA imaging has the advantage of homogeneous intrinsic labeling that leads to robust signals with low animal to animal variability. In addition, FA imaging can be used with older animals. These are important advantages over other in vitro imaging techniques that depend on the loading of exogenous dyes. The ability of FA imaging to reveal hyperactive auditory brainstem circuits in vitro is an important advancement in the field of tinnitus. Although hyperactivity may or may not be the cause of tinnitus (29, 30), hyperactivity provides a reliable marker of tinnitus that has also been seen in the inferior colliculus and the auditory cortex (13, 23, 31–33). Given that FA imaging allows for measurement of circuit activity levels at different auditory nuclei within the same animal, FA imaging experiments in vitro are expected to provide information about the development of tinnitus. Determining the path (bottom up or top down) and the exact time course of the development of hyperactivity may allow for manipulations that can delay or stop the progression of tinnitus.

Similar autofluorescent intrinsic signals have also been recorded from activity-dependent changes in the redox state of nicotinamide adenine dinucleotide molecules NADH and NADPH [together as NAD(P)H] (34, 35). Despite the advantages offered by intrinsic signals, FA and NAD(P)H responses are slow and therefore, not ideal for cellular and microcircuit resolution. Previous studies have revealed that FA signal is primarily related to postsynaptic activity, although a late part of the response may be related to glial activity (16, 18, 20). FA responses were only partially inhibited by CNQX and AP5 in the DCN (Fig. 5), indicating

that antidromic/presynaptic activity as well as postsynaptic activity contribute to the FA autofluorescence responses. Additionally, activation of metabotropic glutamate receptors may contribute to the remaining FA signal. Our results are consistent with FA cortical slice responses (16). Although FA signals are not suitable for cellular resolution, they can reveal specific hypotheses about altered microcircuit and neurotransmitter properties associated with tinnitus that can be assessed with whole-cell recording and more sensitive circuit mapping techniques

Our results provide information about the tinnitus-associated changes in the balance of excitation and inhibition in the DCN. Previous studies have suggested that tinnitus-related hyperactivity is mediated by the lack of glycinergic inhibition, which is evidenced by altered in vivo response of DCN principal neurons to increasing levels of sound and changes in the expression of specific glycine receptor subunits (11, 12, 28). However, no antagonists in animals with behavioral evidence of tinnitus were used in these studies. Here, we used specific antagonists to dissect the role of glycine and GABARs in the spread of FA signal. Application of these antagonists revealed that a reduction in GABAergic inhibition is the main cause for increased DCN surround signals in tinnitus mice. We used electrical stimulation for all of our experiments, and therefore, we hypothesize that changes in electrically evoked activation of synaptic GABARs enhance the spread of FA signals in tinnitus mice. However, our results cannot exclude the role of changes in tonic GABA inhibition in mediating the enhanced FA signal in tinnitus mice. Our proposed mechanism is consistent with the effect of several GABA-enhancing drugs that reduce the perceptual loudness of tinnitus in some patients (36). Our results do not exclude the contribution of the previously observed changes in the expression of glycine receptor subunits (28) in determining the expression of other neural correlates of tinnitus such as increased synchrony or altered tonotopy. Additionally, our results cannot exclude changes in intrinsic properties of principal neurons or changes in the excitability of axons. Finally, our studies indicate that no changes in excitatory neurotransmission mediate enhanced signals in tinnitus mice. Although previous studies have suggested that an increase in excitatory inputs in the DCN after noise damage could lead to hyperactivity (15), our study provides an experimental test of the role of excitation and inhibition in the same animals with behavioral evidence of tinnitus.

In conclusion, we present an application of FA imaging that reproduces the main in vivo neural correlates of tinnitus in a reduced in vitro slice experimental paradigm that allows for mechanistic studies. FA imaging reveals that decreased GABAergic inhibition changes the spatially dependent balance of excitation and inhibition in the DCN, thus leading to more robust and more spatially spread evoked responses. In vitro FA imaging studies in animal models of tinnitus could significantly change our understanding of the circuit and cellular changes associated with the triggering, development, and establishment of chronic tinnitus.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh and Marine Biological Laboratory. Methods for inducing and behaviorally testing for tinnitus, preparing brain slices, stimulating, recording, and analyzing activity-dependent FA signals are provided in *SI Materials and Methods*. FA movies are available in *SI Materials and Methods*.

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