Bimodal Stimulus Timing Dependent Plasticity in Primary Auditory Cortex is Altered After Noise Exposure With and Without Tinnitus

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Abstract

Central auditory circuits are influenced by the somatosensory system, a relationship that may underlie tinnitus generation. In the guinea pig dorsal cochlear nucleus (DCN), pairing spinal trigeminal nucleus (Sp5) stimulation with tones at specific intervals and orders facilitated or suppressed subsequent tone-evoked neural responses, reflecting spike-timing-dependent plasticity (STDP). Furthermore, after noise-induced tinnitus, bimodal responses in DCN were shifted from Hebbian to anti-Hebbian timing rules with less discrete temporal windows, suggesting a role for bimodal plasticity in tinnitus. Here, we aimed to determine if multisensory STDP principles like those in DCN also exist in primary auditory cortex (A1), and whether they change following noise-induced tinnitus. Tone-evoked and spontaneous neural responses were recorded before, and 15 min after bimodal stimulation in which the intervals and orders of auditory-somatosensory stimuli were randomized. Tone-evoked and spontaneous firing rates were influenced by the interval and order of the bimodal stimuli and in sham-controls Hebbian-like timing rules predominated as was seen in DCN. In noise-exposed animals with and without tinnitus, timing rules shifted away from those found in sham-controls to more anti-Hebbian rules. Only those animals with evidence of tinnitus showed increased spontaneous firing rates, a purported neurophysiologic correlate of tinnitus in A1. Together, these findings suggest that bimodal plasticity is also evident in A1 following noise damage and may have implications for tinnitus generation and therapeutic intervention across the central auditory circuit.

Key Words: Multi-sensory integration, Primary auditory cortex, Spike-Timing-Dependent Plasticity, Somatosensory, Tinnitus.
Tinnitus, the phantom perception of sound in the absence of a physical sound stimulus, has been linked to somatosensory innervation of the central auditory circuitry (Roberts et al. 2010). Somatosensory convergence with auditory neurons as early in the pathway as the dorsal cochlear nucleus (DCN; Kanold and Young, 2001; Shore 2005; Zhou et al. 2007) is a potential etiology for tinnitus following noise exposure (Kaltenbach and McCaslin, 1996; Koehler et al. 2011; Dehmel et al. 2012b; Koehler and Shore, 2013b). Somatosensory-auditory bimodal stimulation results in long-lasting changes in neural firing rates in DCN (Dehmel et al. 2012b) that are stimulus-timing dependent, (Koehler and Shore, 2013a), consistent with in vitro results showing spike-timing dependent plasticity (STDP) at parallel-fiber synapses (Tzounopoulos et al. 2004). Furthermore, application of noise over-exposure leading to tinnitus altered the stimulus timing dependent rules from Hebbian to anti-Hebbian, with broader windows of enhancement than in sham-controls or noise-exposed animals without tinnitus (Koehler and Shore, 2013b). These data implicate alterations in DCN bimodal STDP as an underlying mechanism in tinnitus generation.

Since the first description (Markram et al. 1997; Zhang et al. 1998), STDP has been demonstrated across neural structures (Caporale and Dan, 2008; Dahmen et al. 2008; Larsen et al. 2010). In primary auditory cortex (A1) shifts in neuronal frequency selectivity were pairing order and interval dependent, similar to STDP (Dahmen et al. 2008). Similarly, A1 firing rates were increased when a low-frequency tone was preceded by a high-frequency tone, while reversing the pairing order led to no changes in neural firing rates. These studies underscore the importance of the relative timing of sensory input in shaping neural responses and suggest STDP as a key mechanism of plasticity in the auditory system.

While it is evident that somatosensory stimulation can modulate auditory responses in A1 (Allman et al. 2009; Basura et al. 2012; Ghazanfar et al. 2005; 2008; Lakatos et al. 2007; Meredith...
and Allman, 2012; Meredith et al. 2012; Schroeder and Foxe, 2002; Schroeder et al. 2001, 2003), it is not known if these effects are stimulus-timing dependent or if they adhere to similar timing rule changes, reflecting STDP as in DCN following noise exposure and tinnitus (Koehler and Shore, 2013a; b).

The present study tested the hypothesis that stimulus timing dependent plasticity as observed in DCN (Koehler and Shore, 2013a) following bimodal stimulation is also observed in A1, and that it is modified following noise damage (Koehler and Shore, 2013b). In A1, bimodal stimulation in sham-animals resulted in stimulus timing dependent responses similar to those in DCN. Noise-exposure shifted timing rules from Hebbian towards anti-Hebbian in animals with and without tinnitus and to greater enhancing rules in animals with tinnitus. Animals with tinnitus demonstrated frequency-specific increases in spontaneous firing rates (SFRs), a tinnitus neural correlate (Eggermont, 2015). These findings demonstrate that A1, like the DCN, is also modulated by STDP principles that reflect changes in synaptic strength following noise damage.

**Materials and Methods**

**Animals**

Experiments were performed on mature, female, pigmented guinea pigs (n = 16; 250–350g; Elm Hill colony). The guinea pigs used in this study were the same animals used in a recently published report (Koehler and Shore, 2013b), in which electrodes were simultaneously placed in A1 and DCN. All procedures were performed in accordance with the National Institutes of Health *Guidelines for the Use and Care of Laboratory Animals* and approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).
Experimental Design/Noise Exposures

The purpose of this study was to evaluate the effects of noise-exposure in a tinnitus model on stimulus-timing dependent plasticity of A1 tone-evoked responses and spontaneous firing rates (SFRs) following bimodal stimulation. While STDP in neocortex was initially characterized by manipulating pre- and post-synaptic activity with timed current injections in slices (Markram et al. 1997), sensory stimulation has been used in vivo to induce stimulus-timing-dependent plasticity, the macromolecular correlate of STDP (Dahmen et al. 2008). Thus, while stimulus-timing dependent plasticity in vivo in the present data is not, by traditional physiologic definitions, STDP, the responses are consistent with the key features of STDP. Following a 2-hour unilateral noise exposure (97dB noise with ¼ octave band centered at 7kHz) to the left ears guinea pigs were tested semi-weekly before and after noise using gap-prepulse inhibition of acoustic startle (GPIAS) measures to test tinnitus (Turner et al. 2006; Dehmel et al. 2012a). Animals were anesthetized with ketamine (40mg/kg) and xylazine (10mg/kg) during the noise exposure as ketamine has been shown to have no obvious untoward impact on A1 neural frequency tuning (Huang et al., 2013). Ten animals were noise-exposed 3-6 weeks following baseline gap detection testing. Six to 8 weeks later, each animal was subjected to a second exposure to the same narrowband noise as prior data has suggested that tinnitus development is more likely following repeated noise exposures (Dehmel et al. 2012a,b). The remaining animals were grouped as sham-controls and were only subjected to anesthesia with no noise exposure.

Auditory brainstem response (ABR) thresholds were obtained before initiating gap detection and immediately following the first and second noise exposures to ascertain threshold shifts and one week after noise exposure to measure for threshold recovery (Fig. 1; with permission from Koehler and Shore, 2013b). Four to six weeks after the second noise exposure,
single-unit SFRs, rate level functions and bimodal stimulus timing-dependent plasticity were evaluated in acute A1 recording preparations and compared between sham-controls and noise-exposed animals that did and did not show evidence of tinnitus.

**Gap-Prepulse Inhibition of Acoustic Startle (GPIAS)**

For determining if noise-exposed animals developed tinnitus, the GPIAS for testing tinnitus was utilized as previously described (Dehmel et al. 2012a; Koehler and Shore, 2013b). Animals were placed on a piezoelectric force plate to measure movements elicited by a loud broadband noise (startle stimulus: 115dB; 200-20kHz). Each trial consisted of a background noise with and without a 50ms silent gap or pulse placed 50ms before the startle stimulus (Fig. 2; with permission from Koehler and Shore, 2013b). The background noise (60dB) was either broadband or bandpass-filtered noise with a 2 kHz band and lower cutoff frequencies of 4, 8, 12, 16 or 20kHz. The intervals between each trial were randomly selected between 18 and 24 seconds. Startle response amplitudes were found to only mildly decrease (10%) over multiple testing periods in sham and noise-exposed animals. For each session, the normalized startle response was calculated as the ratio \( \frac{A_G}{A_{NG}} \) where \( A_G \) is the mean amplitude of the startle response from 10 trials with gap on one day and \( A_{NG} \) is the mean amplitude of the startle response from 10 trials with no gap on the same day. To evaluate the normalized startle responses within each frequency band for indications of tinnitus, the distribution of all responses was analyzed using Gaussian mixture modeling (Statistics Toolbox; MATLAB release 2012b; MathWorks). The normalized startle responses were placed in the tinnitus group when the probability that the observation belonged to the elevated distribution was >0.55. Using the threshold from the Gaussian mixture model, the distributions of startle responses from all animals were separated into tinnitus and no tinnitus groups. For statistical comparison, animals with no exposure were assigned to the sham-
control group, while noise-exposed animals with no tinnitus were assigned to the Exposed-No
Tinnitus (ENT) and those exposed with evidence of tinnitus were assigned to the Exposed-
Tinnitus (ET) groups. Lastly, pre-pulse inhibition was evaluated in a similar manner as gap
detection. All of the animal groups showed no differences in response to pre-pulse inhibition
before and after noise-exposure. This finding suggests that baseline temporal processing was
unchanged following noise damage and thus, any changes in gap detection were likely a result of
the tinnitus percept “filling the gap” and not due to temporal processing or hearing loss.

Surgical Approach and Neural Recordings

Following anesthesia (ketamine; 40mg/kg and xylazine; 10mg/kg), animals were then held
in a stereotaxic device (Kopf) with hollow ear bars for sound delivery. Rectal temperature was
monitored and core temperature maintained at 38 ± 0.5°C with a thermostatically controlled
heating pad. Supplemental anesthesia (0.25–0.5mls of initial dose; IM) was given approximately
every 30 min, after performing a digital toe-pinch test to elicit paw withdrawal. Craniotomies
were performed over the ipsilateral (to the sound source) cerebellum and contralateral auditory
cortex to identify the middle cerebral artery as described previously (Basura et al. 2012). After
completing neural recordings, the guinea pig was sacrificed by intra-peritoneal injection of sodium
pentobarbital followed by brain removal for histologic analysis and electrode reconstruction to
confirm probe placement. To mark the electrode tracks, the recording and stimulating electrodes
were dipped in Fluorogold (2%) before being inserted into the brain. After being immersed in 4%
paraformaldehyde for 48 hours followed by immersion in a 20% sucrose solution (Zhou & Shore,
2006), the brain was cryosectioned at 40μm, placed on slides and examined under
epifluorescence.
Electrode Placement

To stimulate spinal trigeminal nucleus (Sp5) neurons, a concentric bipolar stimulating electrode (FHC, Bowdoin, ME) dipped in Fluorgold for post-mortem histologic probe placement confirmation was placed into the left Sp5, using stereotaxic coordinates (0.28cm left of midline, 0.25–0.3cm caudal to the transverse sinus, 0.9cm below the surface of the cerebellum). Five biphasic (100μs/phase) current pulses (5 pulses, 100/s) at 1000Hz were delivered to Sp5 using a custom isolated constant current source. The current amplitude was set to the highest level (50–70μA) that did not elicit movement artifact. A four-shank, 32-channel silicon substrate electrode (250μm between sites, 400μm between shanks, 177μm² site area; NeuroNexus Technologies, Ann Arbor, Michigan, USA) was used to record activity from the contralateral (to sound source and Sp5 electrode) A1 single- and multi-unit clusters. To achieve optimal placement, four-shank probes in the contralateral A1 penetrated approximately 2mm below the cortical surface based on previously published data (Wallace et al. 2000; Basura et al. 2012). We were able to sample from locations within a small best frequency (BF) range without moving the probe from the brain. When necessary, the electrode was repositioned until robust responses to acoustic stimulation were obtained. After achieving final probe placement the electrodes were connected by a 32-channel pre-amplifier and digitizer to a TDT data-acquisition system.

Auditory and Somatosensory Stimulation

Neural activity in response to unimodal tones or Sp5 stimulation was recorded before and 15 minutes after the bimodal stimulation protocol (Fig. 3). Tone signals (50ms duration) gated with a cosine window (2ms rise/fall time) were generated using Open Ex and an RX8 DSP (TDT, Alachula, FL) with 12 bit precision and sampling frequency set at 100kHz. Auditory stimuli were delivered to the left ear through a shielded speaker (DT770, Beyer) driven by an HB7 amplifier.
(TDT, Alachula, FL) coupled to the hollow ear bar using Tucker-Davis Technologies (TDT) system III hardware for digital-to-analog conversion and analog attenuation. Digital signals were generated and delivered to the TDT hardware by TDT software on a PC. Tones were calibrated using a 1/4-inch condenser microphone (Bruel and Kjaer; Mic: 4136; Preamp: 2619; Power Supply: 2804) coupled to the ear bar with a 0.2 mL tube. Equalization to correct for the system response was performed in the frequency domain using digital filters implemented in TDT hardware. The stimulus variable sequences were generated in pseudorandom order in MatLab. The maximum system output was 85 dB SPL.

Experimental Design/Bimodal Stimulation

First, before any bimodal stimulation, each unit's receptive field was assessed by presenting 50ms tone stimuli every 200ms with levels selected from 0-85 dB SPL in 5 dB steps and frequencies selected from 200 Hz to 23 kHz in 0.1 octave steps. The bimodal stimulation protocol consisted of 300 trials of 50ms tones combined with electrical activation of Sp5 presented at 2Hz. The tone level (range 65-75dB) and frequency (varied per animal based on best frequency; range 4000-14600Hz) for the bimodal stimulation protocol were fixed for the duration of the recording and were selected to reliably elicit unit responses to sound from most recording sites. All unimodal (BF tone alone without bimodal pairing) tones and rate level functions were at the same frequency used for bimodal stimulation. The entire recording block lasted for 30–35 min with unimodal (either BF tone or Sp5 stimulation alone) recordings at each time point lasting for 5–7 min and the bimodal stimulation protocol lasting for 4–5 min. The recording block (Fig. 3) was repeated for each bimodal interval (BI) tested. The BI was defined as the time elapsed between the tone stimulus onset and the Sp5 stimulus onset. Negative values indicate when tone preceded Sp5 stimulation and positive values denote when Sp5 stimulation preceded tone. To
measure stimulus timing dependence, the change in SFR and tone-evoked firing rates before and after bimodal stimulation was measured while varying the BI. The recording parameters were repeated and the sequences in which the various bimodal parings were tested were randomized from -20, -10, 0, 10, or 20ms. To ensure that neural responses after bimodal stimulation were not influenced by the lingering effects of the most recent pairing in the randomized set, two time points that showed the greatest neural suppression (0ms) and enhancement (+10ms) were each run in separate sham-controls alone and the results recorded 15 min after pairing and compared to the data generated from the randomized animals. To determine the precise timing of the bimodal effect on the circuit, a correction factor was calculated by subtracting the onset of the unimodal (tone or Sp5 alone) response from the respective bimodal pairing. The resultant correction factor is shown in figures in parentheses directly below the BI. Tone-evoked and SFRs to unimodal tone or Sp5 stimulation were presented at the same level as in the bimodal stimulation protocol (300 trials, 5 trials/sec) at 15 min after bimodal stimulation (Fig. 3). Responses at 15 min were used for all bimodal plasticity measurements. The responses to tones were determined by calculating the mean firing rates over the 50ms block corresponding to the tone duration. SFRs were measured by calculating the mean firing rate over 2.5 min of spiking activity in the absence of sound.

Timing rules were classified as Hebbian-like, anti-Hebbian-like, suppressing or enhancing by comparing the mean change in firing rate (i.e., the firing rate before bimodal stimulation subtracted from the firing rate after bimodal stimulation) when Sp5 stimulation preceded sound and Sp5 stimulation followed sound. As such, Hebbian-like timing rules were defined as a decrease in neural firing rates when sound preceded Sp5 and an increase in firing rate when Sp5 preceded sound while anti-Hebbian was the mirrored effect, respectively (Fig. 4A, B).
Enhanced or suppressed units were defined by a significant (2.5 SD relative to background) increase or decrease in firing rate, respectively not showing Hebbian or anti-Hebbian-like timing rules. For comparison between sham, ENT and ET groups, SFRs were measured from the initial recording block prior to the onset of the bimodal stimulation protocol and 15 min after bimodal pairing or repeated unimodal (Sp5 or tones) stimuli.

**Spike Detection and Sorting**

Recordings were made in a sound-attenuating booth. Voltages from each recording site were digitized by a preamp PZ2 (Fs = 12kHz, TDT) and band-pass filtered (300Hz to 3kHz). An online spike-detection threshold was set independently for each recording channel to 2.5 SDs above the mean background noise voltage (RZ2, TDT). Time stamps and associated waveform snippets were saved to a PC using Open Explorer (TDT, Alachua, Fl, USA) and were sorted based on shape and cluster analysis with fixed variance (Plexon Offline Sorter). Single- and multi-units were identified by waveform shape and amplitude. The waveform shapes, amplitudes, and response properties of sorted units were confirmed with pairwise cluster statistics (p>0.05; Plexon Offline Sorter) and were consistent over the duration of the recording protocols.

**Data analysis**

Post-experiment data analysis was performed in MatLab. All units were characterized by best frequency and threshold. Response maps were constructed by computing the tone-evoked firing rate during the 50ms tone stimulus minus SFR measured during the last 50ms of each trial (200ms duration). Enhancement or suppression was considered significant when the firing rate was greater than 2.5 SDs above or below the mean spike rate of all trials with no sound. Post-stimulus time histograms (PSTHs) were constructed for each unit from 300 trials with the tone level 10–30dB above threshold and frequency within 0.1 octave of the identified BF. The effect of
Sp5 stimulation on the firing rate of the response to BF tones during and after bimodal stimulation was assessed using percent difference in firing rates. The bimodal effect (15 min after pairing) on the response to tone was calculated as follows: $100 \times \frac{(FRu2-FRu1)}{FRu1}$ where $FRu1$ is the average firing rate in response to unimodal stimulation (tones) presented before bimodal stimulation and $FRu2$ is the average firing rate in response to unimodal stimulation 15 min after bimodal stimulation. Average firing rates of unimodal responses were computed from 300 trials of tone stimulation. Average firing rates of bimodal responses were computed from 300 trials of bimodal stimulation.

**Statistics**

Statistical analysis was conducted on single-units across all layers of A1 15 min after bimodal stimulation. Significant bimodal plasticity was identified using a paired measurement Student’s $t$ test for the number of spikes measured on each trial before and after bimodal stimulation. Timing rules were constructed and classified as Hebbian, anti-Hebbian, suppressing or enhancing. The proportion of timing rules from each group (sham, ENT and ET) was evaluated for significance using a 2x2 or 2x3 Chi squared test. A 2-way ANOVA with a Tukey-Kramer *post hoc* test was used to ascertain differences between mean population-timing rules (SPSS 19.0).

**Results**

*Noise exposure induced temporary threshold shifts (TTS) and tinnitus as measured by GPIAS*

Guinea pigs for each group (sham, ENT and ET) used in this study for A1 recordings were the same animals that underwent simultaneous recordings in DCN with previously published results (Koehler and Shore, 2013b). Noise exposure induced an immediate TTS as demonstrated by ABR thresholds in the noise-exposed ear only that recovered to baseline by 1 week after exposure (Fig 1; reproduced from Koehler and Shore, 2013b). Maximum threshold elevation was
35dB at 9kHz after the first exposure and 19dB at 10kHz after the second exposure with thresholds elevated in a band from the exposure frequency to 2 octaves above that frequency. Thresholds measured on ABR in sham-controls were not elevated above baseline.

GPIAS was used to assess each animal for frequency-specific tinnitus. Animals displayed normal gap detection during baseline startle testing with expected reduced responses when a gap was present. Baseline startle responses were determined by comparing the startle amplitude with (AG) and without (ANG) gap pre-pulse inhibition. Impaired gap detection, indicative of tinnitus, was identified by significantly elevated normalized startle responses. Approximately 60% of exposed animals were found to have tinnitus in the 12–14kHz band, with half at 4 – 8, 8 – 10, or 16–18kHz bands (Fig. 2; with permission from Koehler and Shore, 2013b; see Materials and Methods). Animals with tinnitus were placed in the ET group and those without evidence of tinnitus in the ENT group and non-exposed animals were designated as sham-controls.

Animals with tinnitus show shifts from tone-evoked Hebbian plasticity to anti-Hebbian plasticity

Bimodal plasticity, the persistent change in neural responses following paired bimodal stimuli, was evaluated in vivo by comparing tone-evoked firing rates and SFRs before and 15 min after bimodal stimulation (Sp5 paired with tone) for single units in A1. The order (tone-preceding Sp5 or Sp5-preceding tone) and BI (0-20ms between stimuli; Fig. 3) of Sp5 and tone stimuli in the pairing protocols were randomly varied. Bimodal plasticity was considered stimulus timing-dependent when the tone-evoked and spontaneous firing rates that were facilitated or suppressed following bimodal stimulation were also dependent on the order and timing of the bimodal stimuli. To determine the effects of tinnitus on bimodal stimulus timing-dependent plasticity, tone-evoked responses in sham (n = 126 units), ENT (n = 46 units), and ET (n = 120 units) animals were measured before and 15 min after bimodal stimulation at each BI and pairing order. Bar
graph distributions of the timing rules demonstrate a preponderance of Hebbian timing rules in sham-controls (Fig. 4C). In both ENT and ET groups, a significant increase in the number of units showing anti-Hebbian timing rules (* to respective sham aH) is observed, along with a decrease in those showing suppressive rules. A significant decrease in the number of Hebbian, but a significant increase in the number of enhancing rules is observed in the ET group (Fig. 4C).

Similarly, in sham-controls, the mean timing rule (Fig. 4D) was predominantly Hebbian, with enhancement of tone-evoked firing rates when Sp5 preceded sound stimulation (+10ms) and suppression when Sp5 stimulation followed sound stimulation. Mean population timing rules for sham-controls were Hebbian, similar to those seen in the DCN (Koehler and Shore, 2012b). However, in ET animals, enhancement occurred at BIs of -20 and -10ms, and suppression occurred at a BI of zero, providing an anti-Hebbian-like plasticity, similar to that in DCN ET animals (Fig 4D). Conversely, ENT animals showed enhancement at BIs of zero and +10ms, and no suppressive plasticity (Fig 4D). The shift from Hebbian in sham animals to anti-Hebbian plasticity in ET animals is consistent with that seen in DCN. However, the shift from Hebbian to bimodal enhancement in ENT animals stands in contrast to the narrow, anti-Hebbian timing rules seen in ENT units in the DCN. An ANOVA revealed significant main effects between sham and ET, F (1.2) p<0.015 and not between sham and ENT F(1.02); p=0.4. BIs for which there were significant differences were confirmed by a Tukey–Kramer's post hoc test.

To ensure that the results of bimodal stimulation 15 min after pairing were not influenced by the lingering effects of the repeated pairing in the randomized protocol, separate sham-controls in which only one BI per animal was presented (0ms or +10ms) were conducted. The means for results from these individual sham-animals (n=88 single units) revealed no differences from those seen with the same BI in the randomized set, indicating that the residual effects of the
previous pairing in the randomized protocol did not influence the overall effect (Fig. 4D).

**Animals with tinnitus show increased frequency-specific SFRs and shifts from Hebbian to less discrete timing rules**

SFRs measured prior to the presentation bimodal stimuli were significantly elevated in the ET group in frequency regions with TTS and evidence for tinnitus (<12kHz; Fig. 5A) but not above the tinnitus frequencies (>12kHz; Fig. 5B). The percent change in SFR was again measured 15 min after various BIs and pairing orders in the three experimental groups. Bar plots reveal mostly Hebbian units for sham-controls and mostly Hebbian and some suppressive units in ENT animals but equivalent numbers of units with anti-Hebbian and Hebbian timing rules in the ET group (Fig. 5C). An ANOVA with sham and ET revealed significant main effects between BI and exposure groups $F(1.78); p<0.0001$; and with ENT BI $F(1.98), p<0.001$ (Fig. 5C).

As with the tone-evoked results, the mean population timing rules of SFRs in single-units from sham ($n = 94$) animals predominantly showed suppression when sound preceded Sp5 stimulation (BI 0ms) and enhancement when Sp5 preceded sound (+10ms) consistent with Hebbian timing rules. There were significant differences between the ENT timing rules ($N=20$ single units) and sham-controls at BIs of 0 and +10ms (Fig. 5D). ET animals ($n = 20$ single-units) exhibited no evidence of plasticity at any of the BIs. This likely reflects a high degree of variability in the responses in this group as the bar graphs showed that individual units did show plasticity but this plasticity was distributed across all timing rule types, as shown in Fig. 5C.

**Bimodal stimulation induces more plasticity than unimodal stimulation in A1**

To assess whether unimodal (tone or Sp5 alone) stimulation also produced equivalent persistent responses to bimodal stimulation, tone-evoked responses were measured during protocols in which the bimodal stimulus was replaced by a tone alone or Sp5 stimulation alone. Compared to bimodal stimulation, responses to tone-alone stimulation did not significantly alter
neural firing rates (either mean increases or decreases) 15 min after stimulation (Fig. 6).

Unimodal Sp5 stimulation produced an increase in tone-evoked firing rates in sham-controls only and had no significant effect in the ENT or ET groups. In all groups, bimodal stimulation at 0 and +10 significantly suppressed or enhanced tone-evoked firing rates as compared to unimodal stimulation, respectively (Fig. 6).

**Discussion**

The hypothesis of the present study was that bimodal plasticity in A1 is stimulus-timing dependent and displays similar timing rule changes found in DCN (Koehler and Shore, 2013b) following noise-induced tinnitus. Extracellular *in vivo* A1 single-unit responses to tones and SFRs measured before and after bimodal stimulation (paired Sp5-tone) demonstrated stimulus timing-dependence similar to that shown in DCN for sham-controls (Koehler and Shore, 2013a).

Furthermore tone-evoked bimodal plasticity timing rules were altered following noise-induced tinnitus: they were more likely to be Hebbian in sham-control animals and anti-Hebbian-like and enhancing in noise-exposed animals with tinnitus (ET), and somewhere in between in animals without tinnitus (ENT). SFR timing rules remained primarily Hebbian in shams but shifted to multiple timing rule types in animals with tinnitus and to Hebbbian and suppressive in animals without tinnitus (ENT). The responses in A1 following bimodal stimulation in sham-controls are consistent with timing rules in DCN (Koehler and Shore, 2013). However, there are some differences in plasticity changes after tinnitus that are observed in A1 and DCN following noise exposure. The changes in A1 appear to be more complex, and demonstrate multiple changes in timing rules in the tinnitus animals. Thus, while A1 appears to demonstrate “already processed” bimodal plasticity in normal animals, additional mechanisms of plasticity change following noise damage appear to be involved in A1.
**A1 Neurons exhibit bimodal STDP**

To date, few studies investigating STDP in A1 have been conducted. Of those, stimulus-timing-dependent plasticity properties in A1 (Kilgard and Merzenich, 2002; Dahmen et al. 2008) are similar to those seen in other sensory cortices (Larsen et al. 2010) including visual (Froemke et al. 2006; Meliza and Dan, 2006) and somatosensory (Nevian and Sakmann, 2006), showing Hebbian and anti-Hebbian-like timing rules. In the present study, suppression or enhancement of A1 neural firing following combined auditory-somatosensory stimulation that is pairing order and timing-dependent, demonstrates that STDP principles in A1 are not limited to auditory-auditory only paradigms (Dahmen et al. 2008; Kilgard and Merzenich, 2002). It is not surprising that non-auditory systems may also modulate auditory circuits since repeated pairing of tones with nucleus basalis, locus coeruleus, or ventral tegmental stimulation increases neural responses to the same tone in A1 (Bakin and Weinberger, 1996; Edeline et al., 2011; Froemke et al., 2007; Kilgard and Merzenich, 1998a; Kilgard et al. 2001). The present study adds support to the contribution of multiple non-auditory systems to A1 processing.

Standard STDP protocols involve single excitatory-post-synaptic potentials (EPSPs) paired with single post-synaptic back-activating action potentials (BAPs; Bi and Poo 1998; Froemke and Dan 2002). Induction of long-term potentiation (LTP) is typically achieved by pre-before-postsynaptic pairing, while post-before-presynaptic pairing can induce long-term depression (LTD), resulting in a Hebbian-like learning rule (Froemke and Dan 2002). In our model, if A1 is receiving ‘already-processed’ inputs from DCN in which the known pre- and post-synaptic sources are identified (Tzounopoulos et al. 2004, Koehler and Shore, 2013b), tone preceding Sp5 (negative values on figures) stimulation would represent post-before-pre (Sp5) synaptic activity resulting in robust suppression of neural firing that is preserved 15 min after pairing, reflecting
long-term depression (LTD). Conversely, Sp5 stimulation preceding tone (positive values on figures) represents pre-before-post spiking activity, leading to neural facilitation that also persisted 15 min after pairing consistent with long-term potentiation (LTP). Karmarkar and Buonomano (2002) demonstrated these principles in A1 slices where repetitive pairing of pre-before-post spiking activity at +10ms intervals produced LTP and post-before-pre intervals at -40ms produces LTD at layer II/III cortical synapses. Our results showing predominantly Hebbian-like timing rules from sham-control animals in A1 are consistent with these data. However, variability in population timing rules following bimodal pairings in cortex demonstrate that pre-before-post-synaptic stimulation can also lead to less change (Markram et al. 1997; Sjostrom et al. 2001; Kampa et al. 2006; Nevian and Sakmann 2006), LTP (Feldman 2000; Froemke and Dan 2002), or LTD (Zhou et al. 2005) depending on the identity, nature and location of the synapse. This is exemplified in our data. ENT units showed greater enhancement in neural firing (BIs of 0 and +10) than ET units that displayed a modest, yet significant anti-Hebbian response profile (BIs of -20 and -10ms; Fig. 4D). This variability may reflect molecular differences in A1 neurons/synapses between ET and ENT groups such as the activity related gene Arc/Arg3.1. This cytoskeletal protein is mobilized after LTP activity and has been proposed to influence post-synaptic AMPA receptor expression that is required for LTP (reviewed by Knipper et al., 2013). Interestingly, A1 Arc/Arg3.1 following noise-exposure was only mobilized in animals without tinnitus and was not changed in those with tinnitus (Ruttiger et al., 2013; Singer et al., 2013). This suggested that increased cortical firing following moderate deafferentation following noise maybe the result of enhanced glutamate sensitivity. That study, however, showed more permanent hearing loss in 'tinnitus' animals, suggesting that the Arc changes could be a reflection of the hearing loss and not the tinnitus. In our data, all noise-exposed groups only display TTS at the
time of testing. It would therefore be interesting to investigate molecular changes that occur in both groups during the threshold shifts before recovery occurred.

_Noise exposure leads to temporary threshold shifts, tinnitus and changes in bimodal stimulus-timing dependent plasticity_

Narrowband noise-exposure (7 kHz) resulted in unilateral TTS and tinnitus in the same animals that also underwent simultaneous recordings from DCN (Koehler and Shore, 2013b). In A1, increased SFRs following noise exposure in cats (Komiya and Eggermont, 2000; Eggermont, 2007), a documented neural physiologic correlate of tinnitus (reviewed by Eggermont 2015), were significantly increased only in the tinnitus bands (<12kHz) as compared to sham and noise-exposed animals that did not show evidence of tinnitus. A1 SFRs outside the tinnitus bands (>12kHz) did not show significant increases in any group. The correlation of increased SFRs with GPIAS measures of tinnitus further validate the use of GPIAS as a method to identify tinnitus in guinea pigs after noise exposure (Dehmel et al. 2012a; b Koehler and Shore, 2013b; Turner et al. 2006).

Tone-evoked timing rules in ET animals shifted from primarily Hebbian-like in sham-controls to primarily anti-Hebbian-like and enhancing rules, with a significant decrease in suppressing timing rules. Animals without tinnitus displayed an equal number of Hebbian and anti-Hebbian-like timing rules without a significant change in enhancing timing rules, in between sham-control and ET response profiles. Timing rule classifications for SFRs also shifted for ENT and ET animals as compared to sham-controls, where timing rule curves showed a lower magnitude plasticity at all BIs with a widening of the temporal windows. While this effect was observed after 15 minutes, it is possible that significant changes in plasticity may be seen if recordings were taken for a longer period after pairing. These results confirm that somatosensory...
inputs have a significant long-term influence on A1 neural activity following noise exposure and tinnitus. These findings are consistent with the neural modulation and changes in bimodal plasticity in DCN following noise exposure and tinnitus and suggest that mechanisms of auditory-somatosensory integration at the brainstem have influence across the central auditory circuit in tinnitus (Koehler and Shore, 2013). This is important to consider given the known extensive cross-modal reorganization of the auditory cortex following noise-exposure in which 84% of sampled auditory cortex neurons began to respond to somatosensory stimulation with 76dB or greater threshold shifts (Allman et al. 2009). The authors of the latter paper acknowledge that their findings may be due, at least in part, to deafness-induced increases in somatosensory inputs to the DCN (Shore et al. 2008; Zeng et al., 2012), the first station of the central auditory relay, which may generate upstream changes seen at the cortical level.

**Bimodal stimulation induces more plasticity than unimodal stimulation in A1**

In the present study, with the exception of a modest increase in neural firing rate following Sp5 stimulation alone in sham-controls, unimodal stimulation (tone alone or Sp5 alone) did not significantly suppress or enhance tone-evoked or SFRs, as shown with bimodal stimulation. Sustained plasticity in A1 following bimodal stimulation may reflect principles of temporal coincidence where multi-sensory interactions are strongest when both (tone-Sp5) modalities are presented (Lakatos et al. 2007). Applying our bimodal plasticity protocol to that model would therefore suggest that somatosensory stimulation (when preceding tone; +10ms) enhances auditory processing by resetting neural oscillations so that concurrent auditory inputs arrive during a high-excitability phase and are thus amplified/enhanced (Lakatos et al. 2007). Alternatively, those auditory inputs less intimately associated with convergent somatosensory stimuli may have weaker multisensory association (0ms pairing; when both stimuli are presented...
simultaneously) and reset neural oscillations to low-excitability phase, resulting in neuron suppression.

*Mechanisms of STDP in A1*

Whether Hebbian or anti-Hebbian-like timing rules predominate depends on a number of factors including the dendritic location of the synapses (Letzkus et al. 2006; Sjostrom and Hausser 2006) and order of activity in pre- and post-synaptic cells (Karmarkar and Buonomano 2002; Karmarkar et al. 2002). In some circumstances, the timing rule may be converted from anti-Hebbian to Hebbian in layer II/III cortical pyramidal cell synapses, depending on the timing of pre- and post-synaptic activity. While one STDP learning rule does not “fit all” excitatory synaptic connections, variability in responses in most cases can be explained by differences in levels of post-synaptic depolarization and subsequent Ca2+ influx during the pairing protocol (Zilberter et al. 2009). This concept is supported by data showing that the balance between excitatory and inhibitory synapses dictates neural STDP in A1 *in vitro* (D’amour and Froemke, 2015). When the excitatory to inhibitory synaptic ratio is higher, more Ca2+ channel and NMDA receptor activation occurs during spike pairing due to the increased level of depolarization. Alternatively, lower ratios may mean that inhibition more effectively limits NMDA-based depolarization limiting excitation. In the present study, the order and timing of bimodal stimulation (Sp5 and tone) may modulate synaptic ratios, ultimately leading to STDP and the observed timing rules. Future studies will be dedicated toward identifying specific timing rules to anatomic layer and cell types across A1.

*STDP Across Auditory Circuits*

A variety of learning rules reflecting STDP have been observed in the brainstem (Tzounopoulos et al. 2004; Koehler and Shore, 2013a) and cortex (Egger et al., 1999; Letzkus et al.)
Bimodal auditory-somatosensory plasticity in DCN modulates spontaneous and tone-evoked activity that is stimulus-timing dependent (Koehler and Shore, 2013a). Timing rules from that \textit{in vivo} study reflected recordings found \textit{in vitro} (Tzounopoulos et al. 2004), suggesting that bimodal stimulus-timing-dependent plasticity in DCN neurons reflects STDP. In 40% of DCN units from sham-controls that exhibited stimulus-timing dependency, auditory responses were facilitated after bimodal pairing in which Sp5 stimulation preceded tones but was suppressed if tones preceded Sp5 stimulation, thus exhibiting primarily Hebbian-like timing rules (Koehler and Shore, 2013a). These results are consistent with the present findings in A1 sham-controls suggesting consistent mechanisms from brainstem to cortex. This comparison suggests that auditory-somatosensory plasticity works in a similar fashion at both brain levels or that A1 inherits plasticity that has occurred in DCN. Support for a consistent mechanism that displays a variety of timing rules ranging from Hebbian to anti-Hebbian may reflect the diversity of principal cell types within DCN that ultimately dictate physiologic control of bimodal plasticity. For example, DCN neurons with less inhibitory influence (Type I receptive fields) were more likely to display Hebbian-like learning rules, while those units with greater inhibitory influence (Type III/IV) more often displayed anti-Hebbian-like stimulus timing-dependence (Koehler and Shore, 2013a). This is important to consider when interpreting the current A1 results that show evidence of primarily Hebbian STDP similar to DCN in sham-controls. Responses in A1 may therefore reflect greater "processed" sensory information through less inhibitory units (Type I) from DCN conveyed along the non-lemniscal pathway and recorded from layer II/III thalamocortical inputs, versus "non-processed" circuits that require cortical integration (Basura et al. 2012) with different timing rules recorded from remaining A1 layers. Within A1, synapses in layer II/III represent a major site of intra-cortical processing for inputs.
arriving from the thalamus where STDP rules could be converted from one mode (Hebbian-like) to another (anti-Hebbian-like) depending on the post-synaptic activity (Zilberter et al. 2009).

Following noise exposure and tinnitus, differences in the proportion of timing rules were observed between DCN and A1. In DCN, increased suppressive plasticity was found in the ENT group that significantly decreased in the ET group. A1 timing rules showed a significant decrease in suppressive units in both noise-exposed groups. While DCN showed a decrease in all units except suppressive rules in ENT animals, both regions showed an increase in anti-Hebbian units in the ET groups. While a consistent mechanism of plasticity may exist in sham-controls between the two regions, different timing rule shifts in A1 suggest that mechanisms underlying multisensory plasticity in A1 and DCN are variably affected by noise exposure and tinnitus. Further evidence for separate multi-sensory processing in A1 is evident in our data. Maximal changes in neural responses in A1 occurred at BIs of 0 and +10ms versus different BIs (-10 to -20ms and +20ms; Koehler and Shore, 2013b) in DCN suggesting that discrete processing at the cortical level may also exist. Future studies will investigate changes in timing rules specific to cortical layer and cell types across A1 that may help better identify where bimodal plasticity may be “already processed” by thalamocortical projections versus layers that are “cortically processed”.

STDP and tinnitus

Although the timing rules and neural changes in A1 in the present study were similar to those in DCN in sham-controls, differences were seen between the two regions after noise exposure in animals with and without evidence for tinnitus. When compared to the findings from DCN in the same animals under the same recording conditions, common changes in timing rules (shifts from Hebbian to anti-Hebbian) were observed in noise-exposed animals with tinnitus (Koehler and Shore, 2013b). Changes in A1 STDP following noise exposure and tinnitus suggest
that processing following bimodal stimulation may be implicated in tinnitus generation and can be potentially harnessed to ameliorate the percept. The observation that STDP is altered in both DCN and A1 following noise-induced tinnitus creates inroads for therapeutic design to target STDP mechanisms within both regions to simultaneously and non-invasively ameliorate neural tinnitus correlates and ultimately, perception.

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Author Contributions

GB, SK and SES conception and design of the research; GB and SK performed the experiments; GB and SK analyzed the data; GB and SES interpreted results of the experiments; GB prepared the figures and drafted the manuscript; SK and SES edited the manuscript.
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**Figure Legends**

**Figure 1.** Auditory brainstem responses (ABR) reveal a temporary threshold shift (TTS) following noise exposure. ABR threshold shifts in noise-exposed animals in the exposed-ear (A) and unexposed ear (B) and from sham-control animals from both ears (C, D; figure reproduced with permission from Koehler and Shore, 2013b). ABR thresholds were measured following the initial noise exposure (green), following the second noise exposure (pink), and just prior to in vivo A1 unit recordings (gray). Dashed lines represent ABR thresholds immediately following noise exposure. Solid lines indicate recovered TTS 1 or more weeks following noise exposure. Shaded bands represent 95% confidence intervals.

**Figure 2.** Gap-prepulse inhibition of acoustic startle (GPIAS). **A.** Schematic outlining the startle-based gap-detection assay for tinnitus (figure reproduced with permission from Koehler and Shore, 2013b). Top row reveals no gap with the bottom two rows showing gap trials (50ms gap, 50ms before the startle stimulus). Each trial consisted of continuous 60dB background noise (gray bar) with a 10ms, 115dB startle pulse embedded (black bar). The animal responds to the startle stimulus, with the amplitude of the response shown by the height of each arrow. In noise-exposed animals without tinnitus (ENT), the gap leads to suppression of the startle response (middle row). In animals with noise-induced tinnitus, the gap is filled by tinnitus (pink), and no gap-induced reduction in startle response (white arrow). **B–F.** Gaussian mixture model analysis separating startle distribution into normal and tinnitus. **B.** Histogram of the normalized startle responses (white line) separated into two distributions: no evidence for tinnitus (black bars) and evidence for tinnitus (red bars). **C.** The probabilities that normalized startle values belong to tinnitus or no tinnitus distributions. **D.** Histogram of the distribution of post-exposure normalized startle
observations for sham-controls. **E.** Histogram of the distribution of normalized startle observations for baseline (pre-exposure) observations from sham and noise-exposed animals. **F.** Histogram of the distribution of post-exposure startle observations from noise-exposed animals. **D–F.** Percentage of observations placed into the tinnitus group is shown on each figure.

**Figure 3.** Bimodal plasticity protocol where spontaneous neural firing (Spont) and pure tone (Tone) responses were measured before and 15 min after the bimodal pairing protocol. Bimodal pairing consisted of repeated presentations of Sp5 stimulus (solid line) and a pure tone burst (sinusoid) with varied bimodal interval (BI) and order. The BI is defined by the onset time of the tone stimulus and the temporal interval before Sp5 stimulus onset and vice versa. **A.** Tone alone trial consisting of 50ms of silence, 50ms of BF tone burst, 50ms of silence; repeat X 50. **B.** Silence for 2.5 minutes. **C.** Bimodal trial consisting of one bimodal pair of Sp5 and 50ms of BF tone; 300 repetitions. **D.** Silence for 2.5 minutes. **E.** Tone alone trial consisting of 50ms of silence, 50ms of BF tone burst, 50ms of silence; repeat X 50.

**Figure 4.** Bimodal plasticity shifts from Hebbian to anti-Hebbian-like and enhancing timing rules with less discrete temporal windows in animals with tinnitus and to mostly anti-Hebbian timing rules in guinea pigs without tinnitus. **A.** Two examples of single-unit anti-Hebbian-like timing rules from a sham-control (left) and noise-exposed (right) guinea pig. A schematic at the top of the panel demonstrates the relationship of the Sp5 stimulus (vertical line) and the tone stimulus (sinusoid). At each BI a correction factor was calculated where the timing of the unimodal stimulus onset was subtracted from the bimodal stimulus onset (correction factor listed in parentheses below the respective BI). **B.** Two examples of single-unit Hebbian-like timing rules
from a sham-control (left) and noise-exposed (right) animals. C. Bar graph showing the percentage of single units that showed Hebbian (H), anti-Hebbian (aH), enhancing (E), and suppressing (S) and undefined (U) timing rules from sham-controls (left), ENT (middle), and ET (right) animals. Within the ET group a larger percentage of anti-Hebbian and enhancing rules were seen as compared to shams that predominantly showed Hebbian timing rules. Animals in the ENT group also showed a greater number of anti-Hebbian timing rules as compared to sham-controls. Significant differences were found between sham, ENT, and ET groups (overlying bar denotes individual comparison (tabs) that is statistically significant as compared to the reference (*p<0.05; Tukey–Kramer’s post hoc test; asterisk under bar denotes comparison to sham-controls for that respective timing rule). D. Mean timing rules showing bimodal plasticity of tone-evoked firing rates for units from sham-control (dark line), ENT (gray line), and ET (dashed line) animals. Schematic above represents the order of Sp5 and tone stimuli. The vertical line indicates Sp5 stimulation with the sinusoid representing tone stimulus onset. Mean timing rules were calculated for BIs and stimulus order from across all layers of A1 with error bars that indicate SEM. At each BI a correction factor was calculated where the timing of the unimodal stimulus onset was subtracted from the bimodal stimulus onset (correction factor listed in parentheses below the respective BI). To demonstrate the effects of bimodal trials separate from the randomized protocol on sham-control responses using the maximal suppressive (0ms) and enhancing (10ms) pairings, the mean change in firing rates (open circles; n=88 single units) and SEM are plotted in relationship to sham-controls at the same BI and order from responses obtained during the randomized set. Note similar responses to each maximal pairing when stimulated alone without a randomized protocol. Significant differences were found between sham-controls, ENT, and ET groups (*p<0.05; Tukey–Kramer’s post hoc test as compared to sham controls).
controls with ET being significantly different at -20 and -10ms and ENT at 0ms; and ($p<0.05$ as compared to the ET group with sham and ENT both significantly different from ET at -20, -10 and +20ms and the ENT group being different from ET at 0ms).

**Figure 5.** Spontaneous firing rates (SFRs) are significantly increased in the ET group but not in ENT animals. **A.** Mean SFRs for units with best frequencies $<12$kHz. Note the significant increase in the ET group as compared to sham ($*p < 0.003$). **B.** Mean SFRs for units with best frequencies $>12$kHz. **C.** Bar graph showing the percentage and distribution of timing rules for SFRs from sham-controls (left), ENT (center) and ET (right) following bimodal stimulation. Overlying bar denotes individual comparison (tabs) that is statistically significant as compared to the reference ($*p < 0.05$; Tukey–Kramer's *post hoc* test; asterisk under the bar denotes comparison to sham-controls for the respective timing rule). **D.** Mean percent change in SFRs and timing rules revealing bimodal plasticity for single-units from sham-controls (black line; 94 units), ENT (gray line; 20 units), and ET (dashed line; 20 units) animals. Mean timing rules were calculated for all measurements across all layers of A1. Error bars indicate SEM. Significant differences were found between sham-controls, ENT, and ET groups ($*p <0.05$; Tukey–Kramer’s *post hoc* test as compared to sham-controls).

**Figure 6.** Bimodal stimulation induces more plasticity than unimodal stimulation A1. Bar graph shows the effects of tone (light gray) and Sp5 (dark gray) alone on the percent change in neural firing rates in all three (sham-control, ENT and ET) groups in A1. The graph also shows the direct comparison of the unimodal responses to the bimodal responses where maximal enhancement (+10ms; upward stripes) and suppression (0ms; downward stripes) were observed. With the
exception of a modest increase in neural firing rate in sham-controls following Sp5 alone, neither unimodal stimulus significantly altered neural firing rates alone. Bimodal stimulation results in greater levels of enhancement and suppression of neural firing depending on BI (overlying bar denotes individual comparison between unimodal and bimodal stimulus (tabs) that is statistically significant as compared to the bimodal stimulus*\(p<0.05\); Tukey–Kramer’s *post hoc* test; asterisk under bar denotes comparison to other bimodal stimulus; *\(p<0.05\); Tukey–Kramer’s *post hoc* test).

**Abbreviations:** A1, primary auditory cortex; BF, best frequency; PSTH, peri-stimulus time histogram; Sp5, spinal trigeminal nucleus; STDP, spike-timing-dependent plasticity.
Figure 1

A. Left

B. Right

C. Noise Exposed

D. Sham

Threshold Shift (dB SPL) vs. Frequency (kHz)

- Post Exposure 1
- Recovery 1
- Post Exposure 2
- Recovery 2
- Final
null