Title: Footprints of inhibition in the response of cortical delay-tuned neurons of bats

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Running head: delay-tuning in homoharmonic bats

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Abstract

Responses of echo-delay-tuned neurons that encode target-distance were investigated in the dorsal auditory cortex of anesthetized short-tailed fruit bats (*Carollia perspicillata*). This species echolocates using short downward frequency modulated (FM) biosonar signals. In response to FM-sweeps of increasing level, 60 out of 131 studied neurons (47%) displayed a “paradoxical latency shift”, i.e. longer response latency to loud sounds and shorter latency to faint sounds. In addition, a disproportionately large number of neurons (80%) displayed non-monotonic responses, i.e. weaker responses to loud sounds and stronger responses to faint sounds. We speculate that the observed paradoxical latency shift and non-monotonic responses are extracellular footprints of inhibitory processes evoked by loud sounds, and that they could represent a specialization for the processing of the emitted loud biosonar pulse. Supporting this idea is the fact that all studied neurons displayed strong response suppression when an artificial loud pulse and a faint echo were presented together at a non-optimal delay. In 24 neurons, iontophoresis of bicuculline (an antagonist of A-type γ-Aminobutyric acid receptors) did not remove inhibitory footprints but did increase the overall spike output, and in some cases it also modified the response bandwidth and shifted the neuron’s “best delay”. We suggest that inhibition could play a dual role in shaping delay tuning in different auditory stations. Below the cortex it participates in delay tuning implementation and leaves a footprint that is measurable in cortical responses, while in the cortex it provides a substrate for an *in situ* control of neuronal selectivity.
Echolocating bats use the time interval from biosonar pulse emission to the arrival of echo (defined as echo-delay) to infer target-distance. In the central auditory system, echo-delay is encoded by delay-tuned neurons that respond to pulse-echo pairs presented at specific delays (Feng et al. 1978; O’Neill and Suga 1979; Suga et al. 1978).

Bats species have been classified into two large groups depending on the properties of their delay-tuned neurons. In bat species such as *Eptesicus fuscus*, *Myotis lucifugus* and *Carollia perspicillata*, the delay tuned neurons are best driven by homoharmonic (HM) pulse echo-pairs, that is, pairs of sounds in which both pulse and echo have a comparable frequency composition (Sullivan 1982a; Dear et al. 1993; Hagemann et al. 2010). On the other hand, neurons from bat species such as *Pteronotus parnellii*, *Pteronotus quadridens* and *Rhinolophus rouxi* respond better to heteroharmonic (HT) pulse-echo pairs in which the pulse and echo do not overlap in the frequency domain (Suga et al. 1978; Schuller et al. 1991; Hechavarria et al. 2013). From here on, for the purpose of simplicity, bats that possess neurons driven by HM pulse-echo pairs will be referred to as HM bats, while bat species in which HT neurons are more abundant will be referred to as HT bats.

Delay tuning is implemented in central neurons and the mechanisms for this implementation have been intensively investigated in HT bats. HT delay tuning is first created in the inferior colliculus (IC) through interplay between excitation and inhibition (Wenstrup et al. 2012). Inhibition delays the excitatory response to the fundamental harmonic of the emitted pulse, so that at the neuron’s optimal delay, this response is temporally aligned with an excitatory response evoked by the upper echo harmonics (Portfors and Wenstrup 1999). Inhibition also suppresses excitatory responses to non-optimal echo-delays (Nataraj and Wenstrup 2005; Sanchez et al. 2008).
inhibitory processes that underlie delay tuning implementation in the IC of HT bats leave a footprint that appears in the form of suppression side-fields in the delay tuning curves (that represent echo-delay vs. spike-rate) of IC and cortical delay-tuned neurons (Nataraj and Wenstrup 2005; Edamatsu and Suga 1993).

It is widely accepted that inhibition plays a key role in the implementation of HT tuning. However, if inhibition also plays a role in the implementation of HM tuning it is still unknown. In this paper, we present a systematic characterization of the response of cortical delay-tuned neurons of the short-tailed fruit bat, *Carollia perspicillata* (a HM species, Hagemann et al. 2010). In particular, we were interested in characterizing response features that could indicate the presence of an underlying inhibition.

We searched for three types of inhibitory footprints in the spike response of the neurons: (I) side suppression fields in the delay tuning curves; (II) paradoxical latency shift (PLS), that is, a longer response latency to loud sounds and shorter latency to weak sounds (Sullivan 1982b); and (III) non-monotonic responses, that is, stronger responses to faint sounds and weaker responses to loud sounds. PLS is known to be created by a combination of low-threshold excitation and high-threshold inhibition (Galazyuk and Feng 2001) and therefore it can be considered as an extracellular inhibitory footprint that appears in response to loud (pulselike) sounds. Likewise, non-monotonic responses are known to depend on high-threshold inhibition (Tang et al. 2008).

We recorded from cortical neurons and not from midbrain neurons as did previous studies on delay tuning (i.e. Portfors and Wenstrup 1999; Wenstrup and Leroy 2001; Nataraj and Wenstrup 2005; Sanchez et al. 2008; Galazyuk et al. 2005) because we reasoned that cells in the cortex inherit the entire repertoire of inhibitory features that could result from a subcortical implementation of delay tuning, regardless of the nuclei where this implementation takes place.
In addition, recording from cortical neurons offers the possibility to measure inhibitory features that could arise from an *in situ* reimplementation of delay tuning at the cortical level.

To explore the possibility of a reimplementation of delay tuning at the cortical level, in a number of neurons we blocked $\gamma$-Aminobutyric acid (GABA)-A mediated inhibition. The role of GABA mediated inhibition in temporal processing remains controversial. In the inferior colliculus of HT bats, the inhibition that is fundamental for delay tuning implementation is mainly of glycinergic origin while GABAergic inhibition seems to be important only for controlling the spike output of the neurons (Nataraj and Wenstrup 2005; Sanchez et al. 2008). In contrast, at least in some collicular neurons of HM bats, blocking GABAergic inhibition completely removes a form of inhibitory delay tuning, in addition to removing the PLS and non-monotonic response of the neurons (Galazyuk et al. 2005). We hypothesized that if delay tuning is to some extent reimplemented in the dorsal auditory cortex, then the GABAergic system could provide the main source of inhibition for delaying the response to the emitted pulse. This idea is based in the fact that the cortex contains a large amount of GABA receptors while glycine receptors are poorly expressed at the cortical level (Krnjević 1974; Emson and Lindvall 1979; Frostholm and Rotter 1985).

We describe that about 80% of the studied cortical delay-tuned neurons in *C. perspicillata* displayed PLS and/or non-monotonic responses, while all of them presented side suppression fields in their delay tuning curves. We also show that delay tuning, and the observed PLS and non-monotonic responses are not created *in situ* in the cortex by GABA-A mediated inhibition.

**Methods**

**Surgical procedures**
The study was conducted in 18 adult *C. perspicillata* of both sexes (10 females and 8 males).

Animals were taken from a breeding colony in the Institute for Cell Biology and Neuroscience, Goethe University, Frankfurt am Main (Germany). Bats were anesthetized subcutaneously with a mixture of ketamine (10 mg kg\(^{-1}\) Ketavet) and xylazine (38 mg kg\(^{-1}\) Rompun). A longitudinal midline incision was made through the skin overlying the skull and the underlying temporal musculature was reflected from the incision along the midline.

Using a scalpel blade, a small hole (~1 mm) was made in the skull above the FM/FM area (i.e. the area where delay-tuned neurons are found). The position of the FM/FM area was determined from the pattern of blood vessels and landmarks in the scalp (see Hagemann et al. 2010). The bat’s head was fixed using a custom-made metal rod (1 cm length, 0.1 cm diameter) that was glued to the skull using dental cement (Paladur, Heraeus Kulzer GmbH).

In 12 bats, electrophysiological recordings started right after finishing surgery and lasted between 6-18 hours (acute experiments). In another six bats, repeated recording sessions were performed every second day, for a time period of no more than eight days (chronic experiments). In the chronic experiments, after the recording sessions, the hole in the skull of the bats was covered with sterile bone wax and the animals were returned to their individual cages. In all cases, during the experiments animals were kept lightly anaesthetized, that is, a dose 0.01 ml of the mixture of anaesthetics was injected every second hour. The animal use in this study complies with all current German laws on animal experimentation (experimental permit # F104/57) and it is in accordance with the Declaration of Helsinki.

*Recording single-cell activity*
The experiments were conducted in a sound proof chamber. The neuronal activity was recorded using glass electrodes (impedance 8-20 MΩ). Glass electrodes were built by pulling (P-97 Flaming/Brown type micropipette puller, Sutter Instrument) borosilicate micropipettes (GB120F-10, Science Products). Pulled micropipettes were filled with a solution of potassium chloride (3mol*L⁻¹). A silver wire was inserted into the micropipette and connected to the preamplifier of a custom-made DC-coupled recording amplifier with a 10-fold amplification. The recorded signals was AC-coupled, filtered between 300 and 3000 Hz and amplified (20 dB amplification) using an SR650 dual channel filter (Stanford Research Systems, Inc.). Recorded signals were finally digitized (DAP 840, Microstar laboratories, Sampling Rate = 278.8 kHz), down-sampled to 22.43 kHz and stored in a computer for offline analysis.

**Iontophoresis of bicuculline methiodide (BMI)**

Custom-made piggyback electrodes were used for combining extracellular recordings with the local iontophoresis of the GABA_A antagonist bicuculline methiodide (BMI, Sigma Aldrich Chemie GmbH). Piggyback electrodes were constructed following the method described by Havey and Caspary (Havey and Caspary 1980). A four-fold multichannel capillary (Hilgenberg GmbH, Germany) was pulled using a vertical pipette puller (model 700c, David Kopf Instruments). The tip of the pulled four-fold multichannel capillary was broken under the light of a microscope (Olympus BH-2, magnification 125x) down to a diameter of ~ 5µm. A recording electrode (similar to the simple glass electrodes described in the preceding text) was glued to the four-fold multichannel capillary. The recording electrode was positioned so that its tip was parallel to the tips of the four iontophoresis capillaries. The tip of the recording electrode protruded between 5 and 10 µm from the tip of the iontophoresis pipettes.
In the piggyback electrodes, the recording electrode and two of the iontophoresis pipettes were filled with a solution of sodium chloride (NaCl, 1 mol*L\(^{-1}\)). The other two iontophoresis pipettes were filled with a 10 mM solution of BMI. The pH of the BMI solution was adjusted to 3 using hydrochloric acid. During the iontophoresis experiments, spike activity was recorded using the same custom-made recording amplifier that was used for extracellular recordings with simple glass electrodes (see preceding text). Iontophoresis of BMI was controlled using a Neurophore BH-2 system (Harvard Apparatus, Medical Systems Research Products). One of the iontophoresis pipettes filled with NaCl was used to balance the Neurophore BH-2 system. A retention current of -20 nA was used to avoid undesired leakage of BMI. Ejection currents were set to values between 5-20 nA depending on the apparent effect of the drug on the neuron. To check that the balancing module of the Neurophore BH-2 system was working properly, in some neurons an ejection current of +20 nA was applied to one of the iontophoresis pipettes filled with NaCl. The latter produced no apparent increase in the cell’s spike activity.

**Acoustic stimulation and response characterization**

Acoustic stimuli were generated by a D/A board (DAP 840, Microstar laboratories, Sampling Rate = 278.8 kHz), attenuated (PA5, Tucker Davis Technologies), amplified (Rotel power amplifier, RB-850) and delivered from a calibrated speaker (ScanSpeak Revelator R2904/7000, Avisoft Bioacoustics, Berlin, Germany). Sound pressure level (SPL, expressed as dB SPL re 20 µPa) was controlled online using the calibration curve of the speaker. The calibration curve was obtained with a ¼-inch Microphone (Brüel&Kjaer, model 4135) that was connected to a custom-made microphone amplifier. The speaker was placed in front of the bat’s right ear, at a distance of 15 cm.
The response of each neuron was studied using 2-ms artificial FM-sweeps (0.5 ms rise/fall time) whose frequencies mimicked the second or third harmonics in the biosonar call of *C. perspicillata* (92-46 kHz and 122-74 kHz, respectively). The response of delay-tuned neurons was tested using two different stimulation protocols: (I) single FM-sweeps of variable sound pressure levels and (II) pairs of FM sweeps that mimicked call-echo pairs with different delays.

Single FM-sweeps of randomly changed SPLs (i.e. between 20-95 dB SPL, 5 dB steps, 50 trials each) were used for calculating rate-level functions (i.e. stimulus SPL vs. spike-rate), latency-level functions and dot raster displays that vary as a function of SPL (i.e. spike-times relative to the stimulus onset vs. stimulus level). In each rate-level function, we considered that a given SPL effectively evoked a “response” if the number of spikes represented at least 35% of the maximum spike-rate observed in the rate-level function. Following this criteria, the minimum threshold of a given neuron was calculated by interpolation at the rising slope of the rate-level function as 35% of the observed maximum response. In the neurons in which the effects of BMI were tested, response threshold was calculated independently for the pre-drug, BMI and recovery conditions.

Neurons were classified according to the shape of their rate-level functions into monotonic (i.e. neurons in which the spike-rate typically increased with increasing SPL until it reached a plateau) and non-monotonic responders (i.e. neurons in which there was a drop in the response at high SPLs). Non-monotonic responders were further classified into SPL-tuned neurons that failed to respond to high SPLs and “notch” neurons in which the spike activity dropped in response to a given SPL and then raised again with further SPL increments.

For the construction of latency-level functions, response latency was measured only at SPLs that evoked a significant response (following our definition of response, that is, ≥ 35% of the maximum spike-rate). Response latency was measured in the post-stimulus time histograms.
(PSTH) at the onset slope of the neuronal response, as the time at which 50% of the maximum of each PSTH was reached. PSTHs were constructed with a bin-size of 1 ms. Based on the shape of latency level-functions we distinguished between neurons that displayed PLS (i.e. response increased with raising SPLs) and those that did not. The amount of PLS was measured as the longest latency shift observed for succeeding increments in SPL.

The second stimulation paradigm (i.e. presenting artificial pulse-echo pairs with different delays) was used to calculate delay tuning curves. For characterization of delay tuning curves, FM pairs of fixed levels presented at random delays were used (i.e. delays between -2-28 ms, 2 ms steps, negative values indicate that the FM sweep considered as “echo” was presented before the one considered as “pulse”, see following text). In a pair of FM sweeps, the “pulse” was at least 5 dB louder than the “echo”. Pulse- and echo-level were chosen taking into account characteristics of the rate-level functions calculated with single FM-sweeps of increasing levels. Only SPL values larger than the response threshold were considered for defining pulse and echo levels. In neurons showing non-monotonic rate-level functions, the pulse-SPL was set to an SPL value that evoked a local minimum in the rate-level function. For example, in neurons that were tuned to SPL, the pulse-SPL was set to match the upper-threshold of the neuron, that is, the sound level at which the neuron stopped to respond. Similarly, in the neurons with notch rate-level functions, the pulse-SPL was set to a value equal (or close) to the SPL that evoked the notch in the response.

For both tuned and notch neurons, the echo-SPL was fixed between 5 and 20 dB below the pulse-SPL and it was chosen so that it matched a local maximum in the rate-level functions. In neurons with monotonic rate-level functions, pulse- and echo-SPL were arbitrarily fixed to 80 dB SPL and 65 dB SPL, respectively. Those values were taken from a previous study on delay tuning in the auditory cortex of *Carollia* (Hagemann et al. 2010).
In the delay tuning curves, the best delay (BD) was considered to be the echo-delay that evoked the maximum response. “Non-optimal delays” were those for which the response was below 50% of the response observed at the best-delay. “Optimal delays” were those for which the response was above 50% of the maximum response. We defined two different types of delay-tuning curves: excitatory curves and suppression curves (also known as inhibitory curves). In neurons with excitatory delay tuning, there was a range of optimal delays that was surrounded by non-optimal delays. On the other hand, in the suppression curves, there was a range of non-optimal delays that was surrounded by optimal delays. In the excitatory delay tuning curves, the bandwidth of the curve was calculated by interpolation, as 50% of the maximum response observed in the neuron.

All statistical tests were conducted using either Statistica (release 8, Statsoft Inc.) or the statistics toolbox for Matlab. Parametric tests were used whenever the tested parameter distributions fulfilled the basic parametric assumptions, i.e. normality (tested with the Shapiro-Wilk W test for normality), and homogeneity of variance (tested with Levene’s test). Non-parametric tests were used whenever the parametric assumptions were not fulfilled.

**Interactions at the best delay and non-optimal delays**

To look for the presence of side suppression fields in the delay tuning curves we compared the response to the pulse and echo presented individually with the responses to pulse and echo presented at different delays. The strength of suppression (and facilitation) was quantified as an interaction index ($ii$) defined as: $ii = (Rpe - Rp - Re) / (Rpe + Rp + Re)$. In the formula, $Rpe$ represents the response to pulse and echo presented together with a given delay, and $Rp$ and $Re$ are the responses to the pulse and echo presented individually. This $ii$ was proposed by Dear and Suga (1995) and has been successfully used for quantifying suppression and facilitation in the...
inferior colliculus of the mustached bat (Portfors and Wenstrup 1999; Nataraj and Wenstrup 2005). The value of the $ii$ varies between 1 and -1, with 1 indicating the strongest possible facilitation, and -1 indicating the strongest suppression. Following the same criteria used in other studies (i.e. Portfors and Wenstrup 1999; Nataraj and Wenstrup 2005; Sanchez et al. 2008), a response was classified as facilitated if the $ii$ was >0.09, corresponding to an increase in response of 20% above the summed responses to the artificial pulse and echo. A response was considered to be “suppressed” if the $ii$ was <-0.11, which corresponds to at least a 20% decrease relative to the expected response.

**Results**

To characterize basic response properties of homoharmonic delay-tuned neurons, we recorded the response of 131 units in the dorsal high-frequency fields of the auditory cortex of *C. perspillata*. All of these neurons displayed delay tuning in response to pairs of FM sweeps (see below). We will start by describing the temporal response pattern of delay-tuned neurons obtained in response to individual FM-sweeps of increasing SPL.

**Responses to individual FM-sweeps**

In response to single FM-sweeps, cortical neurons displayed one of four arbitrarily defined types of temporal response patterns, and one of three types of rate-level functions. Basic response properties of cortical delay-tuned neurons are summarized in the form of a graphical table in figure 1. Sixty of 131 studied neurons (45.8%) displayed PLS, i.e. longer response latency to loud sounds and shorter latency to faint sounds. *Unit CP42* (Figure 1a) is a typical example PLS neuron. In this unit, response latency increased from 23 to 33 ms as sound pressure level increased from 45 dB SPL (i.e. the unit threshold) to 95 dB SPL (the maximum SPL tested...
In the population of PLS neurons, median responses latencies ranged between 14 and 39 ms for levels between 35 and 105 dB SPL, with a systematic increase in median response latency for increasing SPLs (Figure 2a). At high SPLs (i.e. between 80-90 dB SPL), the response latency of PLS neurons was significantly longer than that of all other types of studied neurons (see Fig 2e-f). In the PLS neurons, the amount of paradoxical latency shift ranged from 2 to 37 ms, with a median of 11.5 ms and an interquartile range (IQR, i.e. the difference between the 75th and 25th percentiles) of 8 ms. The rate-level function of the vast majority of PLS neurons (49/60 PLS neurons) was characterized by a “notch” at intermediate SPLs (Figure 1b). Unit Cp42 gives a typical example for a notch-type of rate-level function (curve represented in black in Figure 1b). The response of this unit reached a maximum at 50 dB SPL firing with an average spike-rate of 1.3 spikes/trial. In response to FM-sweeps of 60 dB SPL (that is, the notch level), the response dropped to 0.46 spikes/trial and it raised up to 1.26 spikes/trial as level increased to 85 dB SPL. Another 11 PLS neurons either were “tuned” to SPL (i.e. 3 units, represented in Figure 1c) or had monotonic rate-level functions (i.e. 8 units, represented in Figure 1d).

Forty cortical neurons (40/131, 30.5%) completely failed to respond to high SPL values (that is, the response was below 10% of the response at the best delay) and did not display PLS. These units were tuned to a narrow range of SPLs and they were defined as “strictly-tuned” to differentiate between them and units that were also SPL-tuned but that did respond to some degree (above 10% of the maximum response) to high SPL values. Unit CP105 (Figure 1e) is a typical example of strictly-tuned response. This unit responded to levels between 55 and 65 dB SPL with latencies between 21 and 23 ms. In the population of strictly-tuned neurons, the median response latency varied between 20 and 26 ms at sound levels from 35 to 95 dB SPL (Figure 2b). The rate-level function of Unit CP105 is marked in black in figure 1g to illustrate the typical rate-level function of a strictly-tuned neuron. The response of this neuron reached a maximum at 60
dB SPL with a firing rate of 1.2 spikes/trial. At levels above 65 dB SPL the response dropped to values between 0.06 and 0 spikes/trial.

In seven out of 131 studied neurons the temporal response pattern was classified as “complex”. These units had a complex temporal response pattern characterized by responses that were broken up into two clusters of spikes separated in the time domain. *Unit Cp51* is a typical example of a complex response pattern (Figure 1i). The response latency of this unit decreased from 18 to 15 ms as level increased from 55 to 95 dB SPL. At levels above 70 dB SPL, the unit’s response was broken up by a temporal gap during which the unit fired almost no spikes. The population of neurons with complex responses had the shortest latency of all studied cortical neurons with median latencies ranging between 14 and 17 ms at levels between 55 and 95 dB SPL (Figure 2c). The difference in latency between complex neurons and other neuronal types was more evident at low sound pressure levels (i.e. between 50-60 dB SPL, Figure 2e). Neurons with a complex response pattern had notch rate-level functions (2 neurons, Figure 1j), were tuned to SPL (3 neurons, including *Unit Cp51* (black curve in Figure 1k)), or had monotonic rate-level functions (2 neurons, Figure 1l).

The remaining 24 neurons (24/131, 18%) did not display PLS, nor were SPL-tuned or had “complex” temporal response patterns. The response of these units was characterized by a systematic decrease in response latency with rising sound pressure levels. These units were defined as “non-specialized” because their response resembles that of neurons in early stages of the auditory processing. *Unit Cp15* (Figure 1m) is a typical example of non-specialized temporal response pattern. In this neuron, response latency decreased from 26 to 20 ms as stimulus level increased from 60 to 85 dB SPL. Overall, the median response latency of the population of neurons with non-specialized responses was slightly shorter at levels below 50 dB SPL, with
values between 19 and 26 ms (Figure 2d). At levels above 50 dB SPL median latency varied
between 23.5 and 29 ms. Sixty-seven percent (16/24) of the units with non-specialized temporal
response patterns displayed monotonic rate-level functions (Figure 1p), while the remaining 33%
(8/24 units) had the notch-type of rate-level functions (Figure 1n). The rate-level function of unit
UnitCP15 is highlighted in figure 1p to exemplify a typical monotonic response. In this unit,
spike-rate increased systematically from 1.41 to 4 spikes/trial as stimulus level increased from 60
dB SPL (the unit’s minimum threshold) to 80 dB SPL.

Altogether, 80% (105 of 131) of delay-tuned neurons studied in the dorsal auditory cortex of C.
perspicillata showed some specialization for the processing of SPL in their rate-level functions,
i.e. 59 neurons had the notch-type of rate-level function while another 46 were tuned to SPL. In
the neurons with notch responses, the notch (i.e. the SPL value that evoked the maximum
response drop) occurred between 60 and 90 dB SPL with a peak at 75 dB SPL (Figure 3a). The
maximum response of notch neurons was found at sound levels between 45 and 105 dB SPL with
peak values at 65 and 95 dB SPL. In the neurons that were tuned to SPL, the upper threshold
(SPL value at which the neurons stopped to respond) varied between 65 and 105 dB SPL with a
peak at 85 dB SPL (Figure 3b). The maximum response of SPL-tuned neurons occurred at sound
levels between 45 and 90 dB SPL with a peak at 75 dB SPL.

Responses to pairs of FM sweeps: interactions measured at the best delay

In response to pairs of FM-sweeps, all 131 neurons displayed delay selectivity that was classified
as either excitatory or inhibitory. Neurons with excitatory delay tuning curves typically
responded well to a certain range of echo delays and this “excitatory delay range” was
surrounded by delays that were less effective in driving the neurons. On the other hand, “purely
inhibitory tuning” (or pure suppression curves) typically consisted on a lack of responses to a
certain range of echo delays and strong responses to the delays longer and shorter than those delays evoking the suppression. From the 131 neurons studied here, only three presented purely inhibitory tuning, therefore in the following, we focus only in those neurons with excitatory responses. For the purpose of clarity, it is worth specifying that in the excitatory tuning curves, a lack of responses does not necessarily indicate response “suppression”. Similarly, an “excitatory” response does not necessarily indicate response “facilitation”. As mentioned in the methods section, if there was suppression or facilitation in response to a given echo-delay was determined by comparing the response observed at that particular pulse-echo delay, with the sum of responses to pulse and echo presented individually.

The typical response of a neuron with excitatory delay tuning is shown in figure 4. The response of Unit CP74 was tuned to SPL for FM-sweeps that covered the frequency range of the third biosonar harmonic (Figure 4a and b). This unit responded strongly to FM-sweeps at levels between 75 and 85 dB SPL, i.e. at 75 dB SPL, the neuron fired with an average spike-rate of 1.36 spikes/trial. In response to FM-sweeps of 90 dB SPL the response dropped to 0.24 spikes/trial. FM-sweeps of 90 and 75 dB SPL were presented together with a variable delay to calculate the neuron’s delay tuning curve (Figure 4c and d). In the delay tuning curve, the maximum response (1.96 spikes/trial) was reached when the 75 dB SPL signal was presented 12 ms after the 90 dB SPL signal (Figure 4d). This delay was defined as the best delay (BD). The response obtained at 12-ms-delay was 123% of the sum of responses to individual FM-sweeps of 75 and 90 dB SPL, yielding an interaction index of 0.1 that indicates a weak “facilitation”. In the delay tuning curve of Unit CP74 (Figure 4d), the facilitated response was surrounded by suppression. The delays that were shorter than the best delay were defined as “front delays” while those that were longer were defined as “back delays”. In the front suppression field, the maximum suppression occurred
at 0-ms-delay with the response dropping to 0 spikes/trial \((ii = -1, \text{the maximum possible})\) suppression). In the back suppression field, maximum suppression started at 22-ms-delay with the response also dropping to 0 spikes/trial.

Among 128 neurons with excitatory delay tuning, the best delay varied between 2 and 25 ms (Figure 5a). Thirty neurons had best delays equal to or shorter than 6 ms, while another 98 neurons were tuned to longer delays. The peak value in the best delay histogram was at 12 ms, which is consistent with the fact that the majority of delay-tuned neurons in this study were from the middle portion of Carollia’s chronotopic map. There were no differences regarding best delay between neurons with notch-, tuned- and monotonic rate-level functions (one-way ANOVA: \(p=0.67\), Shapiro-Wilk W test for normality: \(0.05<p<0.15\), Levene’s test for homogeneity of variance: \(p=0.56\)). From here on, neurons tuned to echo delays shorter than or equal to 6 ms will be referred to as short-delay tuned neurons, whereas neurons tuned to delays longer than 6 ms will be referred to as long-delay tuned neurons. The distinction between short- and long-delay tuned neurons made here is similar to that used in previous studies on HT bats (see: Portfors and Wenstrup 1999; Nataraj and Wenstrup 2005). Although this distinction is arbitrary from a functional point of view, it is useful for the purpose of a straightforward comparison with data from previous studies.

In the neurons tuned to short delays (i.e. those with best delays < 6 ms), at the best delay, the value of the interaction index that measures the strength of facilitation varied between 0.81 and -0.38 (median = 0.20, IQR= 0.29) (Figure 5b). In the majority of neurons tuned to short delays (24/30, 80%), the \(ii\) calculated at the best delay was larger than 0.09 indicating that the response observed at the best delay was at least 20% larger than the response expected from the sum of responses to the pulse and echo presented individually, which indicates facilitation. The response
of Unit Cp141 is shown in figure 5c to illustrate the maximum degree of facilitation observed in neurons tuned to short delays. This unit responded with 0.06 and 0 spikes/trial when presented with artificial FM3-sweeps of 65 and 85 dB SPL, respectively. When the echo (65 dB SPL signal) was presented after the pulse (85 dB SPL signal) with a delay of 4 ms, the response of Unit CP141 raised up to 0.56 spikes/trial. This value represents the 1080% of the sum of responses to the individual pulse and echo.

In a minority of neurons tuned to short delays (6/30, 20%), the response was not facilitated at the best delay, that is, in these units the ii was < 0.09. Among the neurons tuned to short delays, the lowest ii was observed in Unit CP105 (Figure 5d) with an ii of -0.34. In response to FM2-sweeps of 70 dB SPL, Unit CP105 displayed almost no response (i.e. it fired an average of 0.03 spikes/trial). However, it responded strongly to individual FM2-sweeps of 60 dB SPL, with an average spike-rate of 1.2 spikes/trial. In the delay tuning curve, the maximum response (0.6 spikes/trial) occurred when the FM2-sweep of 60 dB SPL was presented 3 ms after the 70 dB SPL signal. The response observed at 3-ms-delay represents 50% of the response to the 60 dB SPL signal alone, indicating that the response was suppressed when pulse and echo were presented together.

In another 98 neurons the maximum facilitation occurred at delays above 6 ms (long delay units). The amount of maximum facilitation observed in neurons tuned to long delays was not different from that observed in neurons tuned to short delays (Mann-Whitney U Test, p =0.61, Shapiro-Wilk W test for normality: 0.02<p<0.30, Levene’s test for homogeneity of variance: p= 0.98). In the neurons tuned to long delays, the ii calculated at the best delay varied between 0.75 and -0.35 (median = 0.20, IQR= 0.31, Figure 5f). Most of the neurons tuned to long delays (i.e. 68/98, 69%) were facilitated at the best delay. The response of Unit CP107 (Figure 5f) is an example of
This unit did not respond to loud FM2-sweeps of 80 dB SPL (the pulse), and responded only with 0.3 spikes/trial when presented with FM2-sweeps of 65 dB SPL (the echo). In the delay tuning curve, the response reached a maximum of 1.43 spikes/trial when the 65 dB SPL signal was presented 12 ms after the 80 dB SPL signal. The response observed at 12-ms-delay represents 476% of the sum of responses to the pulse and echo alone, yielding an $ii$ of 0.65.

Thirty out of 98 neurons tuned to long delays (31%) had responses that were not facilitated at the best delay. This type of responses is illustrated in figure 5g. Unit CP97 (Figure 5g) responded strongly to FM2-sweeps of 65 and 85 dB SPL with spike-rates of 1.18 and 1.06 spikes/trial, respectively. In the delay tuning curve, the maximum response was reached at 12-ms-delay with the neuron firing 1.33 spikes/trial. The response at the best delay was slightly larger than the responses to the individual 65 and 85 dB SPL signals, but it represented 47% of their sum. Thus, the $ii$ calculated at the best delay was -0.25, indicating either suppression or that the response was saturated at the best delay.

**Interactions at non-optimal delays**

The interaction index also was used to characterize the response to non-optimal echo-delays. Non-optimal delays were classified into front or back-delays depending on their relation to the best delay. We looked for footprints of inhibition at non-optimal delays by calculating the delays that caused the maximum suppression in the front and back delay fields.

In the front delay field, maximum suppression was observed at 0-ms delay in 26 out of 30 short-delay neurons (87%), and 42 out of 98 long-delay neurons (43%) (Figure 6a). The strength of front suppression did not differ between short- and long-delay-tuned neurons (Figure 6b, Mann-Whitney U Test, $p=0.54$, Shapiro-Wilk W test for normality: $0.0001<p<0.04$, Levene’s test for homogeneity of variance: $p=0.25$). For example, in the front suppression field, the median $ii$
calculated at the point of maximum suppression had values of -0.70 (IQR=0.40) and -0.73 (IQR=0.31) in short- and long-delay-tuned neurons, respectively. Among both short- and long-delay-tuned neurons about 20% of units had an $ii$ of -1 that indicates the maximum possible suppression.

There was a significant difference between short and long-delay-tuned neurons regarding the delay of maximum suppression in the back delay field (Figure 6c, Mann-Whitney U Test, $p < 10^{-5}$, Shapiro-Wilk W test for normality: $0.0002 < p < 0.17$, Levene’s test for homogeneity of variance: $p = 0.001$). Short-delay neurons had shorter back-suppression delays than long-delay neurons. For example, in short-delay neurons the median back-suppression delay was 16 ms (IQR=10 ms) while in long-delay neurons it was 22 ms (IQR=4 ms). There was also a significant difference between short and long-delay-tuned neurons regarding the strength of suppression in the back delay field (Figure 6d, Mann-Whitney U Test, $p = 0.001$, Shapiro-Wilk W test for normality: $0.0004 < p < 0.01$, Levene’s test for homogeneity of variance: $p = 0.15$). Suppression in the back delay field of short-delay-tuned neurons was stronger than that in the back delay field of long-delay-tuned neurons, with median $ii$ values of -0.8 and -0.58, for short- and long-delay neurons, respectively. Thirteen out of 30 (43%) short-delay neurons had $ii$ values of -1 at the back-suppression delay while only 12 out of 98 long-delay units (12%) reached the maximum possible suppression in the back delay field.

**Relation between best delay and magnitude of PLS**

In 60 neurons that displayed both PLS and excitatory delay tuning we calculated the correlation between the amount of PLS and the best delay of the neurons. An example neuron displaying both PLS and excitatory tuning is shown in Figure 7a-d. *Unit CP109* displayed a PLS of 9 ms in response to FM2-sweeps of increasing SPL (Figure 7a). For example, in response to 65 dB SPL
sounds, the neuron fired with a latency of 39 ms. Spike-rate dropped below the threshold criteria in response to levels between 70 and 85 dB SPL and raised back in response to stimuli of 90, 95 and 100 dB SPL. At levels between 90 and 100 dB SPL, the response latency remained essentially constant between 48 and 49 ms (i.e. ~ 9 ms longer than the latency at lower SPLs).

In addition to showing PLS (Figure 7a), Unit CP109 displayed a clear notch-type of rate-level function with the notch at 75 dB SPL and the best level at 65 dB SPL (Figure 7b). The delay tuning curve of the unit was calculated by varying the temporal position of a FM2-sweep of 65 dB SPL (the echo) relative to a 75 dB SPL sweep (the pulse, Figure 7c and d). In this situation Unit CP109 displayed a clear excitatory delay tuning curve with a best facilitatory delay at 12 ms. Therefore the best delay of Unit CP109 slightly deviates from the best delay that could be expected from the PLS displayed by this neuron (i.e. BD=12 ms vs. PLS= 9 ms). Overall, in the entire sample of 60 PLS neurons, there was a moderate correlation between the BD and PLS amount, that is, the linear correlation coefficient had a value of 0.49 (Figure 7e). It is worth mentioning, that the strength of the correlation between PLS and BD reported here should be looked at carefully, because in most neurons, the sound amplitudes used for calculating the delay tuning curves were not exactly the same as the amplitudes evoking the PLS (see the example neuron in Figure 8).

Effects of BMI application on the response of delay-tuned neurons

In 24 cortical neurons we studied the effect of BMI iontophoresis on the response to sounds of increasing SPL and in the delay tuning curves. In all 24 neurons, blocking GABA\textsubscript{A} transmission did not change the temporal response pattern of the neurons nor did it change their type of rate-level function.
To exemplify this behavior, the response of a PLS-neuron studied using FM2-sweeps is shown in Figure 8a-d. In the pre-drug condition, the unit’s response was characterized by a “progressive” paradoxical latency shift (Figure 8a), i.e. the latency of this unit increased progressively from 20 to 29 ms as stimulus level increased from 75-100 dB SPL (at lower levels latency decreased from 29 to 20 ms as stimulus level increased from 45 to 75 dB SPL). The unit also was tuned to SPL firing strongly at levels from 70 to 85 dB SPL (Figure 8d). Local iontophoresis of BMI did not remove the progressive PLS of this neuron (Figure 8b), nor did it remove the amplitude tuning (Figure 8d) but it did increase the overall spike-rate of the neuron. For example, at the best level (that is, 80 dB SPL) the neuron fired a mean of 0.6 spikes/trial in the pre-drug condition but during BMI application the mean spike-rate (also at 80 dB SPL) increased to 1.6 spike/trial. Twenty minutes after stopping BMI iontophoresis, the neuron still showed a progressive PLS (Figure 8c) and its spike output was quite similar to that in the pre-drug condition (Figure 8d).

An effect of BMI application on the response strength was also observed in the delay tuning curves. The latter is illustrated in the delay tuning curves of units $Cp_p29$ and $Cp_p28$ (Figure 9 a and b, respectively). These two units clearly were tuned to echo delay before, during and after BMI application, and in both neurons BMI iontophoresis produced a noticeable increase in the response strength. For example, in unit $Cp_p29$ (Fig. 9a), the maximum spike-rate fired in the pre-drug condition was 0.6 spikes/trial, but the unit fired with 2.0 spikes/trial after 10 min of continuous BMI iontophoresis. The spike-rate decreased back 0.5 spikes/trial, 32 min after stopping BMI release. In the same unit (unit $Cp_p29$, Fig. 9a), neither the best delay nor the 50% bandwidth of the delay tuning curve shifted as a consequence of BMI iontophoresis. The best delay remained stable at 2 ms as did the Bw50.
As observed in unit $Cp_p29$ (Figure 9a), in unit $Cp_p28$ (Figure 9b) BMI iontophoresis also caused a large increase in response strength, i.e. from 0.7 (pre-drug) to 2.4 spikes/trial (BMI).

However, unlike in unit $Cp_p29$ (Figure 9a), in unit $Cp_p28$ (Figure 9b), BMI application did shift the neuronal best delay and Bw50 of the delay tuning curves. For example, the neuronal best delay decreased from 8 ms (pre-drug) to 4 ms after 10 min of BMI iontophoresis, and then shifted back to 10 ms, 35 min after stopping BMI release. Also, the Bw50 increased from 6 ms (pre-drug) to 10 ms when BMI was applied, and decreased to 8 ms after stopping BMI release.

Overall, in our sample of 24 neurons, none of the neurons lost its delay tuning as a consequence of BMI iontophoresis. We observed strong evidence for a BMI-induced increase in neuronal spike-rate. For example, BMI iontophoresis increased neuronal response strength at the best delay in 22 out of 24 studied neurons (Figure 10a). In nine neurons (9/24, 38%) there was a large increase in spike-rate, that is, in these neurons, after BMI iontophoresis response strength was at least twice as large as the response observed in the pre-drug condition (Figure 10a, neurons marked with solid black lines). In another 13 neurons (13/24, 54%), BMI iontophoresis induced only moderate increments in spike-rate. For example, in this neurons, response in the BMI condition increased by less than two times when compared with the response in the pre-drug condition (see Figure 10a, neurons marked with solid grey lines). Only in one neuron the spike rate was not affected by BMI application and in another neuron BMI release caused a decrease in spike-rate at the best delay from 0.46 to 0.3 spikes/trial (Figure 10a, neurons marked with dashed lines).

BMI-induced increments in spike-rate were observed not only in individual neurons but also in the averaged data obtained from all 24 studied neurons (Figure 10b). For example, the average response at the best delay, at the front and back-suppression delays, and in response to the
individual pulse and echo, was larger for the BMI condition than for the pre-drug condition; although in response to the echo, differences between average responses were not statistically significant (Wilcoxon matched-pairs test, p=0.26). Our data also suggests that GABA_A receptors seem to play a role in controlling neuronal best delay and response bandwidth. For example, BMI iontophoresis induced shifts in BD in 20 out of the 24 studied units (Figure 10c). In 11 units there was only a slight BD-shift (i.e. the BD shifted by +/- 2 ms). However, in the remaining nine units (including the neuron in Figure 9b) the shift was more pronounced reaching values up to +/- 6 ms. There was no correlation between the neuronal best delay and the magnitude of BD shift. In eight of the 24 studied neurons (33%), BMI iontophoresis increased response bandwidth by more than 2 ms (the temporal resolution of the delay-tuning curves described in this study).

Discussion

Homoharmonic (HM) delay tuning is poorly understood in comparison to heteroharmonic (HT) delay tuning. In HT bats, inhibition is a building block for the central implementation of delay tuning (Wenstrup et al. 2012). The working hypothesis in this paper was that inhibition also could play a role in the implementation of HM delay tuning. Therefore we looked for footprints of neural inhibition in the response of cortical delay-tuned neurons of the HM species _C. perspicillata_. Our main findings are: (I) that in the response of cortical delay-tuned neurons of homoharmonic bats there are abundant footprints of level-dependent inhibition; (II) that these footprints of inhibition are not the result of cortical GABAergic influences and (III) that the cortical GABA_A system provides a substrate for modulating basic properties of delay tuning _in situ_.

Origin of inhibitory footprints
In the response of HM neurons, evidence for level-dependent inhibition appears in the form of a PLS, non-monotonic rate-level functions or a combination these two features (see figure 1). That PLS and non-monotonic responses indicate an underlying inhibition is supported by the response “suppression” observed in the delay tuning curves (see example neurons in figures 4 and 7). Presenting a weak (echolike) sound alone triggers a strong spike-response. This response is suppressed if the echolike sound is preceded by a loud (pulselike) sound and the delay between the two sounds deviates from the neuron’s optimal delay.

Inhibitory features are overly represented in the cortex but they are not created in situ, or at least not as a result of GABA_A-mediated inhibition. Characterizing inhibitory footprints by studying cortical responses, instead of their site of origin, still could provide valuable information about basic properties of inputs that participate in the implementation of delay tuning in stations downstream. Of course, the latter is assuming that inhibitory footprints are not further modified between their site of origin and the cortex.

HM neurons in the cortex of *Carollia* display one of four types of temporal response patterns: PLS, strictly-tuned, complex and non-specialized responses (see figure 1). At least the first three suggest that delay tuned neurons of HM bats receive a combination of low threshold-excitation and high-threshold inhibition. The idea is that responses to weak sounds are mainly excitatory because at low-SPLs, only a low-threshold excitation is activated. At higher SPLs, an inhibitory input is activated and suppresses a portion or all of the excitatory response. It has been shown that PLS is created when, in response to loud sounds, a high-threshold inhibition suppresses only the early portions of a low-threshold excitation (Galazyuk et al. 2005; Galazyuk and Feng 2001). In principle, a longer lasting inhibition that at high-SPLs suppresses the entire excitatory response could produce a “strictly-tuned” response pattern. Also, a complex response could originate when
the inhibition is sandwiched within the excitatory response. The rate-level functions that
ccompany the different response patterns could be classified as tuned, notch or saturated,
depending on how much of the excitatory response is able to override the inhibitory suppression
at different sound levels.

*Mechanisms underlying HM delay tuning*

Several studies have emphasized the idea of a causal relation between PLS and homoharmonic
delay tuning. It is said that in PLS neurons, excitatory responses to the loud call and faint echo
coincide in time at the optimal delay (because of the PLS) thus causing response facilitation
(Sullivan 1982b; Berkowitz and Suga 1989; Galazyuk et al. 2005; Feng 2011). The latter is
supported by the finding that the amount of PLS of a neuron is correlated with its best delay,
although the strength of this correlation varies between studies, i.e. R=0.71 (*M. lucifugus*,
Berkowitz and Suga 1989) vs. R=0.49 (*C. perspicillata*, this study). At non-optimal delays, there
is no temporal alignment of excitatory responses to the call and echo and therefore the resulting
response would be below the response at the best delay.

The data presented in this article suggests that, for several reasons, the implementation of HM
delay tuning could be more complex than previously thought. (1) At non-optimal delays, the
excitatory response to the echo is largely suppressed. This response suppression likely reflects an
underlying inhibition and therefore inhibition should be considered in any model that tries to
explain HM tuning. (2) Only 45% of the neurons tuned to HM pulse-echo pairs display PLS. (3)
Even HM neurons that do display PLS are clearly tuned to echo-delay when the pulse level is
fixed at a value that does not evoke a latency shift (see the example neuron in Figure 7). The last
two pieces of evidence described in the preceding text (2 and 3) suggest that PLS is not
necessarily required for the implementation of HM delay-tuning or at least, that PLS is not the only mechanism by which HM tuning can be implemented.

One interpretation to the extensive representation of inhibitory footprints in the response of HM delay tuned neurons could be that inhibition, more specifically pulse-evoked inhibition, is instrumental for HM delay-tuning implementation. The latter would explain why inhibitory footprints are found in the vast majority of HM delay-tuned neurons (~80% of the neurons in this study, see figures 1 and 6). If it is assumed that inhibition is indeed important for HM delay-tuning implementation, then one could conclude that HM and HT delay tuning are not so different after all. This thesis is supported by the fact that similar suppression and facilitatory interactions occur in HM and HT neurons, that is, in both types of neurons, a facilitatory response at the best delay is surrounded by strong side-suppression-fields (see example neurons in figures 4 and 7).

Previous studies have discussed that in HT neurons, inhibition evoked by the fundamental harmonic of the pulse could lengthen the latency of the excitatory response to the pulse so that at the best delay it coincides in time with a short-latency excitation evoked by the echo (for studies on HT tuning see: Nataraj and Wenstrup 2005; Gans et al. 2009; Sanchez et al. 2008; Portfors and Wenstrup 1999; Wenstrup et al. 2012). Pulse-evoked inhibition could play a similar role in the implementation of HM tuning. Also, as in HT bats, in HM bats pulse-evoked inhibition could be instrumental for suppressing responses to non-optimal delays.

In spite of the large similarities between HM and HT tuning regarding inhibition and suppression, it seems that there are also some small differences between these two types of neurons. For example, in HM neurons, we did not find differences in the strength of front suppression between neurons tuned to short and long delays (see figure 6). However, in HT bats, front suppression in
neurons tuned to long delays is stronger than in neurons tuned to short delays (Portfors and Wenstrup 1999; Nataraj and Wenstrup 2005).

Roles of cortical GABAergic inhibition

The data presented in this paper suggests that cortical GABAergic inhibition is neither responsible for the implementation of inhibitory footprints, nor is it for the implementation of delay tuning in cortical neurons (see figures 8 and 9). It seems that cortical neurons inherit these properties from auditory stations downstream. However, because we blocked GABAergic transmission in a relatively small number of delay-tuned neurons (i.e. N=24), the possibility of a reimplementation of delay tuning in the cortex cannot be completely ruled out. Recent studies have shown that cortical GABAergic circuits play a key role in shaping and even re-implementing certain types of selectivity that are not preset in the cochlea, such as binaural selectivity or the selectivity to FM-sweep direction and rate (Razak and Fuzessery 2010; Razak and Fuzessery 2009).

Overall, iontophoresis of BMI in the cortex has three main effects: (I) in most neurons it increases the strength of the response (that is, the spike-rate); (II) it could shift the best delay; and (III) in a few neurons it could also shift the response bandwidth (see example neurons in figure 9). Regardless of the mechanisms by which these changes occur (discussed below), the data presented here supports the idea that the cortex possesses a substrate for an in situ modulation of neuronal tuning (Ma and Suga 2001; Sakai and Suga 2001; Suga et al. 2002; Xiao and Suga 2004).

Increments in spike output after BMI application could be explained by an increase in neuronal excitability. BMI could increase neuronal excitability either by blocking ionotropic GABA_A
receptor channels (which reduces chloride conductance), or by blocking calcium-activated potassium channels (Khawaled et al. 1999), or by a combination of those two. Controlled changes in neuronal excitability are known to occur during different attention states (Moran and Desimone 1985; Spitzer et al. 1988) and they also could be important for modulating the response of other cortical neurons and neurons in subcortical nuclei (Ma and Suga 2001; Sakai and Suga 2001; Xiao and Suga 2004; Tang et al. 2007).

On the other hand, shifts in neuronal best delay and bandwidth could result from modulating the cortical GABAergic system, although possible contributions of calcium-activated potassium channels cannot be ruled out. Previous studies (i.e. Xiao and Suga 2004) have suggested that cortical GABA_A shifts response bandwidth and best delay of cortical neurons and that this changes are important for reorganizing the cortical target-distance map during associative learning (Tang et al. 2007; Xiao and Suga 2004).

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Figure 1. Classification of response types in AC neurons of *C. perspicillata*. The figure is organized in the form of a graphical table. **a-d)** Neurons that displayed paradoxical latency shift (PLS). **e-h)** Neurons that were “strictly” tuned to SPL (i.e. they completely failed to respond to high SPLs). **i-l)** Neurons that displayed a “complex” temporal response pattern. **m-p)** Non-specialized responders. The 1st figure column (**a, e, i, m**) shows dot raster plots of four example neurons displaying different types of temporal responses. Dots represent the timing of spikes relative to the stimuli onset. Vertical lines in the upper left corner of each raster-plot represent the temporal position and duration of acoustic stimuli. In the 2nd, 3rd and 4th figure columns, normalized rate-level functions are given. The 2nd figure column (**b, f, j, n**) shows the notch-type of rate-level function; the 3rd column (**c, g, k, o**) corresponds to SPL-tuned responses; and the 4th column (**d, h, l, p**) corresponds to monotonic responders. The “response” of each neuron to different SPLs was normalized relative to its maximum response. In notch neurons, SPL values were normalized relative to the neuron’s “notch” level (i.e. SPL that evoked a substantial drop in spike activity). In SPL-tuned neurons and monotonic neurons, SPL was normalized relative to the neuron’s best level. Rate-level functions represented in black correspond to the example neurons shown in the 1st figure column. For the highlighted rate-level functions, real (instead of normalized) information about certain points of interest in the curve is given in the format of Cartesian coordinates, that is, [amplitude (dB SPL); response (spikes/trial)].

Figure 2. Population latencies of four different types of neurons. **a):** PLS neurons, **b):** neurons that were strictly-tuned to SPL, **c):** neurons with a complex response, **d):** neurons with non-specialized responses. For each type of neuron, “population latencies” are shown in the form of...
boxplots calculated from the response of all the neurons that displayed a given type of response. On each box, the central mark is the median, lower and upper edges are the 25th and 75th percentiles, respectively; and the whiskers extend to the most extreme data points not considered outliers. e-f) statistical comparison of population latencies of the four classes of neurons at sound pressure levels between 50-60 dB SPL (e) and 80-90 dB SPL (f). Population latencies were compared using a Kurskal-Wallis analysis of variance and median test and a multiple comparison of mean ranks for all tested groups. Solid lines indicate significant differences and dashed lines indicate non-significant differences. Asterisk symbols (*) indicate the levels of significance, i.e. **: \( p < 0.01 \), and *: \( 0.01 < p < 0.05 \).

**Figure 3.** Properties of notch and SPL-tuned neurons. a) Histogram of notch-level and best-level in “notch” neurons. b) Histograms of upper threshold level and best level in SPL-tuned neurons. Histograms are shown as 1D and colour-coded 2D histograms.

**Figure 4.** The response of a typical delay-tuned neuron. a) Dot raster plot obtained in response to FM3-sweeps of variable SPL. Dots represent spike times relative to stimulus onset. The timing of the stimulus (onset and duration) is represented by the vertical gray line in the upper left corner of the raster plot. b) The rate-level function of the neuron. Note that it was tuned to SPL. The response to sweeps of 75 and 90 dB SPL is indicated in the form [amplitude (dB SPL); response (spikes/trial)]. FM-sweeps of 90 and 75 dB SPL were used to mimic pulse-echo pairs for calculating delay tuning in this neuron. c) Dot raster plot obtained in response to combinations of pulse (90 dB SPL sweep) and echo (75 dB SPL sweep) presented with variable delays. The timing of pulse and echo is represented with black and gray rectangles, respectively. d) Delay tuning curve of the neuron. The delays that evoked the strongest front-suppression, maximum facilitation and strongest back-suppression are indicated. Delay and response values at those
delays are given in the form [delay (ms); response (spikes/trial)]. The interaction index ($ii$) calculated at the front suppression delay, delay of maximum facilitation and back suppression delay are also given.

**Figure 5.** Response properties at the best delay. 

a) Histogram of best delays (BD). White bars correspond to neurons tuned to short-delays (i.e. best delay < 6ms or = 6ms) and black bars to neurons tuned to long delays (i.e. best delay > 6ms). 

b) Histogram of interaction index ($ii$) measured at the best delay of short-delay-tuned neurons. Neurons that fall within the gray shaded area displayed no facilitation or response suppression at the best delay (the $ii$ was between -0.11 and 0.09). Note that the majority of neurons fall to the right of the shaded area, indicating that most of them were facilitated.

c) The response of an example neuron tuned to short delays showing strong response facilitation. For this neuron the response to the individual pulse (black dot) and echo (gray dot) is provided in the form [level of the pulse or echo (dB SPL); response (spikes/trial)]. The response at the best delay is also provided in the form [echo delay (ms); response (spikes/trial)].

d) The response of an example neuron tuned to short delays that showed response suppression at the best delay.

e) Histogram of $ii$ measured at the BD in neurons tuned to long delays.

f) Example neuron tuned to long delays that displayed strong response facilitation.

g) Example neuron tuned to long delays that displayed response suppression at the best delay.

**Figure 6.** Properties of front and back response suppression. 

a) Histograms of front suppression delays in neurons tuned to short delays (white bars) and in neurons tuned long delays (black bars). 

b) Histogram of interaction index ($ii$) values measured at the front suppression delays in short- and long-delay-tuned neurons.

c) Histograms of back suppression delays in short- and long-delay-tuned neurons.

d) Histogram of interaction index values measured at the back suppression delays in short- and long-delay-tuned neurons.
suppression delays in short- and long-delay-tuned neurons. For each histogram, median values and interquartile ranges are given.

Figure 7. Relation between the amount of paradoxical latency shift (PLS) and the best delay (BD). a-d) Responses to single and pairs of FM-sweeps (a-b, and c-d, respectively) in a typical PLS neuron. Conventions are similar to those in Figure 4. Note that this neuron displayed a PLS of ~9 ms (see a) and that it had a notch type of rate-level (see b). Also note that when the pulse was fixed at 75 dB SPL (i.e. the notch value) and the echo was fixed to 65 dB SPL (the best SPL) the neuron displayed a clear delay tuning with a best delay at 12 ms (c-d). e) Correlation between PLS amount and best delay in 60 PLS neurons. The dashed gray line represents the best possible fit and the black line represents the actual fitting line calculated with a linear regression model. The equation of the best fitting line and the Pearson’s linear correlation coefficient (R) are given.

Figure 8. Effects of local application of bicuculline methiodide (BMI) on the response of a PLS neuron. a-c) Dot raster-plots in response to single FM-sweeps of variable SPL before (a), during (b), and after stopping the release of BMI (c). Conventions are similar to those in Figure 4. The response plotted in b was measured after 12 min of continuous BMI release (+20 nA ejection current). The response represented in c was measured 20 min after stopping BMI release. d) Shows the rate-level functions obtained in the pre-drug, BMI and recovery conditions. The response measured in these three situations at 80 dB SPL is given in the form [amplitude (dB SPL), response (spikes/trial)].

Figure 9. Effects of BMI in the delay tuning curves of two neurons (a and b, respectively). a) The response of a neuron in which BMI iontophoresis increased response strength but did not affect the neuronal best delay or the 50% bandwidth (Bw50) of the delay tuning curves. Responses at the best delay are given in the form [best delay (ms); response (spikes/trial)]. For
this neuron, the response in the BMI condition was measured after 10 min of continuous BMI release (+10 nA ejection current). The recovery was measured 32 min after stopping BMI release.

b) The response of a second neuron in which response strength also increased after BMI application. In this neuron BMI iontophoresis did affect the neuronal best delay and Bw50. Effects of BMI were measured after 10 min of continuous BMI application (+10 nA ejection current). The response recovered 35 min after stopping BMI release.

Figure 10. Quantitative effects of BMI iontophoresis in 24 neurons. a) Change in response strength at the best delay (BD). Note that BMI evoked an increase in response strength in all but two neurons (marked with dashed lines). Black lines indicate neurons in which spike-rate in the BMI condition was at least two times larger than the spike-rate observed in the pre-drug condition. Grey lines indicate neurons with a moderate change in spike-rate, that is, in these neurons spike-rate increased after BMI iontophoresis but the observed increase was below 200% of the spike-rate in the pre-drug condition. b) Average spike-rates obtained at the front suppression delay, the best delay, the back suppression delay and in response to individual pulse and echo in 24 neurons before (white bars) and during BMI application (black bars). Error-bars represent standard deviations. Significant differences as taken from a Wilkoxon matched pairs test are given (**: p<0.01, and *: 0.01 < p < 0.05). c) Histogram of BD change between pre-drug and BMI conditions in the 24 studied neurons.
a. Distribution of brainstem delay (
BD) measurements for
neurons with short and long BDs.

b. Neurons with short BDs:
Distribution of inhibition index (ii)
values and median ii = 0.20 with
IQR = 0.29.

c. Unit CP141:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [65 dB, 0.06]: ii = 0.57
- [85 dB, 0.0]: ii = 0.81

Unit CP105:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [60 dB, 1.2]: ii = -0.34
- [70 dB, 0.03]: ii = 0.65

d. Unit CP141:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [80 dB, 1.06]: ii = 0.81
- [85 dB, 0]: ii = 0.3

Unit CP97:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [65 dB, 0.3]: ii = 0.65

Neurons with long BDs:
Distribution of inhibition index (ii)
values and median ii = 0.20 with
IQR = 0.31.

e. Unit CP107:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [12, 1.4]: ii = 0.65

Unit CP97:
Mean response [spikes/trial] for
delay of low SPL signal [ms].

- [12, 1.3]: ii = -0.25

The graphs show the distribution of brainstem delay (BD) measurements and the mean response of neurons for different SPL signals and delays, with inhibition index (ii) values and medians with IQR.
Neurons with short BD
median = 0 ms
IQR = 0 ms

Neurons with long BD
median = 2 ms
IQR = 4 ms

Neurons with short BD
median = -0.70
IQR = 0.40

Neurons with long BD
median = -0.73
IQR = 0.31

Neurons with short BD
median = -0.80
IQR = 0.44

Neurons with long BD
median = -0.58
IQR = 0.36

delay of minimum ii in
front suppression field [ms]

delay of minimum ii in
back suppression field [ms]

minimum ii
(front suppression field)

minimum ii
(back suppression field)
**Unit CP109**

- **a**
  - Time [ms] vs. amplitude [dB SPL]
  - PLS=9 ms

- **b**
  - Echo delay [ms] vs. Mean response [spikes/trial]
  - Pulse [65, 0.8]

- **c**
  - Pulse vs. Echo delay [ms]

- **d**
  - Mean response [spikes/trial] vs. Echo delay [ms]
  - Front Supp. [2, 0.06]
  - Max. Facilitation [12, 1.17]

- **e**
  - Best delay [ms] vs. PLS amount [ms]
  - n=60
  - y=0.5x + 6
  - R= 0.49

**Equations:**

- PL5=9 ms
- Front Supp. [2, 0.06] $\beta=-0.86$
- Max. Facilitation [12, 1.17] $\beta=0.19$
- Back Supp. [22, 0] $\beta=-1$

**Regression Line:**

- $y=0.5x + 6$
  - $R=0.49$
Unit Cp_p10

(a) pre-drug

(b) BMI

(c) recovery

(d) Mean response [spikes/trial]

Best Level
pre=[80, 0.6]
BMI=[80, 1.6]
Rec=[80, 0.6]

Mean response [spikes/trial]

amplitude [dB SPL]

Mean response [spikes/trial]

amplitude [dB SPL]