Neuroscience Letters 531 (2012) 69–73

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

# Forskolin induced increase in spontaneous activity of auditory brainstem neurons is comparable to acoustic stimulus evoked responses

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## HIGHLIGHTS

- Forskolin increases intracellular cyclic AMP levels.
- Forskolin also increases the spontaneous activity of the auditory brainstem neurons.
- Increased spontaneous activity was comparable to sound evoked neural response.
- The results are viewed in context of hyperexcitability as a correlate of tinnitus.

## ARTICLE INFO

*Article history:*
Received 29 April 2012
Received in revised form 5 October 2012
Accepted 26 October 2012

*Keywords:*
Hyper-excitability
Hyperacusis
Deafness
Tinnitus
Cyclic AMP
Superior olive complex

## ABSTRACT

Contemporary proposals for the pathophysiology of tinnitus due to cochlear damage underscore increased spontaneous activity of auditory brainstem neurons. One of the several consequences of the cochlear injury is the activation of the ERK pathway, suppression of phosphodiestase E activity, and putatively setting a long-term increase in intracellular levels of cyclic AMP at central auditory neurons. Local application of forskolin also increases intracellular cyclic AMP and spontaneous neural activity. We measured the effects of locally applied forskolin on spontaneous firing rate of isolated neurons in the peri-olivary region of the superior olive complex in anesthetized adult Long Evan rats. Forskolin induced increase in spontaneous neural activity was comparable to supra-threshold tone evoke neural responses. These results are viewed in context of hyperexcitability as a correlate of tinnitus.



## 1. Introduction

Discovery of increased spontaneous activity in central auditory neurons and development of animal models was a major breakthrough that had driven contemporary tinnitus research [3,11,12,19,27]. These studies had identified key central areas of hyperexcitability, suggested critical molecular changes triggering the hyperexcitable state, and delineated the characteristics of abnormal firing patterns. A number of theories provided the rationale for the hyper-excitable state. Impaired balance between excitation and inhibition is the most popular mechanism. Paucity of inhibitory synapses, post-synaptic glycine, and GABA receptors in the cochlear nucleus, superior olive complex, and inferior colliculus were identified in rodent models of tinnitus [2,5,14,26]. Discovery of increased glutamate release, vesicular glutamate transport, and redistribution of glutamate AMPA receptors supported increased neural excitability in rodent models of cochlear injury [16,24]. The changes affecting the intrinsic membrane properties and neural excitability, such as expression profile of ion channels [13,25] or synapse related proteins such as calbindin, parvalbumin, ERK, and SAPK were described in the rodent model [9,10,23]. Nevertheless, the common consensus was that increased spontaneous activity of the central auditory neurons is the correlate of tinnitus.

The fundamental question is whether the rate of increased spontaneous activity is comparable to the auditory tone evoked neural responses that normally trigger the percept of sound. In order to address this question we first measured auditory tone evoked responses of isolated auditory brainstem neurons putatively localized in the peri-olivary nuclei of the superior olive complex. Then we locally applied forskolin, a compound that increases intracellular levels of cyclic AMP and increases the spontaneous neural activity [17,18,20]. Finally, we compared whether the spontaneous firing rate after increasing intracellular cyclic AMP is comparable to the auditory stimulus evoked neural discharge of the same neuron.

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http://dx.doi.org/10.1016/j.neulet.2012.10.053

## 2. Materials and methods

The study was approved by the Animal Investigation Committee at the Wayne State University. Adult Long Evans rats (age: 3–6 months; weight: 499–600 g) were used for the experiments. Detail of animal preparation was outlined previously [20,21].

### 2.1. Stimulus generation and delivery

Binaural stimuli were digitally generated and delivered using the program A/Dvance (McKeller Designs) on a Macintosh Quadra 950 computer with National Instruments boards (NB-MIO-16; DMA-2800). Outputs of the digital to analog converters were bandpass filtered, amplified by an Amcron (Crown) amplifier, and attenuated under computer-control (Medical Associates MA919 attenuators). Stimuli were transduced by impedance-matched headphones (Beyer-Dynamic 600 ohm, B4-132.01, frequency range 0.05–35 kHz). Output of the stimulus system was calibrated off-line using a coupler with a 0.3 ml air space. Signals were measured with a 0.25-in. condenser microphone (Bruel and Kjaer) and a calibrated microphone preamplifier (Bruel and Kjaer 2804). Calibration tables were used to determine intensities in dB SPL for pure-tone stimuli ranging from 0.05 to 32 kHz. Auditory brainstem responses following monaural ipsilateral and contralateral clicks were recorded to verify functional binaural pathways.

### 2.2. Extracellular single unit electrophysiology

Double-barrel piggyback electrodes were used for extracellular single unit recordings and simultaneous local injection of forskolin. The electrodes were fabricated by gluing a pressure ejection pipette to the recording electrode, with the tip of the ejection pipette 150 μm behind the recording electrode tip. The recording electrode was filled with 2% wheat-germ agglutinin horseradish peroxidase (WGA-HRP) dissolved in 2 M sodium chloride. Tip resistance of the recording electrodes was 12–20 MΩ. The pressure ejection pipette was filled with 50 μM forskolin.

Recorded electrical signals were amplified using an A-M Systems (Seattle, WA) 1800 amplifier and Kikisui (Japan) oscilloscope. Recordings were bandpass filtered between 10 Hz and 5 kHz. The spikes were identified on an oscilloscope and converted to pulses using a window discriminator (World Precision Instruments).

### 2.3. Localization and classification of single neurons

Binaural clicks with the contralateral stimuli delayed by 20 ms relative to ipsilateral stimuli were used to discretely observe evoked potentials after stimulation of either ear and to search for the area of interest. In order to approach left superior olive complex the electrode was advanced at 15° angle to the right of the mid-sagittal plane and 49.5° up from the horizontal plane. Robust evoked potentials in response to binaural clicks were observed as electrode approached into the superior olive complex, in many instances in its peri-olivary region. Single auditory neurons were isolated and then characterized by with 50 ms pure tone stimuli with 5 ms rise and fall ramps. The frequency of stimulation producing the greatest spike count (best-frequency) was determined from right and left monaural iso-intensity curves at 50 dB SPL. Iso-intensity curves of monaural ipsilateral and monaural contralateral stimulation were compared to confirm tonotopic alignment for binaurally excited neurons.

Single unit responses to ipsilateral best-frequency pure-tone stimuli at variable sound intensity were recorded. Acoustic intensity dependent modulation of the single unit response, the rate intensity function, was assessed. Neural excitability was monitored by sequentially recording the spontaneous activity and acoustic evoked responses to best-frequency tone delivered at 30 dB above threshold. Peri-stimulus time histograms (PSTH) were calculated from spike time occurrences, detected by voltage threshold (WPI 121 window discriminator), and stored at 10 μs resolution. Commercially available forskolin (Tocris) was dissolved in artificial CSF (NaCl 7.93 g/l, KCl 0.22 g/l, $CaCl_2$ 0.24 g/l, $MgSO_4$ 0.14 g/l, glucose 0.6 g/l, and HEPES 5.96 g/l, pH 7.2) to prepare 50 μM solution. After establishing the baseline neural responses to atleast 250 acoustic stimuli, forskolin was injected with 30 PSI, 250 ms pressure pulses delivered every ten seconds for 1 min. During each pressure pulse approximately 11.5 nl forskolin was injected with a total volume of approximately 70 nl for six pulses over a 1 min period. This volume fills a sphere with a radius of over 280 μm. Details for the calibration of pressure ejection were previously described [21]. Brief application of 50 μM forskolin produced a long-lasting change in the neuronal excitability, whereas lower doses did not exhibit such effects [20]. Therefore 50 μM forskolin was used in current experiments.

### 2.4. Confirmation of location

At the end of tested set of paradigms, the recording sites were marked by iontophoretic injection of HRP-WGA filled in the recording electrodes. Current pulses (5 nA amplitude and 500 ms width) were delivered at 1 Hz frequency for iontophoretic injection. Tracks were marked using the same ejection parameters while slowly withdrawing electrodes. These locations were assessed with histology. After each experiment the animals were overdosed with sodium pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde fixative and 20% sucrose–4% paraformaldehyde. Approximately 50 μm thick frozen sections were cut in the plane of the electrode track, mounted, and reacted for peroxidase tetramethylbenzidine/glucose oxidase chromogen reaction. The sections were counterstained with thionin. Tracings of relevant sections and electrode depths were used to identify the locations of the neurons studied. Fig. 1A depicts an example of electrode track marked at the end of the experiment. In this example, the reconstructed path of the recording electrode, passed through the mid-superior olive complex (superior peri-olivary nuclei, medial superior olive, and ventral nucleus of trapezoid body).

### 2.5. Analysis: computation of activity index 1 (ai)

There was a considerable inter-neuronal variability in the spontaneous or tone evoked responses. Given such disparity amongst recorded neurons it was not appropriate to compare the absolute effects of forskolin on their sound encoding property and spontaneous activity. Therefore, tone-evoked neural activity recorded before the application of forskolin and spontaneous firing rate in the presence of forskolin were normalized with respect to control (pre-forskolin) spontaneous activity. This normalized parameter, activity index (ai) was computed using Eq. (1).

$$ai = \frac{(\text{firing rate during given condition})}{(\text{average pre-forskolin spontaneous firing rate})} \quad (1)$$

The activity index was also computed for forskolin induced changes in the neural activity (*aiFSA*).

### 2.6. Quantitative determination of neural response to threshold-level acoustic stimulus

Neural responses were considered supra-threshold if the neural firing rate was two standard-deviations greater than the mean spontaneous activity. This criterion determined the activity index