An in vitro model for testing drugs to treat tinnitus

Calvin Wu, Kamakshi Gopal, Guenter W. Gross, Thomas J. Lukas, Ernest J. Moore

Department of Biology, University of North Texas, Denton, TX 76203, USA
Center for Network Neuroscience, University of North Texas, Denton, TX 76203, USA
Department of Molecular Pharmacology & Biological Chemistry, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

ABSTRACT

Tinnitus affects approximately 50 million people in the USA alone, with 10 million being highly debilitated. Pharmacotherapy for tinnitus is still in emerging stages due to time consuming clinical trials and/or animal experiments. We tested a new cellular model where induced rapid neuronal firing or spiking was used as a mimic for the type of aberrant activity that may occur in tinnitus. Spontaneously active auditory cortical networks were then exposed to experimental tinnitus drugs linopirdine (Dup966, a potassium channel blocker), t-carnitine (an antioxidant), or selective Ca2+ channel antagonists pregabalin (Lyrica), or gabapentin (Neurontin) at various concentrations. PTZ increased spike rate by 139.6 ± 27% and burst rate by 129.0 ± 28% in auditory cortical networks with a phenotypic high firing of excitable neurons. Reductions of increased activity were observed to varying degrees using the experimental tinnitus drugs. The potency of the drugs was linopirdine (EC50: 176 ± 7.0 μM), t-carnitine (EC50: 1569 ± 41 μM), pregabalin (EC50: 8360 ± 340 μM), gabapentin, with 34.2 ± 7.5% efficacy (EC50: 2002 ± 980 μM). These studies provide proof of principle for the use of auditory cortical networks on microelectrode array as a feasible platform for semi-high throughput application for screening of drugs that might be used for the treatment of tinnitus.

1. Introduction

Noises or ringing in the ears are experienced by people with normal hearing (Thabet, 2009), but most noticeable by individuals who have some type of auditory deficit (Littler, 1965). The malady is known as tinnitus, and affects approximately 50 million people in the USA alone. The sounds perceived are usually high-pitched whistles, but some individuals report hearing other noises such as hissing, buzzing, chirping, clicking, or other internally generated annoying sounds. The basic underlying mechanisms responsible for the generation of tinnitus after otologic insult are largely unknown (Jung et al., 1993; Lanting et al., 2009). While various anatomical structures of the auditory system may be the underlying site of tinnitus generation, the hair cells of the inner ear cochlea may be the most vulnerable structures. One school of thought about the genesis of tinnitus is that the hair cells release an unusually high amount of neurotransmitter substances such as glutamate, which excites afferent fibers of the VIIIth cranial nerve (Zemmer and Ernst, 2003). This over excitation is perceived as a phantom sound (Denk et al., 1997). However, tinnitus may also have its origin in the brainstem or cortex, and thus, the unwanted sounds are perceived also to be originating in the brain (Lockwood et al., 1999; Mühlau et al., 2006). The sensory neural axis of the auditory system (Jastreboff, 1990) such as the dorsal cochlear nucleus (Kaltenbach, 2000, 2006) has been similarly indicated. Nevertheless, the basic mechanisms underlying tinnitus that might lead to better treatment regimens are not well understood and have not been readily forthcoming (Kay and Davies, 1993; Lanting et al., 2009).

Tinnitus is a highly debilitating disorder for many patients and the condition is exacerbated by the fact that no chemotherapeutic agents have been found to alleviate the problem over a long period of time (Baguley, 2002). The problem lies with the fact that the fundamental physiological and biochemical mechanisms or mechanisms underlying tinnitus remain unclear (Kaltenbach, 2000, 2006). It would be of great benefit to millions of tinnitus patients if effective pharmaceutical agents could be found to provide relief for tinnitus. Since pharmacoeutical product development requires 10–15 years and at costs estimated between 500 million and 2.0 billion dollars to bring a drug to market, the concept of “repurposing” or “repositioning” of FDA approved drugs has been suggested (Boguski et al., 2009; Tobinick, 2009). However, a suitable screening assay is necessary if one is to identify drugs that might be used to treat tinnitus before repurposing.

Eggermont and colleagues hypothesized that an increase in neuronal firing may be indicative of tinnitus of auditory cortical neuronal origin (Eggermont, 2008; Kennochi and Eggermont, 1997). The central origin...
is widely speculated also to be due to its role in changing neural plasticity (Lockwood et al., 1998, 2002). We used in vitro auditory cortical networks (collections of neurons) derived from mouse embryos, growing on microelectrode arrays as a platform to induce rapid neuronal firing to mimic aberrant activity that may be similar to tinnitus. The proconvulsant drug pentylentetrazol (PTZ) was applied to the neuronal networks to induce an increase in neuronal firing rate. Four “experimental tinnitus drugs” (pregabalin, gabapentin, linopirdine, L-carnitine) were applied at various concentrations to test their efficacy to reduce the rapid neuronal firing that was hypothesized to mimic the excitatory activity that may occur in tinnitus.

Pentylentetrazol [6,7,8,9-tetrahydro-5H-tetrazolo[1,5-a]azepine] (C₄H₆N₄) (Pentrazol) is a respiratory and circulatory stimulant that in high doses causes convulsions - its mechanism of action is a GABA antagonist. Pregabalin [(S)-3-(aminomethyl)-5-methylhexanoic acid] (C₅H₁₂NO₂) (Lyrica) is an anticonvulsant drug that is used for neuro-pathic pain, partial seizures and generalized anxiety disorder. The mechanism of action is a calcium channel blocker or a GABA agonist. Gabapentin [2-1-(aminomethyl) cyclohexyl acetic acid] (C₆H₁₄NO₂) (Neurontin) was originally thought to be a GABA analog and a treatment for epilepsy, although subsequently it was determined that the former was not substantiated. It was originally developed for use as an adjunctive therapy for epilepsy, but presently is used for neuropathic pain and major depressive disorder. Linopirdine [1-phenyl-3,3-bis(pyridin-4-ylmethyl)-1,3-dihydro-2H-indol-2-one] (C₈H₁₁N₂O₂) (linopirdine) is a psychostimulant/nootropic drug that acts as a potassium channel blocker, and releases acetylcholine. L-Carnitine (3-hydroxy-4-(trimethylaminomethyl)butanoate) (C₄H₁₄NO₃) (carnitine) is a quaternary ammonium compound that is biosynthesized from the amino acids lysine and methionine. One mechanism of action is thought to be an antioxidant within the mitochondria of cells.

Here we present the first observation of the use of the proconvulsant drug, PTZ (Klöcker et al., 1996), to excite mouse neuronal cortical activity recorded in vitro, as a homologue for tinnitus. We further investigated the modulation of the excitatory neuronal activity by drugs of the class: Ca-channel blocker, K-channel blocker, or antioxidant. These drugs exhibited differential effects of reducing over-excitation of neuronal activity and thus suggest a possible chemotherapeutic treatment for tinnitus.

2. Methods

2.1. Isolation of murine auditory cortex

The care and use of animals, as well as all procedures involving animals in this study were approved by and conducted in accordance with the guideline of the institutional animal care and use committee. Mouse embryos (Balb-C/Icr, day E17) were extracted from the dam after CO₂ narcosis and cervical dislocation, followed by a double thoracotomy. Auditory cortices located on the posterolateral surface and depths of the left temporal cortex (TE1, TE2 and TE3) were dissected (-2×2×1 mm) from the embryos under sterile conditions.

2.2. Cell culture and microelectrode array fabrication

The standard culturing process published earlier was used in this study (Gopal and Gross, 1996; Keefer et al., 2001). Briefly, the auditory cortical cells were mechanically minced, enzymatically dissociated, triturated and mixed with Dulbecco’s Modified Minimal Essential Medium (DMEM). Horse serum (10%) and fetal bovine serum (10%) were added to the DMEM. The cell suspension was decorated onto the microelectrode array surface decorated with poly-o-lysine and laminin and incubated at 37 °C in a 10% CO₂, 90% air atmosphere. The age of cultures used in this set of experiments ranged from 21 to 96 days in vitro with an average age of 31±2 days. Fabrication and preparation of microelectrode arrays, as well as recording techniques have been described previously (Gopal and Gross, 2004; Keefer et al., 2001).

2.3. Solutions and drugs

Drug solutions were made on the day of experiments from stock solutions. Solutions and drug applications were made to a constant volume of 1.0–2.0 ml of the experimental bath. Drug concentrations were calculated to minimize bulk volume changes to the experimental bath. Pentylentetrazol, gabapentin, linopirdine and L-carnitine were obtained from Sigma-Aldrich (St. Louis, MO), while pregabalin was obtained from Pfizer Pharmaceutical Company (Groton, CT).

2.4. Electrophysiological recordings and analyses of microelectrode data

Microelectrode arrays were placed in a stainless steel recording chamber mounted on an inverted microscope head stage and maintained at 37 °C. Chamber components were sterilized via autoclaving before each experiment. The original medium was replaced by fresh DMEM stock medium (without serum). The pH was maintained at 7.4 with a continuous stream of filtered, humidified, 10% CO₂ confined by a cap with a heated ITD window to prevent condensation and allow continuous microscopic observation. Evaporation due to constant air flow was compensated by a syringe infusion pump with sterile water. Auditory neuronal activity was recorded with a two-stage, 64-channel amplifier system (Plexon, Dallas, TX), and digitized simultaneously at 40 kHz via a Dell 8600 workstation. Total system gain was set to 1.2×10⁵. Spike identification and separation was accomplished with a real time template-matching algorithm (Plexon, Dallas, TX) to provide single-unit spike rate data. Electrodes/channels were assigned to 64 digital signal processors. Each digital signal processor can discriminate up to four different action potential waveforms or “active” units. Multiple unit data were analyzed off-line using custom programs for burst recognition and analyses (Gopal et al., 2007). Burst patterns derived from spike integration (τ = 100 ms) provided a high signal-to-noise feature extraction that has been shown previously to reveal several modes of neuronal network activity in the form of action potentials (spike activity) of high frequency clusters (burst activity). From this approach, single unit activity was averaged to yield spike rate and burst rate, readily computed from individual recording sites. All data presented are based on quantification of single unit parameters. Data obtained were analyzed using custom and commercially available data analyses software programs (Plexon, Dallas, TX). The primary data display was the real time plotting of total or average network spike production per minute.

2.5. Data analyses

In order to quantify neuronal network responses, the mean spike and mean burst rates were used. One-minute average activity across all discriminated units was derived for each experiment. Bursts were identified for each discriminated unit by integration (Morefield et al., 2000). Burst rates depicted in figures represent an average of all bursts per minute. In order to control for differences in the spontaneous activity among cultures, the PTZ-induced activity was quantified as a percent change from the reference (DMEM stock) activity. Furthermore, tinnitus drug-induced activity was quantified as a percent change from the PTZ-induced activity. Waveform analysis was performed by comparing peak width (P1–P3) and amplitude (P1–P2 and P2–P3) from a total of 10 waveforms. Statistical significance was probed using one-way ANOVA, p<0.05 considered as significant.

3. Results

Data recorded for this research were obtained from 46 separate auditory cortical networks with an average of 47±4 active neurons (range: 10–124 neurons) and an average age of 31±2 days in vitro.
3.1. Effects of pentylenetetrazol on in vitro cultures

Typical responses to sequential addition of PTZ are shown in Fig. 1A. This 28 day in vitro auditory cortical network showed a gradual increase from reference (Ref) in spike rate and burst rate from 0.1 to 0.9 mM concentration of PTZ, reaching maximum plateau at 1.0 mM. Concentrations (1.1–1.5 mM) of PTZ higher than 1.0 mM decreased the spike and burst rates. Each data point on the y-axis reflects the “minute mean” for spike and burst values averaged over 46 active units in the culture. While the application of 200 μM PTZ at 62 min (Fig. 1A) showed a sudden increase in spike rate for the initial moment of drug application, this is interpreted as an artifact due to injection of eddy currents for the pipette and unequal distribution of PTZ, which slowly stabilized as the culture bath reached uniform concentration. In most experiments, the interval between drug injections was approximately 30 min, allowing for at least 15 min of stable activity. Based on these data (n = 6), PTZ of 1.0 mM was determined to maximally induce excitation of neurons in the cultures (Fig. 1B). Hence, 1.0 mM PTZ was used as the standard concentration in all subsequent experiments. The auditory cortical network exhibited an average spike rate increase of 139.6 ± 27%, and an average burst rate increase of 129.7 ± 28% under 1.0 mM PTZ (n = 39; Fig. 1B). Activity increase under PTZ is persistent and remained stable for more than 8 h, and is reversible, i.e. activity returns to the reference level upon medium changes (data not shown). To identify if there were any morphological changes of the neurons with application of PTZ, neurons and neuronal processes were monitored throughout the experiment (Fig. 1C). We found no overt changes in morphology with 1.0 mM PTZ. In all experiments, the action potential waveforms were monitored also during sequential application of PTZ. No statistical significance was found at PTZ of 100 μM, 500 μM, 1.0 mM, or 1.5 mM (ANOVA, P > 0.05).

3.2. Effects of potassium channel blocker linopirdine on PTZ-treated cultures

In the presence of 1.0 mM PTZ, various concentrations of linopirdine were applied. Linopirdine is a potent inhibitor of the excitation by PTZ, displaying an effect at the micromolar concentration range. All neuronal activity ceased at 500 μM, but was reversible with a wash which consisted of a full medium change with fresh DMEM stock (Fig. 2A). Marked horizontal lines depict >20 min of stable activity following each application; these data points were taken into account for the calculation of the concentration–response curve. The EC50 of linopirdine is 176 ± 70 μM (n = 7, Hill coefficient = 1.9) calculated for seven experiments (Fig. 3). Raster plots seen in Fig. 2B display reference, 1.0 mM PTZ and 150 μM linopirdine conditions. PTZ increased spike production and burst coordination. Linopirdine (150 μM) reduced the PTZ-induced excitation with no overt changes in neuronal morphology (Fig. 2C). Fig. 2D displays action potential waveforms under each of the experimental condition. No statistical significance was found among all experimental conditions (ANOVA, P > 0.05).

3.3. Effects of the antioxidant L-carnitine on PTZ-treated cultures

L-Carnitine is an over-the-counter antioxidant supplement which was used here to determine its effects on PTZ-treated cultures. Fig. 4A shows a typical PTZ treated culture responding to various concentrations...
of L-carnitine. L-Carnitine first became effective in reducing the PTZ-evoked activity at a level of 500 μM. At 2.0 mM, the activity was reduced to more than 50% of the reference level; a full medium change after applications of the highest dose brought the activity back to the native reference level. Fig. 3 indicates that the EC₅₀ of L-carnitine was 1569 μM ± 41 μM (n = 8; Hill coefficient = 1.2). Raster plots for L-carnitine application are depicted in Fig. 4B. No overt morphological changes (Fig. 4C) or action potential waveform changes (Fig. 4D) were noticed with the application of L-carnitine.

3.4. Effects of the anticonvulsants gabapentin and pregabalin on PTZ-treated cultures

Gabapentin (Neurontin) was not a potent inhibitor of PTZ-induced activity. No effect is seen at a concentration less than 4.0 mM (Fig. 5A), far beyond the physiological range. The spike rate approached to the pre-PTZ level at the highest concentration tested (12 mM). The concentration–response curve indicated that the efficacy of gabapentin is only 34.2% ± 7.5, thus, the EC₅₀ is 2091 ± 980 μM (Hill slope = 1.2; Fig. 3). At 2.0 mM, gabapentin decreased burst frequency without changing the overall burst pattern, which partly reflects its low efficacy (Fig. 5B). Neuronal morphology as well as action potential waveforms were unaffected by the application of gabapentin (Fig. 5C–D). Even at the high dose of 12 mM, a change in neuronal morphology was not discernible (data not shown).

The dose-dependent activity plot of pregabalin (Lyrica) follows a very similar pattern of gabapentin, its precursor, as the effect can only be seen at a concentration greater than a 2.0 mM (Fig. 6A). Pregabalin, although ineffective in attenuating PTZ induced hyperactivity, shows 100% efficacy with an EC₅₀ of 8360 ± 307 μM (Hill slope = 1.7). The raster display shows that pregabalin still maintains a coordinated burst pattern initiated by PTZ. Pregabalin of 8.0 mM also does not change cell morphology (Fig. 6C) or action potential waveforms (Fig. 6D) over the 4 h experimental time period.

4. Discussion

The effectiveness of the various tested compounds with the potential of eliminating tinnitus was linopirdine > carnitine > pregabalin > gabapentin. Other channel antagonists were not tested since there have been a myriad of off-labeled drugs such as the GABA agonists and NMDA channel blockers used in the investigation of tinnitus (Akula et al., 2009; Elgoyhen and Langguth, 2009) that, for the most part, have proven ineffective. The fact that linopirdine (a potent blocker of the KCNQ4 potassium channel that is expressed in the mouse) was taken off the market for lack of efficacy in improving memory in Alzheimer patients, perhaps other KCNQx antagonists may be useful in this...
instance. Linopirdine's side effects may be due to the inhibition of acetylcholine receptors (Berni et al., 2008) so that with other K⁺ channel antagonists, there may be fewer "off target" effects that may be active. Linopirdine is a potent K⁺ channel blocker of the KCNQ4 sub-type and shows its greatest effect on the M-channel (Liang et al., 2005). The M-channel has been implicated also in the neurogenesis of tinnitus (Holt et al., 2007). In the current study the EC_{50} of linopirdine was 176 μM, which is approaching a physiological range and may thus serve as an adjunctive therapy for tinnitus, but would have to be made available once again to the consumer market. The fact that linopirdine is classified also as a nootropic drug ("smart drug") and is surmised to work by altering the availability of neuronal neurochemicals. This may in fact improve neuronal oxygen supply. However, the efficacy of various nootropic substances in modulating tinnitus remains to be elucidated.

L-Carnitine is an over-the-counter supplement that is classified as an anti-oxidant. Its mode of action is to act as a scavenger of reactive oxygen species, a possible cause of tinnitus in patients (Berni et al., 2008).
Gabapentin were originally developed as GABA receptor antagonists. These drugs may work in modulating tinnitus. Pregabalin and gabapentin are structurally similar, but pregabalin has greater efficacy at smaller dosages in reducing certain types of pain and reducing the effects of electrical discharges in epilepsy patients. Thus, if epilepsy or pain can serve as homologue of tinnitus, one can use these drugs directly antagonizing the effects of PTZ. It has been reported that gabapentin does not bind to the GABA\_A receptor, thus, this might perhaps account for its less potential efficacy for tinnitus patients (Shimizu et al., 2004). Anecdotal reports of patients on a regimen of pregabalin for the management of pain reported an exacerbation of their tinnitus, but some patients reported also reduced tinnitus as a result of taking pregabalin. Thus, our \textit{in vitro} results might suggest that a more effective type of Ca-channel antagonist may be necessary to treat tinnitus.

There is emerging evidence to suggest that dysregulation within the auditory system after insult may underlie aberrant excitation in the form of tinnitus (Hwang et al., 2011a,b). These investigators found altered expression of COX-2 and NMDA receptor genes in the cochlea and midbrain in salicylate-induced tinnitus (Noreña et al., 2010). Hwang and his colleagues induced the “tinnitus-like activity” in a mouse model trained to experience tinnitus. There is evidence to suggest also that the pathophysiology underlying tinnitus may be mediated via receptors or ion channels of the NMDA or GABA receptor type (Hansen et al., 2004), chloride channel types or even auditory efferent pathway neuroactive substances (Puel, 1995). This may be manifested at multiple levels of the auditory neuraxis to include not only the hair cells of the cochlea, but brain stem auditory structures such as the dorsal cochlear nucleus (Kaltenbach, 2006), inferior colliculus (Melcher et al., 2000), auditory cortex (Eggermont, 2008), and thus non-auditory CNS structures such as the limbic system (Lockwood et al., 1998). While our data was obtained \textit{in vitro} and thus would not reveal any information about the auditory system loci, nevertheless, further studies to reveal precisely anatomical loci are worthy of pursuit.

5. Conclusion

In conclusion, we used PTZ, a proconvulsant agent that causes excitation of cortical neurons without inducing cell necrosis or apoptosis. Previous studies have used either salicylates or quinine to induce similar excitatory activity. Drugs of the K\(^+\) channel blocker type, the Ca\(^{2+}\) channel antagonists, as well as an antioxidant were used to attenuate the effects of PTZ. We found a protective effect from neuronal excitation under those conditions that modulated or blocked neuronal electrical activity at the 50\% (ED) level. Most of the drug levels, however, were beyond ideal physiological range, except linopirdine, and thus clinical use of these drugs may cause unwanted side effects. The MEA platform therefore serves as a useful vehicle for screening drugs (i.e., assessing potential toxicity and/or safety) that may reduce the percept of tinnitus. This study fulfills our goal of principle for testing the efficacy of thousands of FDA drugs already available on the market that might attenuate increased ongoing electrical activity of mouse auditory cortical neurons in an experimental condition resembling tinnitus.
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References