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Pharmacological response sensitization in nerve cell networks exposed to the antibiotic gentamicin

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ABSTRACT

Gentamicin is an aminoglycoside antibiotic that is used in clinical, organismic, and agricultural applications to combat gram-negative, aerobic bacteria. The clinical use of gentamicin is widely linked to various toxicities, but there is a void in our knowledge about the neuromodulatory or neurotoxicity effects of gentamicin. This investigation explored the electrophysiologic effects of gentamicin on GABAergic pharmacological profiles in spontaneously active neuronal networks in *vitro* derived from auditory cortices of E16 mouse embryos and grown on microelectrode arrays.

Using the $GABA_A$ agonist muscimol as the test substance, responses from networks to dose titrations of muscimol were compared in the presence and absence of 100 μM gentamicin (the recommended concentration for cell culture conditions). Spike-rate based EC_{50} values were generated using sigmoidal fit concentration response curves (CRCs). Exposure to 100 μM gentamicin exhibited a muscimol EC_{50} ± S.E.M. of 80 ± 6 nM (n=10). The EC_{50} value obtained in the absence of gentamicin was 124 ± 11 nM (n=10). The 35% increase in potency suggests network sensitization to muscimol in the presence of gentamicin. Action potential (AP) waveform analyses of neurons exposed to gentamicin demonstrated a concentration-dependent decrease in AP amplitudes (extracellular recordings), possibly reflecting gentamicin effects on voltage-gated ion channels. These *in vitro* results reveal alteration of pharmacological responses by antibiotics that could have significant influence on the behavior and performance of animals.

1. Introduction

Aminoglycoside antibiotics (AGA) are derivatives of microbial products, and are extensively used in the treatment of bacterial infections (Hoffman et al., 2005). Gentamicin, the most commonly used AGA, is also recommended for use in cell culture given its bactericidal efficacy, stability, and heat resistance (Fischer, 1975; Schafer et al., 1972). It has become evident from the data gathered over the past twenty years in our laboratory, that pharmacological responses from nerve cell networks grown on microelectrode array (MEA) platforms closely mimic the responses of the parent tissue (Gopal and Gross, 1996; Keefer et al., 2001a; Xia and Gross, 2003; Gross and Gopal, 2006; Gross and Pancrazio, 2007; Johnstone et al., 2010; Novellino et al., 2011). Most of these data were gathered without the use of antibiotics. Under certain experimental conditions, however, the addition of antibiotics was deemed necessary. Differences in EC_{50} values, primarily for muscimol, obtained from networks with and without penicillin/streptomycin treatment were seen as early as 2005 (Rijal-Oli, 2005) - but never quantified. This paper represents the first systematic electrophysiologic investigation of antibiotic-related alteration of concentration-response curves of the $GABA_A$ agonist muscimol in the presence of gentamicin.

Gentamicin is a polycationic compound that acts by binding to the bacterial 30 S ribosomal subunit to block the formation of the initiation complex with mRNA. This leads to defective protein synthesis, accumulation of truncated proteins, and ultimately bacterial cell death (Davies and Davis, 1968). Gentamicin is said to be safe for human use, and has been approved for clinical usage in the United States by the Food and Drug Administration (Xie et al., 2011). However, side effects such as ototoxicity, neuromuscular blockade, peripheral neuropathy, encephalopathy (Grill and Maganti, 2011) and nephrotoxicity (Balakumar et al., 2010; Grill and Maganti, 2011) have been reported with AGA use, and gentamicin is considered to be the second most toxic AGA (Xie et al., 2011).

General neurotoxicty of AGAs was shown in human cases with degeneration of spiral ganglion neurons at peak serum concentrations

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of 3.0–7.7 mg/l (Hinojosa and Lerner, 1987), well within the acceptable therapeutic range of ≤10 mg/l (Caputy et al., 1981). Earlier studies have linked intravenous and intramuscular administration of gentamicin to peripheral neuropathy, psychosis, convulsions, and encephalopathy (Wadlington et al., 1971; Kane et al., 1975). Numerous other investigations demonstrated toxic effects of AGAs on various neural tissues (Watanabe et al., 1978; Hodges and Watanabe, 1980; Segal et al., 1999; Robert and Hevor, 2007), including the cochlear nucleus (Xu et al., 2009) at a concentration range of 2.5 mg/kg – 100 mg/kg. Despite these studies, quantitative data on gentamicin-induced neurotoxicity and neuromodulation are still lacking, and the underlying mechanisms remain elusive. In this investigation we hypothesize that gentamicin modulates neuronal responses via the GABAergic system. Muscimol is not broken down by neural tissue and has a longer half-life than GABA, allowing establishment of equilibrium conditions necessary for pharmacological investigations. In this study we used muscimol titrations on networks with and without gentamicin exposure to determine the degree of modulation.

2. Materials and methods

2.1. MEA fabrication

All MEAs used in this study were fabricated in the Center for Network Neuroscience photolithography lab at the University of North Texas. Previous publications describe MEA fabrication techniques in detail (Gross, l979; Gross et al., l985; Gross, 1994). Briefly, 1.0 mm thick indium-tin oxide (ITO) sputtered glass plates (Colorado Concepts Coatings, Loveland), were photoetched, cut to 5.0 cm^2 wafers, and spin insulated with methyltrimethoxysilane. Each plate contained a total of 64 microelectrodes terminating as an 8×8 pattern in a 1.0 mm^2 recording area, with 32 amplifier contact strips on the left and right edges. The electrodes were laser-deinsulated and electrolytically gold-plated to achieve impedances at 1.0 kHz of 0.6–0.8 kOhms.

2.2. Cell culture procedures and electrophysiologic recordings

The Institutional Animal Care and Use Committee of the University of North Texas approved the experimental methods used in this study. Cell culture and recording methods, similar to those used by Rijal-Oli and Gross (2008), were adopted for use in the present study and are described in previous work (Gopal and Gross, 1996). Following CO2 narcosis and cervical dislocation of the mouse dame (ICR CD-1), the 16 d old embryos were removed. The auditory cortex tissues were extracted from these E16 embryos, and dissociated using standard primary culture techniques. Cells (neurons and glia) were seeded at approximately 80 K/100 µl in Dulbecco Modified Minimal Essential Medium (DMEM), supplemented with horse serum (10%), fetal bovine serum (4.0%), and 1.0 µl/L B27 (GIBCO) onto a 2–3 mm diameter adhesion area centered on the recording matrix. After four days *in vitro*, cells were switched to DMEM-10 (10% horse serum) with 1.0% B-27 supplement, and maintained in this medium subsequently with bi-weekly 50% medium changes. The incubation environment was maintained at a temperature of 37 °C and 10% CO_2 in air.

With an average incubation period of 31.9 ± 8.8 days *in vitro* (div), the cultured networks on MEAs were incorporated into stainless steel recording chambers and mounted onto an inverted microscope stage. Details of the recording apparatus were described previously (Gross and Schwalm, 1994; Gross et al., 1995). Cultures were kept at 37 ± 1 °C and the pH was maintained between 7.3 and 7.5, using a continuous 10 ml/min flow of filtered 10% CO2 in air, mixed by a gas flow controller (AFC 2600-PRO, Aalborg, Inc., Orangeburg, NY). Prior to recordings, the original medium was replaced with fresh iso-osmotic DMEM stock medium containing no serum. The osmolarity was maintained between 300 and 320 mOsmol/kg using a syringe pump infusion of ultrapure water at a rate of 35 µl/h for a 1.0 ml chamber well, and 70 µl/h for a 2.0 ml chamber well to compensate for evaporation.

A cap with a heated ITO window placed directly on top of the stainless steel recording chamber confined the 10% CO2 atmosphere and prevented condensation, which allowed simultaneous microscopic observation during recordings. A 64-channel, two-stage amplifier system was used to record extracellular activity (Plexon Inc., Dallas, TX). Analog signals were digitized using 64 processors operating at 40 kHz. Under optimal conditions with good signal to noise ratios, up to four signals could be separated with a real time template-matching algorithm (Plexon Inc., Dallas, TX). Discriminated single unit spike waveshapes were given a time stamp at the first threshold crossing. All spike rate data was obtained from these time stamps. Real-time average or total network spike production was plotted per minute and used as the primary network activity display. Time stamps from all discriminated units were recorded continually and all selected waveshapes were stored for 30 s every 30 min during an entire experiment.

2.3. Off-Line burst identification

After waveshape discrimination, the time stamps were integrated with a simulated RC circuit using an integration constant of 75 ms. Two thresholds were used for burst parameter quantification: T1 was in close proximity to the noise level, and T2 was at approximately 5x T1 (Fig. 1). The first threshold indicated the potential start of a burst, while the second threshold confirmed or negated that signal. T2 had to be reached for acceptance of the integrated profile as a burst. T2 settings were selected based on visual agreement with the NeuroExplorer (NEX Technologies, Madison, AL) 1-D viewer. This program recreates the entire experimental raster plot and allows matching of burst numbers per min between program and visual identification for the same time period. The process is tedious and therefore was done only for the last 10 min of the reference period. To compensate for a burst termination overshoot caused by the decay constant (Fig. 1B), 10 ms were deducted from all burst durations. A gap time of 100 ms was used to separate bursts. If activity remained below T1 for more than 100 ms, two bursts were generated. The gap time was adjustable and selection was generally dependent upon the overall spike pattern.

2.4. Drugs and Solutions

The potent and well-studied $GABA_A$ agonist, muscimol ([methylene-3H (n)]–3-hydroxy-5-aminoethyl isoxazole) was used as the neuropharmocologic test agent. Muscimol is a toxin produced by the Amanita muscaria mushroom (Johnston, 1996), which induces highly repeatable and reversible electrophysiologic responses (Rijal-Oli and

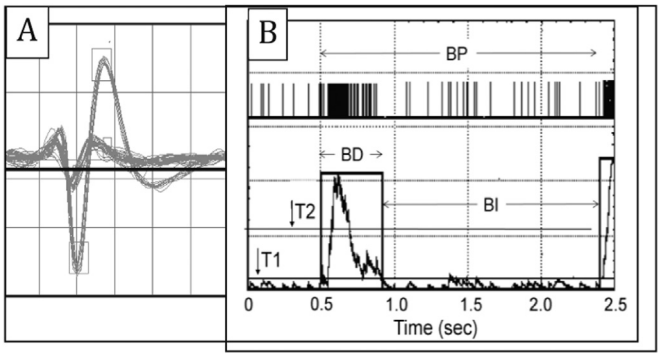

Fig. 1. AP and burst identification. (A) Separation of APs via template matching algorithm (Plexon Inc., Dallas, TX) from 40 kHz digitized waveshapes. (B) After waveshape separation, bursts were identified from integrated profiles (integration constant: 75 ms) with two thresholds (see text). BD: burst duration, BP: burst period, BI: burst interval.