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Dissociation constants for $GABA_A$ receptor antagonists determined with neuronal networks on microelectrode arrays

Sabnam O. Rijal, Guenter W. Gross*

Department of Biological Sciences and Center for Network Neuroscience, University of North Texas, Denton, TX 76203, United States

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ABSTRACT

Changes in spontaneous spike activities from murine frontal cortex networks grown on microelectrode arrays were used to determine the dissociation constants of three $GABA_A$ antagonists: gabazine, bicuculline, and trimethylolpropane phosphate (TMPP). Networks were treated with fixed concentrations of antagonists and titrated with the $GABA_A$ receptor agonist muscimol. Muscimol decreased spike activity in a concentration-dependent manner with full efficacy (100% spike inhibition). A sigmoidal curve fit provided a 50% inhibitory concentration (IC_{50}) of 0.14 ± 0.05 μM (mean ± S.D., $n = 5$). Increasing concentrations of the three antagonists shifted the muscimol concentration response curves (CRCs) to the right with the same efficacy. Schild plot analyses with linear regressions resulted in slopes that are statistically not different from unity and provided *X*-intercepts (dissociation constants) of 0.23, 0.61, and 3.98 μM for gabazine, bicuculline, and TMPP, respectively. Corresponding pA2 values ($-\log K_B$) were 6.64, 6.21, and 5.40. The dissociation constants for gabazine and bicuculline agree well with those obtained with other methods. The TMPP K_B has not yet been reported in the literature. The data suggest that spontaneously active networks on microelectrode arrays can be used as reliable platforms for rapid quantitative pharmacological investigations.

1. Introduction

Spontaneously active murine neuronal networks grown on substrate-integrated microelectrode arrays (MEAs) *in vitro* have been applied for studies of pharmacological and toxicological responses to known and unknown compounds (Gross et al., 1995; Gramowski et al., 2000, 2006; Morefield et al., 2000; Keefer et al., 2001a; Chiappalone et al., 2003). These networks function as physiological sensors as they are capable of generating activity changes in the same concentration ranges that alter functions of an intact mammalian nervous system (Xia et al., 2003; Xia and Gross, 2003; Gopal and Gross, 2004; Shafer et al., 2008). Effective concentrations for a 50% activity change (EC_{50} values) are in the same range as those published for other *in vitro* preparations and frequently overlap with those obtained from animal experiments. It appears that the major receptor, synaptic, and cellular mechanisms responsible for basic pattern generation in central nervous system (CNS) tissues are represented in neuronal primary cell cultures.

There is now little doubt that such cultures provide histiotypic (similar to parent tissue) pharmacological responses (Gross and Gopal, 2006). Primary cultures that are derived from cell pool suspension are well suited for automated cell seeding and offer advantages such as strong cell-surface adhesion, resulting in stable cell-electrode coupling, and large signal-to-noise ratios. In addition, one pregnant mouse (12 embryos) can provide tissue for the seeding of approximately 300 MEAs with spinal cord cells and 300 MEAs each from at least four major cortical regions. The potential generation of over 1000 networks per mouse provides remarkable tissue utilization efficiency for compound screening in the fields of neurotoxicology, drug development, and pharmacology.

Although the full efficiency of culturing with large pools of dissociated cells will not be realized until high throughput multinetwork platforms emerge, it is essential to demonstrate the pharmacological reliability and utility of such systems. The observed high degree of intra- and intercultural repeatability of pharmacologic and toxic responses, suggests expansion of this methodology to more quantitative pharmacological analyses, such as determination of compound binding, a phenomenon best described in terms of dissociation constants.

Muscimol ([methylene-3*H* (*n*)]-3-hydroxy-5-aminoethyl isoxazole) is a potent $GABA_A$ agonist found naturally in the mushroom, *Amanita muscaria* (Johnston, 1996). Muscimol is slowly removed by uptake mechanism, making it more suitable for long-term

* Corresponding author at: Department of Biological Sciences, P.O. Box 305220, UNT Station, University of North Texas, Denton, TX 76203, United States.
Tel.: +1 940 565 3615; fax: +1 940 565 4166.
E-mail address: gwgross@unt.edu (G.W. Gross).

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quantitative studies (Simmonds, 1980). It is more potent then GABA (Johnston, 1996) and displaced stereo-specifically only by those drugs and amino acids that are known to interact with the synaptic GABA receptor (Beaumont et al., 1978). Bicuculline, a phthalide isoquinoline alkaloid and a competitive antagonist of $GABA_A$ receptors, blocks the hyperpolarization effect of GABA (Johnston, 1996). Gabazine (SR 95531 [2-(3′-carboxy-2′-propyl)-3-amino-6-*p*-methoxyphenylpyridazinium bromide]), a pyridazinyl derivative of GABA is a potent competitive antagonist of $GABA_A$ receptors (Wermuth et al., 1987). Trimethylolpropane phosphate (TMPP) is an ethyl bicyclophosphate produced during the partial pyrolysis of certain synthetic, ester-based turbine lubricants supplemented with phosphate-based lubricity additives (Centers, 1992). TMPP induces epileptiform activities in hippocampal CA1 neurons, and binds to the $GABA_A$-benzodiazepine receptors (Higgins and Gardier, 1990; Lin et al., 2001). It shows very low binding affinity for $GABA_B$, nonadrenergic, dopaminergic, or cholinergic receptors (Jung et al., 1995). These compounds were used to demonstrate that primary neuronal cultures can be effectively used for the determinations of dissociation constants.

2. Methods

2.1. Microelectrode arrays (MEAs)

The spike activity of neurons was recorded with 64 substrate-integrated microelectrodes located in a central 1 mm^2 area of 5 cm × 5 cm and 1-mm thick glass plates. Details of MEA fabrication can be found in previous publications (Gross, 1994; Gross et al., 1985). These plates are spin-insulated with methyltrimethoxysilane resin and de-insulated at the electrode tips via a laser shot. MEAs fabricated by the Center for Network Neuroscience (CNNS) feature transparent indium–tin oxide (ITO) substrate integrated conductors with electrode impedances of 0.6–0.8 MΩ at 1 kHz (measured in 0.8–1% saline) after electrolytic gold plating of the exposed ITO. In preparation for cell culture, the hydrophobic surface is flame-activated and treated with poly-D-lysine and laminin for better cell-surface adhesion (Gross, 1994).

2.2. Neuronal cell culture

Frontal cortex tissues were dissociated from embryos of HSd:ICR mice at age of E16-17. The care and use of animals, as well as all procedures involving animals in this study were approved by the institutional animal care and use committee of the University of North Texas and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). Cortices were minced mechanically, enzymatically digested with papain, triturated, combined with Dulbecco's Modified Minimal Essential Medium (DMEM) supplemented with 5% fetal bovine serum and 5% horse serum, and seeded at 80 K cells (all cell types present in the parent tissue) per ml on the MEA in volumes of 100 μl to a 3 mm diameter adhesion island. After 24 h, the cultures were transitioned to medium containing 5% horse serum, maintained at 37 °C in a 10% CO_2 atmosphere, and given half medium changes biweekly. This procedure generally yielded networks with 300–500 neurons per 1 mm^2 over the electrode array. Minimum culture ages for experiments were 3 weeks after seeding. Neurons grown under such condition developed shallow, three-dimensional

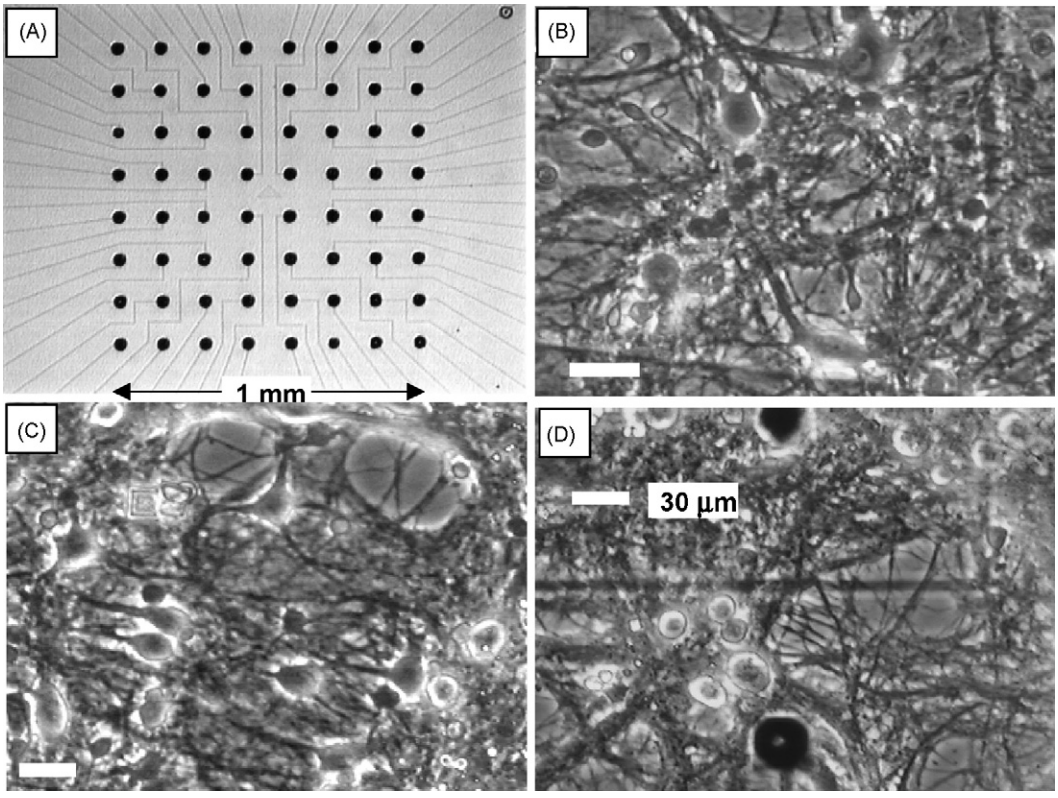

Fig. 1. Frontal cortex neurons in the living state on MEA surfaces seeded at approximately 80 k/ml. Age: 43 days after seeding. Density of surviving neurons is approximately 400 neurons per 1 mm^2. (A) Recording area of an 8 × 8 electrode matrix with equidistant electrode spacing of 150 μm. Conductors are 8 μm wide and consist of transparent indium–tin oxide. Recording craters with electrolytically deposited gold are 30 μm in diameter and provide impedance at 1 kHz of approximately 0.7 MΩ. (B–D) Networks formed on the insulation surface of the MEA (bar: 30 μm). Panel (D) shows two recording sites (arrows).