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XENON-INDUCED INHIBITION OF SYNCHRONIZED BURSTS IN A RAT CORTICAL NEURONAL NETWORK

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Abstract—Xenon (Xe) and other inert gases produce anesthesia via an inhibitory mechanism in neuronal networks. To better understand this mechanism, we measured the electrical signals from cultured rat cortical neuronal networks in a multi-electrode array (MEA) under an applied Xe pressure. We used the MEA to measure the firing of the neuronal network with and without Xe gas pressurized to 0.3 MPa. The MEA system monitored neuronal spikes on 16 electrodes (each 50 × 50 μm^2) at a sampling rate of 20 kHz. The embryo rat cortical cells were first cultured on MEAs without Xe for approximately 3 weeks, at which time they produced synchronized bursts that indicate maturity. Then, with an applied Xe pressure, the synchronized bursts quickly ceased, whereas single spikes continued. The Xe-induced inhibition-recovery of neuronal network firing was reversible: after purging Xe from the system, the synchronized bursts gradually resumed. Thus, Xe did not inhibit single neuron firing, yet reversibly inhibited the synaptic transmission. This finding agrees with the channel-blocker and a modified-hydrate hypothesis of anesthesia, but not the lipid-solubility hypothesis.

Key words: general anesthesia, multi-electrode array, synapse transmission inhibition, xenon molecule.

INTRODUCTION

General anesthesia is thought to be caused by the inhibition of neuronal firing from an agonist and/or an antagonist. One anesthetic is xenon (Xe), an inert gas that produces general anesthesia without causing undesirable side effects (Cullen and Gross, 1951). But the Xe-anesthesia mechanism remains unclear.

The Xe anesthetic effect was first thought to be caused by the cell membrane swelling with Xe (Miller et al., 1965; Miller, 1969; Lever et al., 1971; Koski et al., 1973; O'leary, 1984) due to Xe's high solubility in lipids (Meyer, 1937). On the other hand, Pauling (1961) and Miller (1961) proposed the hydrate hypothesis in which the signals are inhibited by a clathrate hydrate at synapses. Although this hypothesis was opposed by several researchers (e.g., Miller, 1969), it has received support from theoretical (Dorsch, 1970; Dorsch and deRocco, 1973), experimental (Schoenborn et al., 1964; Dorsch and Distefano, 1973) and clinical (Matsumoto, 1995) investigations. Recently, several pharmacological studies suggest that Xe molecules act as the antagonist of glutamate–induced channels. This mechanism differs from that of other general anesthetic agents, such as isoflurane and halothane, which activate the inhibitory γ-aminobutyric acid type-A ($GABA_A$) receptor channels (Franks and Lieb, 1982, 1988, 1994; Mihic et al., 1997). Some researchers claimed that Xe molecules strongly inhibit the excitatory N-methyl-D-aspartate (NMDA) receptor channels (Franks et al., 1998; de Sousa et al., 2000; Ma et al., 2002; Negale et al., 2005), but others suggested the target to be non-NMDA receptor channels (Plested et al., 2004) or both (Dinse et al., 2005; Preckel et al., 2006; Haseneder et al., 2008; Georgiev et al., 2010). Therefore, several general anesthetic mechanisms of Xe gas are under consideration.

The functioning of neurons in culture samples has made possible various studies in the field of neuroscience. When dissociated neurons start contacting each other via synapse formation, neuronal cells and networks show primitive patterns of synchronized activity by groups of neurons. Such patterns of electric activity occur in an early phase of network formation, often in close similarity to those seen *in vivo* (Ben-Ari, 2001; Corner et al., 2002; Khazipov et al., 2004). The pioneering work of Gross (1979), Gross and Schwalm (1994) and of Pine (1980) has evolved to the point where, as later shown by Robinson et al. (1993) and Jimbo et al. (1999), long-term multi-electrode registration can be used to study activity-dependent plasticity at the synaptic level. Dissociated neurons cultured *in vitro* autonomously form complicated networks that spontaneously show synchronized bursts (Kamioka et al., 1996; Opitz et al., 2002; Ito et al., 2010) that are highly variable in terms of their spatio-temporal firing patterns, yet highly correlated among neurons (van Pelt et al., 2004; Chiappalone et al., 2006; Wagenaar et al., 2006). Therefore, neuronal cultures *in vitro* on multi-electrode arrays (MEAs) are a useful tool for modeling the maturation of the neuronal networks and their electrophysiological properties.

We developed a model system for rat cortical neuronal networks *in vitro* on MEAs (Ito et al., 2010) to measure

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Abbreviations: BD, burst duration; DMEM, Dulbecco's modified Eagle medium; DIV, day *in vitro*; $GABA_A$, γ-aminobutyric acid type-A; *IBI*, inter-burst interval; MEA, multi-electrode array; MED, multi-electrode dish; NMDA, N-methyl-D-aspartate; *SA*, spike amplitude; *SBR*, synchronized-burst rate; *SIB*, number of spikes in a burst; *SR*, spike rate; *SW*, spike width; Xe, xenon.

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the development of neuronal electric activity. This system is useful to understand the spatiotemporal single spikes and bursts, not only for single neurons but also for the neuronal network. Here we apply this system to the Xe-anesthesia problem by exposing the neuronal network to pressurized Xe gas. We find that the pressurized Xe gas inhibits the synchronized bursts, but leaves the single spikes essentially unchanged.

EXPERIMENTAL PROCEDURES

Cell culture on MEAs

The sample preparation was almost the same as that in our previous work (Ito et al., 2010), so here we give only a brief description. Dissected cortex was prepared from Wistar rats at embryonic day 17 using the Nerve-Cell Culture System (Sumitomo Bakelite, Tokyo, Japan) as described previously (Mizuno et al., 2004; Banno et al., 2005; Takeuchi et al., 2005). Cortices were dissociated into single cells using dissociation solution (mainly papain), and then resuspended in Neuron Culture Medium [Sumitomo Bakelite; serum-free conditioned medium from 48-h rat astrocyte confluent cultures based on Dulbecco's modified Eagle's minimum essential medium (DMEM)/F-12 with N2 supplement, (Banno et al., 2005; Takeuchi et al., 2005)]. Dissociated neuron was plated with a nominal density of 2500 cells/mm^2 onto a poly(ethylenimine)-coated multi-electrode dish (MED) probe (Alpha MED Scientific, Osaka, Japan). The probe consisted of 64 planar microelectrodes and 4 reference electrodes (Kudoh et al., 2007; Hosokawa et al., 2008; Ito et al., 2010). Each electrode was $50 \times 50\ \mu m^2$ and the electrode spacing was 150 μm. To avoid cell attachment onto reference electrodes, we used a cloning ring with an inner diameter of 5 mm and total area of 19.6 mm^2 (Honma et al., 1998). The rings were removed after adhesion of neurons on the MED probe (approximately 3 h).

The cultures were incubated in the neuron culture medium in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C (that is, the physiological condition). After 3 days in the neuron culture medium (twice a week), half of the medium was replaced with fresh DMEM/serum medium, which consisted of DMEM (Invitrogen-Gibco, Carlsbed, CA, USA) supplemented with 5% fetal bovine serum (Invitrogen-Gibco), 5% horse serum (Sigma–Aldrich, St. Louis, MO, USA), 25 μg/mL insulin (Invitrogen-Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen-Gibco) (Ito et al., 2010). Thus the Neuron Culture Medium is gradually replaced with the DMEM/serum medium during culture. The adhesion, growth, and morphological changes of cultured neurons were observed under a phase-contrast microscope (Olympus, Tokyo, Japan; type CKX-41).

Firing-activity measuring system

The firing activity of each cortical culture was recorded at a sampling rate of 20 kHz using a MED64 extracellular recording system (Alpha MED Scientific), and the A/D conversion was done with the MED64 conductor software. To observe the neuronal network maturation, we measured the firing activities of cultured neurons on the MED probe several times per week. Based on the firing activity exhibiting periodical synchronized bursts, the samples used in the gas-pressurizing experiments were between 20 and 30 days *in vitro* (DIV).

To measure the firing activities under pressurized gas conditions, we constructed a high-pressure vessel equipped with the MED connector (Taiatsu Techno, Tokyo, Japan). Since the measurement system was in a non-humidified incubator, the MED probes of cortical cultures were taken from the cultivation incubator. The pressure was monitored by a pressure transducer (Yokogawa; type FP201-C22-C20A*B) and the pressure could reach 1 MPa. The cell shape was monitored through the pressure-proof glass windows by a macro-zoom lens + CCD camera system (Elmo, Nagoya, Japan; type TVZ610 M-P). The 16 electrodes at the center of the MED probe could be monitored in this vessel. The system was placed in an incubator (Fukushima Industry, Osaka, Japan; type FMU-132I) in which the temperature was controlled within ±1 °C. A type-T thermocouple measured the temperature in the vessel. Pressure and temperature were recorded by a data logger (Graphtec, Yokohama, Japan; type GL400). The whole system is illustrated in Fig. 1.

The experimental procedure has four periods, distinguished by the gas pressure and gas composition. Traces are shown in Fig. 2 and described as follows. Period (I), the control period: After reducing the culture medium to less than half of its original volume (to minimize the time-lag due to the dissolution process of gas in the medium), the MED probe with the cortical culture was put in the high-pressure vessel and incubated at 38.5 ± 1 °C while the firing activity was measured. Period (II), the pressurizing period: Following the control period, Xe gas was introduced to the vessel at a gauge pressure of approximately 0.3 MPa. Because the original atmospheric air had a pressure of 0.1 MPa, the Xe fraction in the gas of the vessel is 0.75 and the total ambient pressure is 0.4 MPa. We applied Xe gas at this pressure because a Xe concentration of 75% in the vessel approximates the minimal Xe concentration effective for human anesthesia of about 71% (Goto et al., 1997). The pressurizing period lasted one hour. Period (III), the pressure-released period: The release valve was opened to reduce the pressure in the vessel to atmospheric pressure (0.1 MPa). However, the gas composition in the vessel is still Xe-rich. Period (IV), the purge period: One hour after the depressurization, we let the purging gas flow for several minutes to flush out Xe in the vessel. After 1-h of measurements in this period, we returned the MED probe to the cultivation incubator. Then, several days after the experiment, we checked the cultured sample for damages. To check the reproducibility of the results, we ran the experiments for eight different cultured samples.

Xe gas used for experiments had a purity of 99.995% (Air-Water, Wakayama, Japan) and the purging gas was air that contained 5% CO_2 (Hokkaido Air-Water, Sapporo, Japan). The purging gas had the same composition as that in the cultivation incubator.

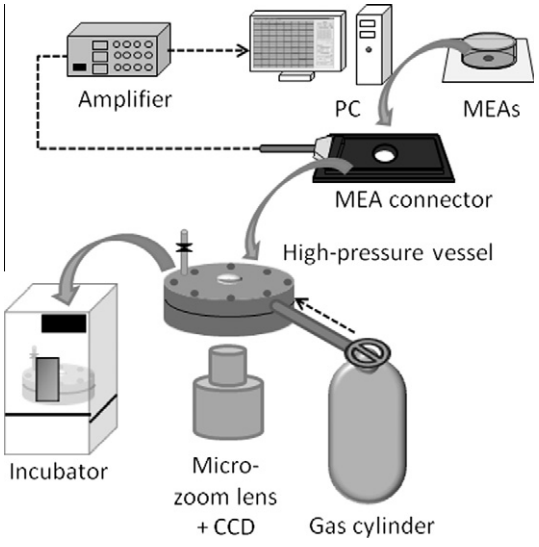

Fig. 1. The experimental system. The cortical neuronal network grown on a multi-electrode array (MEA) is set in the MED connector within a high-pressure vessel in the incubator.