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The effect of hyperbaric air on the electric activity of neuronal *in vitro* networks

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ABSTRACT

Breathing hyperbaric air or gas mixtures, for example during diving or when working underwater is known to alter the electrophysiological behavior of neuronal cells, which may lead to restricted cognition. During the last few decades, only very few studies into hyperbaric effects have been published, especially for the most relevant pressure range of up to 10 bar. We designed a pressurized measuring chamber to record pressure effects on the electrical activity of neuronal networks formed by primary cells of the frontal cortex of NMRI mice. Electrical activity was recorded with multi-electrode arrays (MEAs) of glass neuro chips while subjected to a step-by-step pressure increase from atmospheric pressure (1 bar) to 2 and 4 bar, followed by a decompression to 1 bar, in order to record recovery effects. The effects of pressure on the total spike rates (TSRs), which were averaged from at least 45 chips, were detected in two cell culture media with different compositions. In a DMEM medium with 6% horse serum, the TSR was increased by 19% after a pressure increase to 2 bar and remained stable at 4 bar. In NMEM medium with 2% B27, the TSR was not altered by a pressure increase to 2 bar but increased by 9% at 4 bar. After decompression to 1 bar, the activities decreased to 76% and 101% of their respective control levels in the two media. MEA recordings from neuronal networks in miniaturized hyperbaric measuring chambers provide new access for exploring the neuronal effects of hyperbaric breathing gases.

1. Introduction

Investigations on the influence of hyperbaric gases on cells have been performed with biopsy tissue of human subjects or with experimental animals (Baddeley et al., 1968; Kerem et al., 1995; Fenn, 1967; Turle-Lorenzo et al., 1999; Weltman et al., 2000; Bennett, 1989). Most of the investigations have been carried out between the 1960s and 1980s and not been confirmed ever since, using more advanced technologies. One research focus was the investigation of how micro-bubbles originate and grow in the blood and tissue volumes, for example with Doppler ultrasonography (Balldin and Borgström, 1976; Ikeda et al., 1989; Neuman et al., 1976; Daniels, 1984). A second focus was the investigation of the cognition restriction of subjects under hyperbaric conditions with cognitive tests in pressurized chambers (Baddeley et al., 1975; Biersner and Cameron, 1970; Lewis and Baddeley, 1981; Logie and Baddeley, 1983, 1985), and a third focus was the phenomena of decompression accidents, which have been studied on testing animals, which could be dissected after the experiments. Human subjects were appointed only under controlled conditions and for relatively safe experiments. Nowadays, hyperbaric gases (especially oxygen) are also used in the postoperative or regenerative medicine, i.e. in the treatment of diving accidents and in the hyperbaric oxygen therapy (Chew et al., 1969; Lillehei et al., 1964; Mutschler and Muth, 2001).

Only few cell-physiological experiments have been carried out in the pressure range below 10 bar, which is most important for recreational and commercial diving or underwater work. One reason was the lack of appropriate experimental methods. The experimental equipment used in literature reports was comparatively bulky and expensive (Jackson, 1968; Hochachka and Storey, 1975; Murphy et al., 1980; Castellini et al., 1985, 1992; Dean and Mulkey, 2000; Dean et al., 2003; D'Agostino et al., 2009). Nevertheless, many of the experimental results are not comprehensible and the detected effects only partially understood.

The number of *in vitro* experiments on the influence of hyperbaric gas on the cellular physiology is very limited and only some of them were carried out on the electric activity of neurons (Dean and Mulkey, 2000; Stoetzer et al., 2012; Huang et al., 2000; Sébert, 2010).

It is known, that pressurized inert gases, like nitrogen, helium or argon may cause symptoms that are comparable to a weak narcosis or alcohol intoxication (Bennett et al., 1967; Marshall, 1951; Behnke et al., 1935; Haldane, 1941; Hobbs, 2008). A

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consideration of Bunsen's solubility coefficient of gases in oil showed an increasing narcotic effect for gases of higher liposolubility at 37 °C (Behnke and Yarbrough, 1939; Bennett et al., 1967; Roth and Seeman, 1972). The different liposolubilities of gases result in different changes of the physiological, physical and electrical properties of cell membranes (Seeman and Roth, 1972; Hills and Ray, 1977; D'Agostino et al., 2009), partly similar to those already known from alcohols and anesthetics (Frangopol and Mihailescu, 2001; Yun et al., 2002).

The high liposolubility of nitrogen and its incorporation into the cell membrane increases the lateral membrane pressure, leading to a "swelling" of the cell membrane (Bennett et al., 1967; D'Agostino et al., 2009). For neurons, the induced changes in the geometric and electric properties of their cell membranes may lead to alterations in the signal quality, magnitude, and propagation velocity of the action potentials (Grossman and Kendig, 1988; D'Agostino et al., 2009; Sébert, 2010). Neurons do not only show altered signal propagation properties along their axons or dendrites, but also a delay in the signal coupling across their synapses (Bryant and Blankenship, 1979; Sauter, 1979a,b; Dean and Mulkey, 2000). In networks with a small number of neurons, the electric activity was found to be reduced by the latter effect (Hills and Ray, 1977; Sauter, 1979a,b; Grossman and Kendig, 1988; Hamilton et al., 1995; Dean and Mulkey, 2000; Levett and Millar, 2008; Pendergast and Lundgren, 2009). These properties of nitrogen are probably the reasons for the similarity of the effects to an alcoholic stupor in the human brain, which led to the term "rapture of the deep" for the nitrogen narcosis (Behnke and Yarbrough, 1939; Baddeley et al., 1968; Roth and Seeman, 1972; Davis et al., 1972; Bennett, 1986).

We present a new miniaturized hyperbaric chamber containing a custom made glass neuro chip (GNC; Koester et al., 2010; Reimer et al., 2012) with a multi-electrode array (MEA) (Thomas et al., 1972; Gross et al., 1977; Gross et al., 1985) for detecting the electric activity of neuronal networks under the influence of hyperbaric air. MEAs are common tools for the detection of the spontaneous electric activity of neuronal *in vitro* networks under the influence of certain substances (Gross, 1995; Johnstone et al., 2010; McConnell et al., 2012; LeFew et al., 2013). Their use for animal replacement is currently being tested by many groups. Measuring hyperbaric effects on the electric activity of neuronal networks with the MEA technique in a hyperbaric chamber is a new approach. For this, our small custom made GNC (chip size $16 \times 16\ mm^2$) is especially suitable. We think that the approach may contribute to extend our knowledge on the influence of hyperbaric air or breathing gases on neuronal networks.

2. Material and methods

2.1. Glass neuro chip

The central element of the measuring setup is a custom made GNC (Fig. 1) (Koester et al., 2010; Reimer et al., 2012). It consists of a 16 mm × 16 mm, 1.1 mm thick glass chip carrying 100 nm-thick platinum sensor structures. Their on-chip connectors are passivated with 1.2 μm – thick silicon nitride layers. A 4 mm high glass trough with inner and outer diameters of 8 and 10 mm and a volume of 240 μl was glued onto the glass chip with MED-1511 (Nusil Technology LLC, USA). The GNC is autoclavable and reusable for more than 16 times. Some of our GNCs are in use for more than 4 years. Besides the MEA with 52 electrode pads (diameter 25 μm, inter-pad distance 100 μm) our GNC features an interdigitated electrode structure (IDES) for cell-adhesion measurements (Ehret et al., 1997; Baumann et al., 1999; Koester et al., 2010; Buehler et al., 2011), a resistive temperature sensor (PT1000), two pH sensors, and two ground electrodes (Koester et al., 2010; Reimer et al., 2012). The GNC contact pads (diameter 0.5 mm) at the four edges of the GNC were electrically contacted by gold-plated spring needle contacts (compare to Fig. 1).

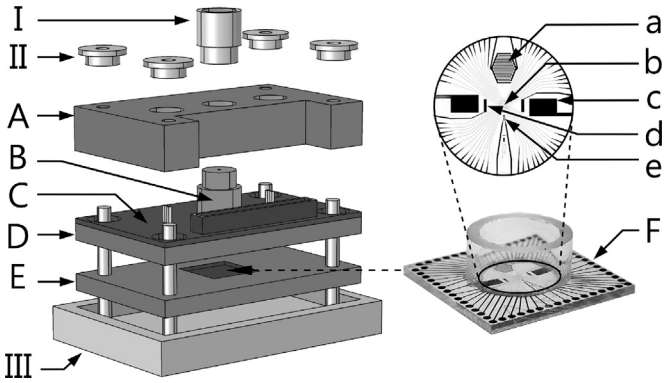

Fig. 1. Sketch of the experimental setup. A: cover; B: high-pressure cylinder; C: circuit board with connectors; D: holder for spring needle contacts with high-pressure cylinder; E: chip holder; F: GNC with a: IDES, b: MEA, c: pH sensors for future use, d: ground electrodes, e: temperature sensor; I: high-pressure connector; II: knurled-head screws; and III: base plate with heating element.

2.2. Experimental setup

The experimental setup consisted of a base plate with the heating element of the temperature control unit, a chip holder, the high-pressure cylinder, a hand pressure pump (WIKA Alexander Wiegand SE&Co. KG, Germany), a home-made head stage (Koester et al., 2010), a Plexon 64-channel amplifier (Plexon Inc., USA) and a PC.

The pressure was applied through a high-pressure connector to the high-pressure cylinder, which was fitted over the GNC trough and sealed with a silicone sealing ring on the chip surface outside the trough (Fig. 1). The pressure was adjusted with a hand pressure pump. The top ends of the spring needle contacts connected the chip pads to a circuit board with an electronic standard connector for the head stage. Its output signals were fed into the Plexon amplifier.

For experiments, the GNC was put into the chip holder and the silicone sealing ring slipped on the trough (Fig. 1). Besides its role as a pressure gasket, the ring prevented moisture from reaching the GNC pads. During the measurements, the GNC was covered with a 0.025 mm-thin membrane of fluorinated ethylene–propylene (FEP–Teflon® film with a water vapor transmission rate of $6.2 \times 10^{-3}\ g/cm^2$ per day at 40 °C, Bohlender GmbH, Germany) allowing for the diffusion of gas, though efficiently reducing the vaporization of water from the culture medium in the GNC (Potter and DeMarse, 2001). This was important for a stable osmolarity during the experiments. The pressure setup was assembled at the base plate using knurled-head screws.

For measurements, the temperature control unit was adjusted to 37 °C (Fig. 1). Data was recorded with a PC (Windows 8.1 pro) with MEA-Server and MEA sort client software (version 1.3, Plexon Inc., USA) as well as VernAC (a generous gift of Prof. G. Gross, University of Texas, Denton, USA) for data recording.

2.3. Measuring parameter

To consider the electric activity of the networks, the total spike number of all units per minute, i.e. the total spike rate (TSR) was used as an integrative parameter for each GNC. Single action potentials were separated by hand, using the MEA sort client software. This software allows for separating up to 4 different