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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).

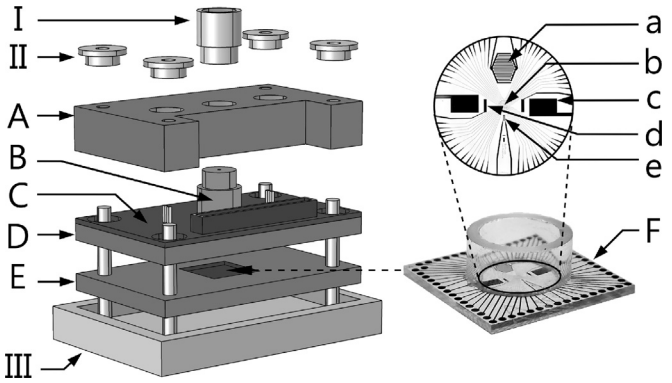

Fig. 1. Sketch of the experimental setup. A: cover; B: high-pressure cylinder; C: circuit board with connectors; D: holder for spring needle contacts with high-pressure cylinder; E: chip holder; F: GNC with a: IDES, b: MEA, c: pH sensors for future use, d: ground electrodes, e: temperature sensor; I: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

waveforms (units) per electrode, which were assumed to originate from individual nerve cells. The waveform and time index (of the AP trigger level) of each action potential were separately recorded. Besides the TSR, the VernAC program provided the mean spike numbers per minute of all active units per chip. For data analysis, only the TSRs were considered.

2.4. Measuring procedure

When the GNCs were taken from the culture, their troughs were full to the line. Before they were fitted into the measuring setup 50 µl cell culture medium were removed. This amount was sufficient to check the osmolarity and pH of the medium with an osmometer (Osmomat 030, Gonotec GmbH, Germany) and a pH meter (Seven2Go S2-meter, Mettler-Toledo GmbH, Germany). After taking the GNCs from the cell culture, their pH and osmolarities ranged from 7.5 ± 0.2 and 323.3 ± 13.25 mOsm/l. From the literature, osmolarity changes below 50 mOsm/l are known to be uncritical for neuronal cell cultures (Potter and DeMarse, 2001), while a decreasing extracellular pH is known to decrease the TSR by approx. 7.7% per 0.1 pH (Balestrino and Somjen, 1988; Jarolimek et al., 1989; Chesler, 1990; Chesler and Kaila, 1992). The checks were repeated 24 h after the GNCs were transferred to the setup. The osmolarities and pH values were protocolled and used to estimate their values after 10 h of measurements, assuming linear changes for both parameters. The recorded TSRs were not used when the estimated changes were larger than 50 mOsm/l or 0.3 pH.

Measurements on the pressure-dependence of the TSRs were finished after 10 h. TSR data recorded later than 10 h were only used to control the long-term stability of the neuronal networks without medium exchange. The pressure-dependent measurements were started at the ambient pressure of 1 bar. After a stable TSR was recognized, the control data was recorded for approx. 30 min, before the pressure was doubled to 2 bar and again increased to 4 bar after constant TSRs were observed. Finally, the system was depressurized to the ambient pressure at a pressure reduction rate of approx. 1 bar/min in correspondence to the ascend velocities recommended in diving tables. Unfortunately, the adjustment of an exact decompression rate was tricky in our setup with the hand driven pressure pump.

2.5. Chip handling and cell culture

2.5.1. Chip preparation

For chip cleaning, the chips were washed with distilled water and incubated for 2 h in a 1% Terg-a-zyme solution (Alconox, Inc., USA) at 37 °C. The chips were manually cleaned with Q-tips, deposited in ultra-pure water (Carl Roth GmbH&Co. KG, Germany) for 12 h, finally autoclaved at 121 °C for 15 min and dried.

For cell seeding, the MEA areas of the chips were coated with 10 µl of a 0.05% polyethyleneimine solution (PEI, Sigma-Aldrich Chemie GmbH, Germany) in borate buffer with a pH 8.5 (Sigma-Aldrich Chemie GmbH). After incubation at 2–8 °C for 12 h, the chips were washed three times with ultra-pure water for 5 min. After drying, a second coating layer of 10 µl laminin solution, diluted 1:60 in Dulbecco's modified Eagle's medium (DMEM, Biochrom AG, Germany), was applied to the MEA surface. Before cell seeding, the chips were stored under 10% CO_2 at 35.5 °C for at least 1 h.

2.5.2. Cell culture media

A D10/10 medium contained 79% DMEM with glutamine, 10% fetal bovine serum (Biochrom AG), 10% horse serum (Biochrom AG) and 1% penicillin/streptomycin (P/S, Biochrom AG). It was used for cell-seeding, while the on-chip cell cultures were conducted in D6 or, alternatively in NMEM+B27 media.

The D6 cell culture medium supported the incubation with 10% CO_2 and consisted of 93% DMEM with glutamine, 6% horse serum, and 1% sodium-pyruvate (Biochrom AG). The NMEM+B27 medium supported the incubation with 5% CO_2. This medium contained 10% DMEM, 1% sodium pyruvate, 1% stable glutamine (Biochrom AG), 2% B27 (Invitrogen, Germany), 1.25% of a 20% glucose solution (final concentration 7 g/l, Carl Roth GmbH&Co. KG), 4% of a 5.5% $NaHCO_3$ solution (final concentration 2.2 g/l, Biochrom AG) and 80% ultra-pure water. While this serum-free medium was specially developed to support the growth and viability of neurons (Brewer et al., 1993; Brewer, 1995), both culturing media were selected for stabilizing the glia and neuron cell numbers at their *in vivo* ratio.

2.5.3. Cell preparation

To our knowledge, neuronal networks have been successfully derived from stem cell lines only by very few groups (Toivonen et al., 2013; Heikkilä et al., 2009) and neuronal cell lines are not yet used on regular basis for MEA experiments. The main problem in all approaches is the stability and reproducibility of the differentiation of specific neurons or neuronal subtypes. Because primary cells are still the gold standard for MEA measurements (Gross et al., 1977; Gross, 1995; Johnstone et al., 2010; Parenti et al., 2013), we chose primary cells of the frontal cortex of embryonic mice for our *in vitro* model.

Neuronal primary cells, were prepared from 16-day old embryos of NMRI mice, which were provided by the “Animal House” core facility at the University of Rostock. For the extraction of the neuronal primary cells of the frontal cortex, the mouse embryos were stored in cold DISGH-buffer (Ransom et al., 1977). After harvesting the brains of the embryos, they were stored in cold DISGH-buffer, the frontal cortex was cut and the olfactory bulbs and meninges were removed.

For enzymatic digestion of the cortices, the DISGH-buffer was removed and the cortices were incubated with 3 ml of the papain–DNase mix for 5 min at 35.5 °C. The mixture of 3 ml papain (Roche Applied Science, Germany) in DISGH-buffer (10 Units/ml) was supplemented with 0.2 mg/50 µl DNase I (Roche Applied Science, Germany). For cell separation, the suspension was gently mixed three times using a pipette, incubated for 5 min and again gently mixed before transfer into two falcon tubes. Each falcon tube contained 3 ml of preheated D10/10 medium. After 10 times of gentle mixing, the cell suspension was centrifuged at 800 rpm for 2 min. The cell pellets were gently resuspended in 1 ml of fresh D10/10 medium and the suspension was let at rest to allow for the sedimentation of debris. The supernatants were pooled in a fresh falcon tube. After determination of the cell count with a Neubauer chamber, the cell concentration was adjusted to 5×10^6 cells/ml. This cell concentration approx. corresponds to half of the mean neuronal density of 1.32×10^4 cells/mm^3 in layer three of the middle frontal cortex of adult humans (Huttenlocher, 1979). We found that seeding higher cell counts may cause problems in cell culturing and does neither increase the signal quality nor quantity. The day of cell preparation was defined as “day *in vitro* zero” (DIV0).

2.5.4. Glia cell culture

In order to improve the viability of the neurons in the GNCs, the excess cell suspensions of the cell preparation were used for culturing glia cells in a co-culture. For this, the cell suspensions were seeded in two cell culture flasks with growth areas of 25 cm^2. After 24 h of cell growth, the cells were washed with preheated PBS-buffer (w/o Ca^{2+}, Mg^{2+}) and the cell culture medium was exchanged. This step was repeated every three to four days. After the cell layer reached a confluence of 70–80%, the cells were

resuspended using trypsin–EDTA (PAN Biotec GmbH, Germany), split 1:3 and cultured in 5 ml D10 medium in two culture flasks. For co-culture, the glia cells were split again and 50,000 cells were seeded in cell culture dishes (diameter 6 cm) using 5 ml of D10 medium. After 3 days of glia cell growth (i.e. DIV0 of neuronal cell culture), the D10 medium was exchanged with 10 ml of NMEM+B27 to allow the glia cells to precondition the medium for co-culture. Small paraffin spacer spots (height and distance of approx. 2 mm and 10 mm) in the cell culture dishes ensured the distance between the bottom of the GNCs and the cell culture dishes.

2.5.5. On chip cell culture

At DIV0, 5 or 10 µl of the neuronal cell suspension were seeded onto the MEAs of the GNCs and the cells were allowed to adhere for 1 h. Then, the D10/10 medium was exchanged by one of the two cell culture media D6 or NMEM+B27. After 24 h (DIV1), the D6 containing GNCs were placed in cell culture. This procedure was repeated with the NMEM+B27 containing GNCs that were placed in the cell culture dishes containing the prepared glia cells. The dishes were filled with the respective media to the complete coverage of the GNC troughs. The GNCs were incubated for 21 days at 35.5 °C with 10% (D6) or 5% (NMEM+B27) CO_2. 1/3 of media was exchanged only twice a week and the osmolarity checked regularly. After DIV21 the neuronal networks on the GNCs show complex and coordinated activity and can be used for measurements (Gross, 1995).

2.6. Gas diffusion time measurements

For measuring the gas diffusion across the cell culture medium volume, oxygen was used as a tracer, because of the lack of optical nitrogen sensors, which can be used in the cell culture. The diffusion coefficients of nitrogen (2×10^{-5} m^2 s^{-1}) and oxygen (2.4×10^{-5} m^2 s^{-1}) are very similar (Lide, 2005). For measurements, fluorescence-based optical oxygen-foil sensors (Presens, Regensburg, Germany) were used. Spots of the foil sensors were glued outside the MEA areas onto the bottom inside the GNC troughs. The oxygen concentrations were measured with an optical fibre from underneath the trough bottoms. To determine the characteristic diffusion time of oxygen across the cell culture media, the measuring data was fitted using a three-parameter exponential rise to maximum function.

2.7. Data analysis

The TSR data analysis was performed using a specially written Matlab® (MathWorks GmbH, Germany) script. To determine the TSRs at the given pressure plateaus, the data records of each network were checked for the stabilization of their TSRs after the pressure was adjusted to a new plateau. Only data recorded after TSR stabilization were stored to matrices. Then all data points were normalized to the average of their respective control values at atmospheric pressure, before they were summarized in pressure classes and stored. For statistical analysis a “one-sample Kolmogorov-Smirnov test” (ks-test) was performed to check the data for a normal distribution at every pressure plateau. For a positive ks-test, i.e. confirmation of the null hypothesis with a significance level of 5%, an “one way ANOVA” test and a “multi comparison” test were used to check for significant differences between the measuring data at the different pressure plateaus. If the ks-test failed, “Kruskal-Wallis” (h-test) and “multi comparison” tests were used to check the measuring data for significant differences at the different pressure levels (compare to asterisks in Fig. 4 and Matlab® documentation: kruskalwallis and multcompare). Sigmaplot (Systat Software GmbH, Germany) was used for data presentation using box-and-whisker plots.

3. Results and discussion

3.1. Pressurized measurements

During experiments, the GNC was thermostated to 37 °C, the averaged body core and brain temperatures of humans.

A critical point in the measurement of neuronal activities is the electric noise of the system. For noise reduction, the silicone sealing ring was important, which ensured the dryness of the spring needle contacts with the GNC. The electric noise was generally below 2 μV_{pp}, allowing for the detection of neuronal signals down to 2.5 µV.

3.2. Osmolatrity and pH shift

The FEP film covering the GNCs could largely prevent water evaporation from the cell culture trough. On average, the osmolarity changed from 323.3 ± 13.25 mOsm/l (mean and standard deviation) to 377.8 ± 38.8 mOsm/l during 24 h, corresponding to an increase of 16.9%. Accordingly the osmolarity increased by approx. 7% within the measuring time of 10 h assuming a continuous evaporation of water. This change is clearly below the critical value of 50 mOsm/l (Potter and DeMarse, 2001).

Within 24 h the averaged pH decreased from 7.5 ± 0.2 to 7.1 ± 1.1 by approx. 6%. From these values, a pH decrease of approx. 0.2 pH steps can be extrapolated within the 10 h of measuring time. Nevertheless, control measurements without pressure changes did not show a significant decrease of the TSR within the measuring time. These considerations let us assume negligible influences of the observed osmolarity and pH changes on the detected TSRs.

3.3. Oxygen diffusion time measurements

At every new pressure plateau, 95% saturation of the new oxygen partial pressure equilibrium was reached throughout the cell culture medium in approx. 70 min (Fig. 2). For the two cell culture media, no differences in the diffusion times of oxygen could be observed. For nitrogen, we estimated an approx. time of 58 min from the differences in the diffusion coefficients.

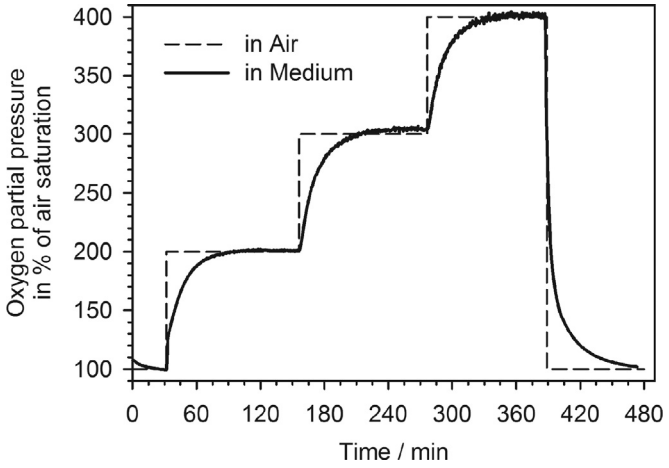

Fig. 2. Time dependent diffusion of oxygen across a layer of cell culture medium (height: 4 mm, volume: 200 µl).

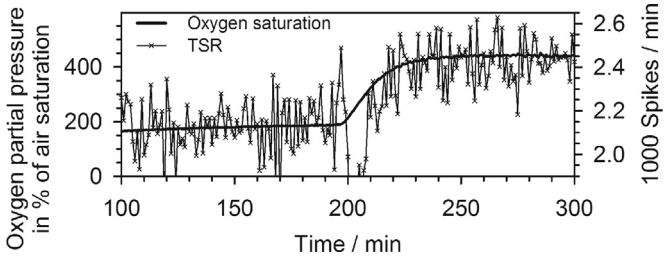

Fig. 3. TSR and oxygen saturation (continuous line) change induced by a pressure step from approx. 2 bar to 4 bar in NMEM+B27 cell culture medium.

3.4. Pressure dependence of the total spike rate

Fig. 3 shows the changing TSR for a pressure increase from 2 to 4 bar in NMEM+B27 cell culture medium. The TSR increase corresponded to the time dependence of the O_2 partial pressure. The observed changes in the TSR can be interpreted as the immediate results of the incorporation of gas into the cellular membrane phase, especially of nitrogen as a partial gas with a 78% volume portion in air.

In a number of cases, a decrease of the TSR as shown at 200 min in Fig. 3 could be observed directly after pressure application. This probably indicates the influence of the hydrostatic pressure on the neuronal network as already shown by Bryant and Blankenship (1979). These authors did not observe the effect in every experiment. In our experiments, the effect was not characteristic for certain networks because once it was observed in a network, it could usually not be reproduced for other pressure-increase steps during the same measurement.

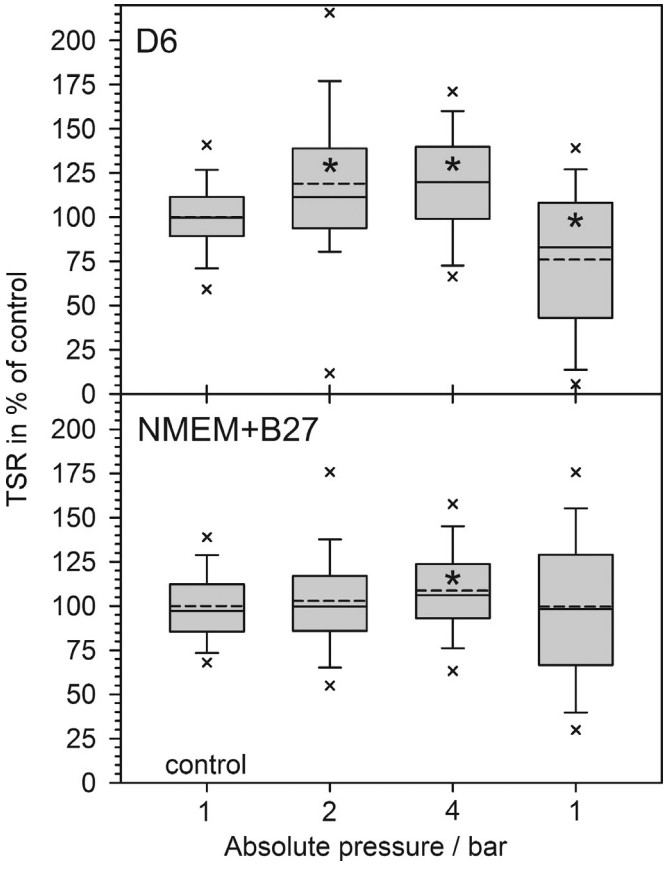

Fig. 4. Box-and-whisker plot of the pressure dependent TSR in different cell culture media. The horizontal solid and dashed lines represent the medians and means, respectively. The crosses mark the 5% and 95% percentiles. Boxes marked with an asterisk are significantly different to their control ($p < 0.05$). The numbers of experiments were 27 (D6) and 18 (NMEM+B27). The quantities for the plots are $n=1480$, 788, 661 and 2796 for D6 and $n=816$, 842, 744 and 2210 for NMEM+B27 (left to right).

The TSRs showed a different pressures behavior in the D6 and NMEM+B27 cell culture media (Fig. 4). In D6 medium a significant ($p < 0.05$) increase of the TSR by approx. 19% could be observed for a pressure increase to 2 bar. A further increase to 4 bar did not result in a further increase of the TSR. Also preliminary experiments at 5 and 9 bar did not show any pressure dependent TSR increase above the 19% already registered at 4 bar. After decompression to 1 bar the TSR decreased to approx. 76% of the control level.

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 (Fig. 4). After decompression to 1 bar, the TSR decreased to 101% of its control level.

Interestingly, the TSRs showed a wider variance at the first pressure plateau (2 bar) than at 4 bar in D6 medium. Especially the variance was increased to higher TSR. We interpret this as an evidence for a beginning diffusion of nitrogen into the cell membrane, which may lead to changes in the physical membrane properties.

In all cases, the TSR showed increased variability after decompression to 1 bar than their respective controls. We interpret this as a decompression effect. One reason probably was that the manually operated pressure pump did not allow for a continuous pressure reduction. This might have induced unwanted decompression effects, for example in one case when all cells died after an accidental zero time decompression. The investigation of such effects is projected in future experiments, which require the implementation of an automated pressure controller.

With 78%, nitrogen has the highest partial volume in air together with a high liposolubility. We therefore attribute most of the observed effects to the incorporation of nitrogen into the cellular membrane phase (Bennett et al., 1967; Bryant and Blankenship, 1979; Dean and Mulkey, 2000). Fig. 3 suggests that the TSR increase is an immediate result of this incorporation. Under high nitrogen partial pressure, a swelling of lipid membranes has been described by Bennett et al. (1967). These authors predicted that a nitrogen partial pressure of 5.1 bar absolute pressure, comparable to a diving depth of 41 m, has the same narcotic impact as a nitrous oxide concentration of 57% under atmospheric pressure.

Our findings on the pressure dependent TSR increase are in line with the results presented by Dean and Mulkey (2000). In their experiments, the TSRs of neurons in rat brain-stem slices increased by a factor of approx. 3 for a pressure increase to 4 bar. Nevertheless, these authors measured the activity of single neurons in a network with the patch clamp technique using hyperbaric oxygen and helium. In an *in vitro* setup with rat superior cervical ganglia cells, Sauter (1979a) showed an increase of the electric trans-synaptic response of single ganglions under the influence of nitrogen (8 bar) while the preganglionic activity of the same fiber remained stable.

It is not clear, whether the increased *in vitro* activities may explain *in vivo* findings, which rather point at a decrease of the neuronal activity. Under hyperbaric nitrogen, Marshall (1951) described a reduction in the brain waves and reflex activity of frogs and mice while humans showed a decreased cognition under hyperbaric nitrogen (Baddeley et al., 1975; Logie and Baddeley, 1985).

Checks of the TSR for up to 24 h suggested a recovery trend of the TSR under atmospheric pressure after the measuring period of 10 h. This effect was already described by Sauter (1979a) and Dean and Mulkey (2000). In our current setup, a more thorough investigation of the long term recovery effect was hindered by the

changes in pH and osmolarity of the cell culture medium beyond the tolerable limits.

Our literature review showed that the differences between *in vivo* and *in vitro* findings cannot be consistently explained. One reason for this situation may be that different neuronal cell types may respond differently to membrane swelling or hydrostatic pressure changes. Even if the pressure effects on the different cell types would be comparably, their different roles within a neuronal network may yield different results for the network activity. The suppression of an inhibitory neuron may for example result in an increased TSR. Currently, we combine a pressure-tight, self-contained perfusion system with the existing MEA setup to ensure stable cell-culture conditions and continuous nutrition supply over measuring periods longer than 24 hours.

4. Conclusion

Our literature review showed that many differences between *in vitro* and *in vivo* findings on hyperbaric pressure effects cannot yet be consistently explained. Neuronal *in vitro* networks on MEA in miniaturized GNC chambers provide new access for electrophysiological measurements under hyperbaric pressure. This new approach combines existing conventional techniques. The small measuring chambers are easier to handle than special patch-clamp setups. A comparison of the new *in vitro* data from small neuronal networks with *in vivo* results may give indications of the mechanisms of hyperbaric air and breathing gas effects, for example on their influence on the electrophysiology of neurons.We observed two different pressure effects in the TSR records of neuronal *in vitro* networks under the influence of hyperbaric air. As a first (and reproducible) effect, TSR was correlated to the applied pressure. This effects depend on the composition of the cell culture medium. One possible explanation for medium-dependent effects may be related to the influence of the medium on the glia-cell proliferation after the seeding of a certain number of neuronal cells. Such influence of different neuronal cell types on the observed pressure effects needs further investigation. A second, apparently sporadic effect was the immediate TSR reaction to alterations in the static pressure, which has already been described in the literature.

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