



Terrestrial Trunked Radio (TETRA) exposure of neuronal *in vitro* networks

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ABSTRACT

Terrestrial Trunked Radio (TETRA) is a worldwide common mobile communication standard, used by authorities and organizations with security tasks. Previous studies reported on health effects of TETRA, with focus on the specific pulse frequency of 17.64 Hz, which affects calcium efflux in neuronal cells. Likewise among others, it was reported that TETRA affects heart rate variability, neurophysiology and leads to headaches. In contrast, other studies conclude that TETRA does not affect calcium efflux of cells and has no effect on people's health.

In the present study we examine whether TETRA short- and long-term exposure could affect the electrophysiology of neuronal *in vitro* networks. Experiments were performed with a carrier frequency of 395 MHz, a pulse frequency of 17.64 Hz and a differential quaternary phase-shift keying ($\pi/4$ DQPSK) modulation. Specific absorption rates (SAR) of 1.17 W/kg and 2.21 W/kg were applied.

In conclusion, the present results do not indicate any effect of TETRA exposure on electrophysiology of neuronal *in vitro* networks, neither for short-term nor long-term exposure. This applies to the examined parameters spike rate, burst rate, burst duration and network synchrony.

1. Introduction

Due to the globally advancing digitalization, the use of mobile communication systems and their mobile devices is continuously growing, which results in an area-wide rise of radiofrequency electromagnetic field (RF-EMF) exposure of the human body (International Telecommunication Union, 2016; Rennhoff and Routon, 2016; Valentini et al., 2007). Possible health effects of RF-EMF on the human body are still in discussion and have not finally been clarified (Apollonio et al., 2013; Frey, 1961).

It is well known that RF-EMF belong to the non-ionizing part of the electromagnetic spectrum. Thus, direct damage of DNA or an indirect damage of the DNA by free radicals does not occur, because quantum energy is not sufficient to free electrons from atoms or molecules (Sheppard et al., 2008). The undisputed and possibly only interaction of RF-EMF with biological cells and tissue is based on dielectric heating and is declared as thermal effect. Which leads to temperature increase in cells and tissue (Apollonio et al., 2013; Independent Advisory Group on Non-ionizing Radiation, 2012; International Commission on Non-Ionizing Radiation Protection, 1998; Sheppard et al., 2008). The occurrence of non-thermal effects within cells, tissue and organism however are still in controversial discussion (Juutilainen et al., 2011; Manna and Ghosh, 2016; Simko et al., 2016; Valentini et al., 2007).

Nevertheless, there is no mechanism known that explains the observed effects with certainty (Apollonio et al., 2013; Sheppard et al., 2008).

The implementation of Terrestrial Trunked Radio (TETRA) in Europe, a digital radio communication standard for authorities and organizations with security tasks (e.g. police forces) gives new reason for discussions regarding possible effects of RF-EMF on human health. The frequency range of TETRA varies between 380 and 400 MHz in Europe and thus has a frequency band below older standards such as Global System for Mobile Communications (GSM-900, GSM-1800) or the Universal Mobile Telecommunications System standard (UMTS) (European Telecommunications Standards Institute, 2002, 2005, 2006). During the gradual introduction of this technology in Europe, there has been an increasing number of reports of possible adverse health effects (Wallace et al., 2010, 2012). Previous studies reported that pulse frequencies around 16 Hz can influence the calcium efflux in neuronal cells (Bawin and Adey, 1976; Blackman et al., 1980). However, these results were controversially discussed (Blackwell and Saunders, 1986; Myers and Ross, 1981; National Radiological Protection Board, 2001). Further publications did not find such effects (Merritt et al., 1982; Shelton and Merritt, 1981). Nevertheless, these results have given cause for concern, because TETRA is pulsed with a comparable frequency of 17.64 Hz. Therefore, Green et al. investigated the impact of the TETRA-specific exposure on calcium efflux of rat cardiomyocytes and neurons

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and concluded there is no effect (Green et al., 2005). Eggert et al. found no evidence that TETRA handset signals produce significant changes on slow cortical potentials (Eggert et al., 2015) and Sauter et al. concluded that there is no indication of negative short-term effects by TETRA handsets on cognitive function and well-being (Sauter et al., 2015). Barker et al. reported that TETRA handset signals do not affect heart rate and blood pressure (Barker et al., 2007). Riddervold et al. concluded that brief exposure to TETRA handsets does not affect human cognitive function, also they did not find evidence for subjective symptoms caused by exposure (Riddervold et al., 2010). Wallace et al. concluded that TETRA base station signals have no impact on cognitive performance, heart rate, skin conductance, and blood pressure of self-reported electrosensitive participants and control participants (Wallace et al., 2010; 2012). In contrast, Nieto-Hernandez et al. found that exposure to the continuous wave signal in a carrier frequency range of TETRA handsets leads to an increase in headaches of participants, but paradoxically no effect was achieved by the exposure to an additional pulse frequency of 16 Hz, described as TETRA-like signal (Nieto-Hernandez et al., 2011). As well Burgess et al. indicates TETRA handset signals affect heart rate variability and neurophysiology of British police officers (Burgess et al., 2016). The effects were measured by electroencephalogram (EEG) and electrocardiogram (ECG). In a previous study of the same involved research groups (Fouquet et al., 2013), it was shown that TETRA signals can produce artefacts in EEG signals under certain experimental conditions. By adjustments of the experiment this was bypassed in Burgess et al. In total, the studies show no uniform result.

Therefore it is important to perform further investigations to clarify the situation. Hence, this study is focused on possible effects of TETRA exposure to neuronal *in vitro* networks, because the impact on the head and its cognitive function is of special concern. For that we employ a neuronal *in vitro* model for systematic TETRA exposure experiments in a well characterized exposure setup. This method allows for an exclusion of possible environmental or systemic impacts on the neuronal networks.

For the first time, we examined the impact of TETRA-RF on the electrophysiology of neuronal networks with common base station signals. Based on parameters of spike rate, burst rate, burst duration and network synchrony we investigated whether short-term exposure affects neuronal *in vitro* networks and furthermore, how the networks respond to long-term exposure up to several weeks.

2. Materials and methods

2.1. Cultivation of cortical neurons

Cryopreserved primary rat cortex neurons (E18) of the strain Sprague Dawley were purchased (Thermo Fisher Scientific, Waltham, USA). MEA-Chips (Multichannel Systems, Reutlingen, Germany) were coated with 0.1% Polyethylenimine (Sigma-Aldrich, St. Louis, USA) and 20 µg/mL Laminin (Thermo Fisher Scientific) (Multi Channel Systems MCS GmbH, 2016). Thawing of the cells and initiation of culture process was prepared as given in a protocol (Thermo Fisher Scientific Corporation, 2014). 125,000 cells were seeded on the coated electrode field of the MEA-Chips and incubated at 37 °C and 5% CO₂. Cells were fed every third day by exchanging half of the medium and showed typical spontaneous spike trains (Fig. 1).

2.2. Electrophysiological recordings

Extracellular recordings were performed by using the non-invasive method of Microelectrode Array (MEA). The system used was the MEA60-System (Multichannel Systems). Cells were cultured on MEAs type 60MEA200/30iR-TiN-Gr (Multichannel Systems). Measurements were performed using LabVIEW 8.6.1 (National Instruments, Austin, USA) at a sampling rate of 10 kHz. Electrophysiological measurements

of neuronal networks were conducted between 14 days *in vitro* (DIV) and 34 DIV, to ensure optimal physiological conditions. Furthermore, the cells were visually screened for morphological changes (Chiappalone et al., 2006; Van Pelt et al., 2005).

2.3. TETRA exposure conditions

TETRA exposure of cortical neuronal networks cultured on MEAs was performed in an incubator-based open transverse electromagnetic (TEM) cell, called stripline. The setup has been extensively described and characterized before, by numerical simulations, RF-EMF measurements, temperature measurements and provides an excellent field homogeneity as shown in previous study (Oster et al., 2016). For the TETRA experiments we applied additional SAR which were determined by temperature measurements and verified by numerical simulations. The respective temperature curves were derived during TETRA exposure at two different exposure level. The SAR was subsequently calculated as the product of the specific heat capacity of the cell media and the time derivative of the temperature at electrode height (Kühn and Kuster, 2006; Merla et al., 2011). This yielded SAR of 1.17 W/kg and 2.21 W/kg. These values were verified by numerical simulations, which are based on the model described in Oster et al. utilizing the software COMSOL Multiphysics (RF Module, Version 3.5a, Comsol Multiphysics, Goettingen, Germany) (Oster et al., 2016).

To get the most realistic exposure scenario we employed a TETRA mobile base station (KaiTec GmbH, 2014). For all TETRA exposure experiments we used a carrier frequency of 395 MHz and Differential Quaternary Phase-Shift Keying ($\pi/4$ DQPSK) modulation. The modulated signal included control sequences, user data modulated with uniformly distributed random values, and training sequences (European Telecommunications Standards Institute, 2010). Time-Division-Multiple Access (TDMA)-scheme of TETRA was used with one allocated timeslot, which results in a pulse frequency of 17.64 Hz (Fig. 2). Experiments of TETRA short-term and long-term exposure were carried out. For short-term exposure, neurons were exposed to SAR values of 1.17 W/kg and 2.21 W/kg for 15 min. Sham experiments were performed in the same setup and position with offline base station.

Electrophysiological recordings were performed immediately before and after exposure and took place between 22 DIV and 34 DIV. TETRA long-term exposure with a SAR of 1.17 W/kg started automatically every 2 h for 15 min beginning with first day of culturing inside the stripline. Electrophysiological measurements started with beginning of electrical activity and lasted for 18 days. Sham exposure was done in a Faraday cage, shielding the samples against RF-EMF, underneath the stripline in the same incubator. Controls were incubated in a separate incubator at same temperature and CO₂ conditions. Electrophysiological derivations occurred daily between exposure times. Experiments were not made under blinded conditions.

2.4. Exposure limits

There is no worldwide obligatory safety standard for occupational exposure to RF-EMF in the range of 100 kHz to 6 GHz. In the European Union maximum values of SAR for occupational exposure to RF electromagnetic fields are defined in the Directive 2013/35/EU of the European Parliament and of the Council (Table 1), based on the recommendations of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) (International Commission on Non-Ionizing Radiation Protection, 1998; The European Parliament and of the Council, 2013). The exposure limit values for electromagnetic fields from 100 kHz to 6 GHz for the general public in the EU vary. In Germany (GER) these limits are explicitly lower than for occupational exposure (Table 1) and were also based on ICNIRP recommendation (International Commission on Non-Ionizing Radiation Protection, 1998). In the United States of America (USA) SAR exposure limit for general public is regulated by the Federal Communications Commission

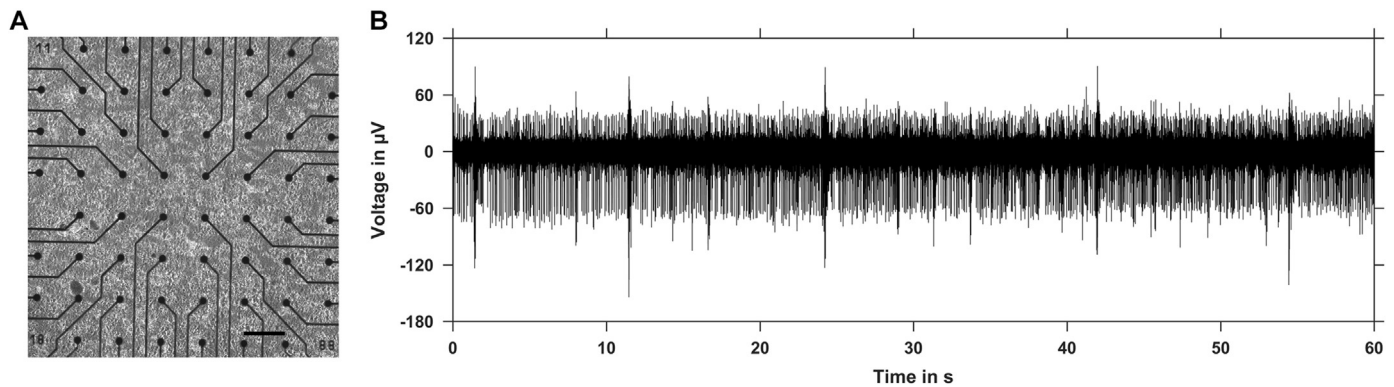


Fig. 1. Cortical neurons generate spontaneous electrophysiological activity. (A) Cortical neurons were cultured on electrode field of MEA to examine their electrophysiological activity. Diameter of electrodes are 30 µm, electrode spacing is 200 µm, scale bar 200 µm. (B) A typical extracellular record of a spontaneous, neuronal spike train, taken from a single electrode of a MEA.

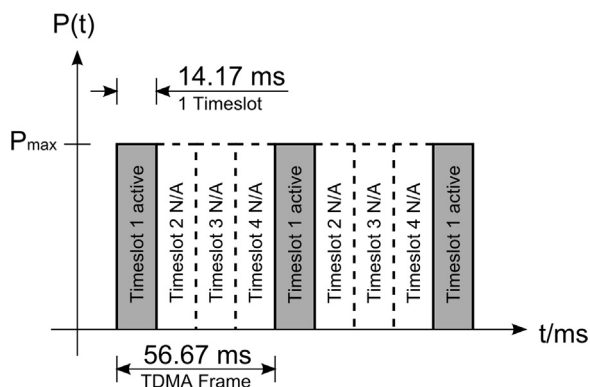


Fig. 2. TETRA Time-Division-Multiple Access with 4 time slots. The modulated signal is on air for 14.17 ms and repeats every 56.67 ms, which results in a pulse frequency of 17.64 Hz.

Table 1
Exposure limits for the head in the EU, USA and Germany.

	Occupational SAR EU & GER	General public SAR GER	General public SAR USA
Exposure limit	10 W/kg	2 W/kg	1.6 W/kg

(FCC) (Table 1) (Federal Communications Commission, 1996). In this study we applied SAR of 1.17 W/kg and 2.21 W/kg which were determined at monolayer cell level and thus are not directly comparable with exposure limits of safety standards, where a mass averaged over 1 g or 10 g occurred (Schmid and Kuster, 2015).

2.5. Temperature measurements

Temperature measurements were performed in close vicinity to the electrode field at the bottom of the MEA-Chip, containing 1.5 mL culture medium. The non-interfering fiber-optic-sensor has a resolution of 0.01 °C (OTG-F, OPSSENS, Quebec, Canada). The temperature increase occurred in steps of 0.1 °C per minute to finally 37.5 °C, controlled by a temperature unit (Multichannel Systems).

2.6. Data analysis

Data analysis occurred offline, applying the MATLAB-based software tool *DrCell* (Nick et al., 2013). Raw data was filtered with a band stop filter to eliminate the 50 Hz power line hum. Spike detection was performed as described in (Nick et al., 2013). Bursts were detected by an algorithm integrated in *DrCell* as described in Baker et al. (2006). In

order to quantify the synchrony of a neural network on an MEA chip, the method of cross-correlation was used (Jimbo and Robinson, 2000). Therefore a pairwise comparison of spike trains was performed. The time bin size in which spikes were declared as synchronous was 40 ms.

2.7. Statistics

Data is presented as mean \pm standard error of the mean (SEM), whereas n refers to the number of used MEA-Chips originating from three independent short-term and long-term experiments. Assessment of the normality of data occurred via Shapiro–Wilk test. Statistical significance of differences between groups was determined using one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Temperature effects

It is well known that RF-EMF exposure causes temperature rise of cells by dielectric heating, called thermal effect. To keep thermal effects low, a common approach is to avoid a RF-EMF induced temperature increase of more than 0.1 °C (Schuderer et al., 2004). To verify this statement for cortical rat neurons and to receive a full picture of temperature effects, we systematically increased medium temperature from 37 °C to 37.5 °C, in steps of 0.1 °C.

It was observed that temperature rise leads to an increase of spike and burst rate (Fig. 3A, B). In contrast, untreated controls showed a constant level over time. Although these results are not statistically significant, a clear trend towards temperature induced increased neuronal activity is observed. Thus we can also consider a threshold of $\Delta T = 0.1$ °C as appropriate for cortical rat neurons.

Concerning burst duration and network synchrony no effects were determined, all values were comparable with respective controls (Fig. 3C, D).

We would like to point out that due to their robustness toward temperature rises, these parameters should be ideal to examine non-thermal effects on neural networks, which must be clarified by future studies. In summary, the results show that the temperature rise has a direct impact on spike and burst rate, in the physiological range of 37–37.5 °C. It must be assumed, that for a temperature rise of more than 0.1 °C, thermal effects can overlay non-thermal effects. Therefore, in the following TETRA exposure experiments we used SAR of 1.17 W/kg and 2.21 W/kg which are below the temperature threshold of 0.1 °C during an exposure of 15 min. Lower SAR of 1.17 W/kg caused a temperature rise of 0.04 °C, the higher SAR leads to an increase of 0.09 °C.

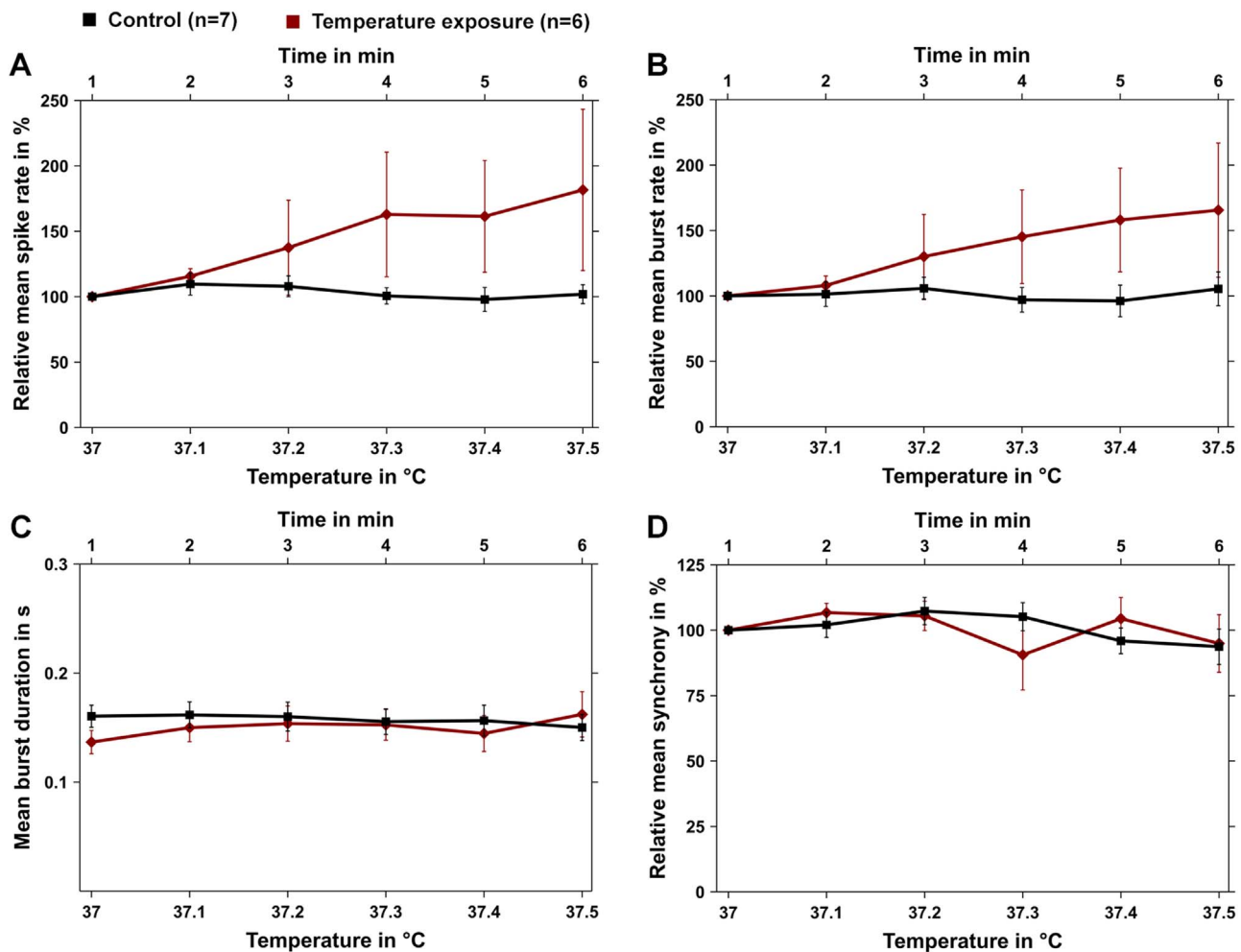


Fig. 3. Effects on electrophysiological parameters caused by temperature increase. (A, B) The temperature increase in the media from 37 to 37.5 °C, over a period of 6 min, occurred in steps of 0.1 °C and leads to an increase in spike and burst rate, compared with control measurements. (C, D) Parameter of burst duration and network synchrony shows no temperature depending alteration. All values are not significant. Mean \pm SEM was calculated from the number of used MEAs (n) of each group. Statistical significance between groups was determined using one-way ANOVA.

3.2. TETRA exposure

To address the question whether TETRA RF-EMF affects the functionality of neuronal networks *in vitro*, we used the non-invasive method of MEA (Fig. 1). Experiments were split in short- and long-term exposure studies with different exposure protocols. These investigations are focused on possible effects on spike and burst rate, burst duration and network synchrony, which are commonly used parameters to evaluate the response of the neuronal networks (Chiappalone et al., 2006; Jimbo and Robinson, 2000; Mack et al., 2014).

3.2.1. Short-term TETRA exposure

To investigate possible effects of short-term TETRA exposure on electrophysiology of cortical *in vitro* networks, exposure at two different SAR levels of 1.17 W/kg and 2.21 W/kg was applied for 15 min. TETRA-induced temperature rise for these conditions was $\Delta T = 0.04$ °C for SAR of 1.17 W/kg and $\Delta T = 0.09$ °C for SAR of 2.21 W/kg. Both values are below the aforementioned threshold of $\Delta T = 0.1$ °C, avoiding thermal effects.

For spike and burst rate no significant differences between sham and TETRA exposed samples were detectable (Fig. 4A, B). The marginal decrease for SAR of 1.17 W/kg is subject to general fluctuations of cell activity (Fig. 4A, B). Likewise the minor increase in burst rate for higher SAR of 2.21 W/kg is related to this (Fig. 4B).

Considering burst duration, each group shows an increase after

sham and TETRA exposure, which is not significant (Fig. 4C). Differences between sham and exposed groups before and after exposure are also not significant. Values of burst duration vary between 0.15–0.18 s. This value range is typical and comparable with results in other publications and is due to general fluctuations between cortical cultures (Chiappalone et al., 2006; Martinoia et al., 2004). Thermal effects have no influence on parameter of burst duration (Fig. 3C).

Regarding the performed test of neuronal network synchrony (Selinger et al., 2004), data showed no significant alterations in synchrony between sham and the two TETRA exposure levels of 1.17 W/kg and 2.21 W/kg (Fig. 4D). As shown before, this parameter is robust against changes in temperature in the range of 37–37.5 °C (Fig. 3D).

Previous studies of Moretti et al. showed that during a 3 min GSM-1800 MHz exposure with an SAR of 3.2 W/kg a reversible decrease in spike and burst rate of cortical rat neurons occurred (Moretti et al., 2013). This effect was accompanied by a temperature increase of 0.06 °C during an exposure of 3 min. Thermal effects are unlikely to influence spike- and burst rate in this temperature range (Fig. 3A, B). These results may be explained by different exposure parameters, which do not permit direct comparison with TETRA exposure experiments. Nevertheless, it should be noted we cannot exclude reversible non-thermal effects during TETRA exposure in our experiments. In summary, no significant alterations in parameters of spike rate, burst rate, burst duration and network synchrony for TETRA short-term exposure have been observed.

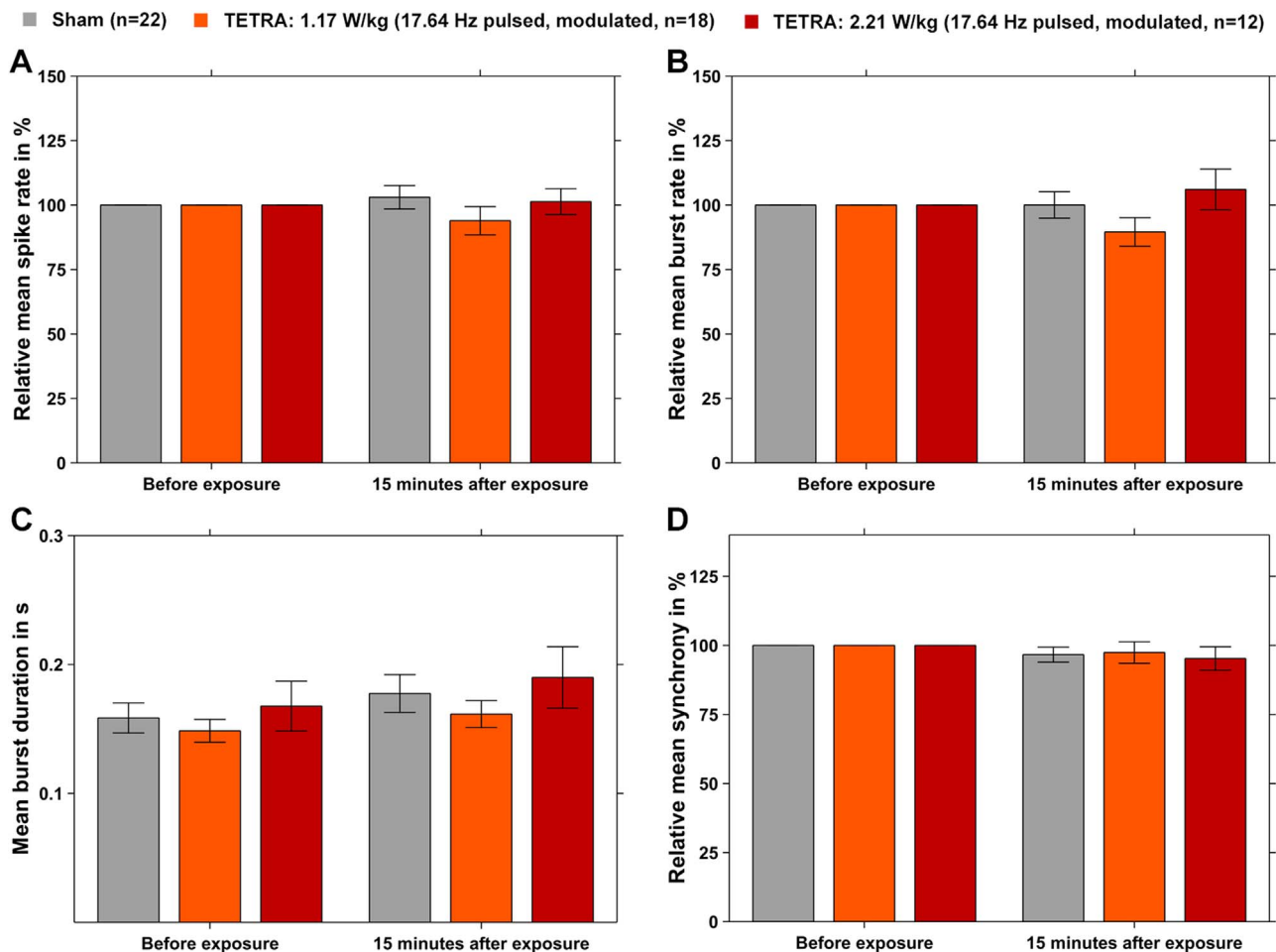


Fig. 4. Electrophysiological parameters of TETRA short-term exposure. (A, B) TETRA short-term exposure causes no significant alterations in spike and burst rate between sham group and the two TETRA exposure levels of 1.17 W/kg and 2.21 W/kg. (C) After TETRA and sham exposure a minor but not significant increase in burst duration of all groups is observed. (D) Parameter of network synchrony also shows no significant alterations between the groups. Mean \pm SEM was calculated from the number of used MEAs (n) of each group. Statistical significance between groups was determined using one-way ANOVA.

3.2.2. Long-term TETRA exposure

In addition to the short-term experiments, it is of particular importance to examine whether TETRA long-term exposure affects electrophysiology of neuronal networks *in vitro*. Therefore we performed extracellular measurements following a specific exposure protocol, for 18 days after electrical activity of cultures. Exposure occurred with an SAR of 1.17 W/kg.

Regarding parameters of spike and burst rate a continuous increase of TETRA exposed group, as well as control, and sham group is observed (Fig. 5A, B). This is related to developmental processes during the *in vitro* maturation of the cortical networks, which results in an increasing number of synapses per neuron (Chiappalone et al., 2006; Ichikawa et al., 1993). The following entry into a plateau phase with a subsequent trend to decrease is due to degenerative processes of neuronal networks. This process leads to a reduced number of synapses per neuron and is consistent with data from further publications (Chiappalone et al., 2006; Ichikawa et al., 1993). The statistical variation of all groups is large and can be explained by inter-sample variations, which particularly appear in long-term experiments. In summary, for spike and burst rate no significant differences between control, sham, and TETRA exposed group were detectable.

Furthermore, control, sham, and exposed group of burst duration show a minor increase over time (Fig. 5C). Values vary from 0.12 s at day 1 of electrical activity up to 0.31 s at day 14 of electrical activity. Again this is due to development and following degenerative processes as described before. Differences between TETRA exposed group,

control, and sham group are not significant.

Neuronal network synchrony shows a continuous increase over time, with a decreasing trend at the end of the investigated time (Fig. 5D). This is due to developmental processes as already described above. In summary, we found no significant differences in network synchrony between the groups at all times.

4. Conclusion

The aim of this study was to investigate whether TETRA-specific short- and long-term exposure affect the functionality of neuronal networks *in vitro*. Neuronal network activity was examined in terms of spike rate, burst rate, burst duration and network synchrony in short and long-term experiments with different SAR levels of 1.17 W/kg and 2.21 W/kg.

We found no indication that short-term or long-term TETRA exposure affects the electrophysiology of the cell cultures significantly (Figs. 4 and 5). Indications for possible non-thermal effects could not be ascertained.

However, we showed that the electrophysiological parameters of burst duration and network synchrony are robust against temperature changes in the range of 37 °C to 37.5 °C. Thus, these parameters could be ideal to examine non-thermal effects on neural networks in future work.

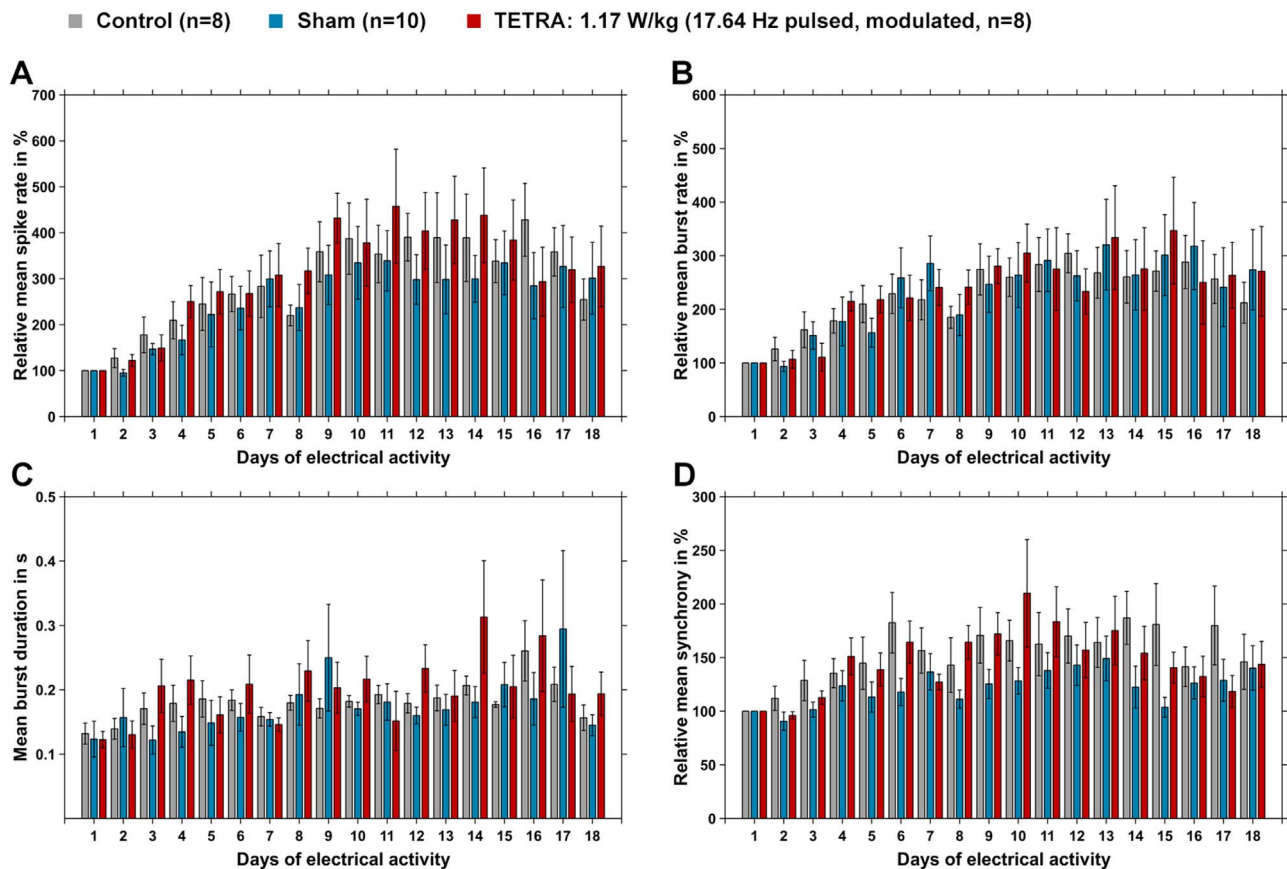


Fig. 5. TETRA long-term exposure causes no significant alterations in electrophysiological parameters. (A, B) No significant differences in spike and burst rate between control, sham and TETRA exposed group can be found for an exposure level of 1.17 W/kg. Both rates increase at the beginning and stabilize to a plateau phase, with finally a decreasing trend. (C) Burst duration of control, sham, and exposed group increase over time with no significant differences. (D) Values of network synchrony of all groups showed a continuous increase in synchrony over time, no significant differences were observed. Mean \pm SEM was calculated from the number of used MEAs (n) of each group. Statistical significance between groups was determined using one-way ANOVA.

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