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Research Article

In Vitro Developmental Neurotoxicity Following Chronic Exposure to 50 Hz Extremely Low-Frequency Electromagnetic Fields in Primary Rat Cortical Cultures

Martje W. G. D. M. de Groot, Regina G. D. M. van Kleef, Aart de Groot and Remco H. S. Westerink[1]

Neurotoxicology Research Group, Toxicology Division, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, Utrecht NL-3508 TD, The Netherlands

[1]To whom correspondence should be addressed at Neurotoxicology Research Group, Toxicology Division, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, Utrecht NL-3508 TD, The Netherlands. Fax: +31-30-2535077. E-mail: r.westerink@uu.nl

ABSTRACT

Exposure to 50–60 Hz extremely low-frequency electromagnetic fields (ELF-EMFs) has increased considerably over the last decades. Several epidemiological studies suggested that ELF-EMF exposure is associated with adverse health effects, including neurotoxicity. However, these studies are debated as results are often contradictory and the possible underlying mechanisms are unknown. Since the developing nervous system is particularly vulnerable to insults, we investigate effects of chronic, developmental ELF-EMF exposure *in vitro*. Primary rat cortical neurons received 7 days developmental exposure to 50 Hz block-pulsed ELF-EMF (0–1000 μT) to assess effects on cell viability (Alamar Blue/CFDA assay), calcium homeostasis (single cell fluorescence microscopy), neurite outgrowth (β(III)-Tubulin immunofluorescent staining), and spontaneous neuronal activity (multi-electrode arrays). Our data demonstrate that cell viability is not affected by developmental ELF-EMF (0–1000 μT) exposure. Depolarization- and glutamate-evoked increases in intracellular calcium concentration ($[Ca^{2+}]_i$) are slightly increased at 1 μT, whereas both basal and stimulation-evoked $[Ca^{2+}]_i$ show a modest inhibition at 1000 μT. Subsequent morphological analysis indicated that neurite length is unaffected up to 100 μT, but increased at 1000 μT. However, neuronal activity appeared largely unaltered following chronic ELF-EMF exposure up to 1000 μT. The effects of ELF-EMF exposure were small and largely restricted to the highest field strength (1000 μT), ie, 10 000 times above background exposure and well above current residential exposure limits. Our combined data therefore indicate that chronic ELF-EMF exposure has only limited (developmental) neurotoxic potential *in vitro*.

Key words: primary rat cortical cultures; in vitro developmental neurotoxicity (DNT); Extremely low frequency electromagnetic fields (ELF-EMF); Single-cell fluorescent microscopy; Neurite outgrowth; Multielectrode arrays (MEA)

Electromagnetic fields (EMF) are an integral part of everyday life. They exist both naturally (eg, the earths geometric field) and from man-made sources, both in home and in the work environment. Extremely low frequency (ELF) EMFs are defined as EMFs with frequencies between 3 and 300 Hz (IARC Working Group, 2002; World Health Organization, 2007). As most electrical appliances operate at a frequency of 50 or 60 Hz (Europe and US, respectively), we are exposed to ELF-EMF on a daily basis. Man-made sources of ELF-EMF include high-tension power lines, electric power systems, and electronic appliances for consumer and industrial use (IARC Working Group, 2002; World Health Organization, 2007). Average daily exposure intensities in the ELF range are typically very low (<0.1 μT), but can reach up to tenths of μT's in close proximity to power lines (IARC Working Group, 2002; World Health Organization, 2007). Exposure resulting from these man-made ELF-EMFs is several orders of magnitude larger than those from natural sources. Because of their low frequency, 50 Hz EMFs are non-ionizing

and unable to break bonds between molecules (Otto and von Muhlendahl, 2007). Moreover, they are not likely to induce thermal effects in humans (Pall, 2013; World Health Organization, 2007), although their low frequency does enable the magnetic field to penetrate deep into tissues (eg, muscle, heart, or brain).

Since the late 1970s, researchers have investigated the possible effects of ELF-EMF on the risk of (chronic) disease, such as multiple types of cancer, cardiovascular- and neurodegenerative diseases (reviewed in Ahlbom *et al.*, 2001; Feychting *et al.*, 2005; World Health Organization, 2007). Of the many endpoints investigated in epidemiological studies of EMF, childhood leukemia is the endpoint for which there is most evidence of an association (Ahlbom *et al.*, 2001; Greenland *et al.*, 2000; Kheifets *et al.*, 2010, but also see Bunch *et al.*, 2014). These epidemiological findings suggest that children may be more vulnerable to ELF-EMF exposure. Moreover, children could be particular prone to effects of ELF-EMF as the developing central nervous system (CNS) is very sensitive to external influences, eg, exposure to chemicals (Rice and Barone Jr, 2000). Also, CNS' function and development critically depend on many voltage-dependent processes (Butz *et al.*, 2009; van Ooyen, 1994), eg, opening of ion-channels and generation of action potentials, that may be influenced by EMF exposure.

There is some evidence supporting potential ELF-EMF effects on neuronal cells, although studies often differ in their experimental setup (eg, the cell type used or the frequency, intensity, and duration of the exposure) and outcomes are therefore often inconsistent, hard to interpret, or even difficult to reproduce. Acute (30 min) and chronic (7 day) exposure to 50 Hz ELF-EMF (100–1000 μT) has previously been shown to increase the production of reactive oxygen species (ROS) and affect calcium homeostasis in undifferentiated pheochromocytoma (PC12) cells (Morabito *et al.*, 2010). However, our more recent *in vitro* study demonstrated that acute (30 min) and sub-chronic (48 h) exposure to 50 Hz ELF-EMF did not evoke consistent effects on ROS production and intracellular Ca^{2+}-concentrations ($[Ca^{2+}]_i$) in naïve or in chemically stressed PC12 cells (de Groot *et al.*, 2014b).

Considering such inconsistencies and the lack of scientific data on possible long-term effects of developmental exposure to ELF, we investigated the effects of chronic ELF-EMF exposure during neuronal development using primary rat cortical neurons. Primary cortical cultures have been investigated for several decades and are widely used in the field of (developmental) neurotoxicology (eg, Briz *et al.*, 2010; Hogberg *et al.*, 2011; McConnell *et al.*, 2012; Robinette *et al.*, 2011; Valdivia *et al.*, 2014; Wallace *et al.*, 2015). As such, cortical cultures have proven to be a valuable tool to study neuronal function and developmental neurotoxicity, especially when investigating functional endpoints. Moreover, they are the current standard for studying neuronal signaling using the multi-electrode array (MEA; Hogberg *et al.*, 2011).

During neurodevelopment, proper regulation of intracellular calcium (Ca^{2+}) levels is of critical importance. Changes in $[Ca^{2+}]_i$ affect many (sub) cellular processes, including proliferation, excitability, motility, plasticity, apoptosis, and gene transcription (Berridge *et al.*, 1999; Clapham, 2007). Moreover, in neuronal cells, the influx of Ca^{2+} through voltage-gated channels is the classical trigger that activates the exocytotic release machinery and initiates vesicular neurotransmitter release (Barclay *et al.*, 2005; Neher and Sakaba, 2008; Westerink, 2006). $[Ca^{2+}]_i$ is thus essential for the regulation of neuronal communication, but it is also involved in neuronal differentiation, neurite outgrowth, and axonal pathfinding (Arie *et al.*, 2009; Leclerc *et al.*, 2011; Neher and Sakaba, 2008; Rusanescu *et al.*, 1995). Consequently, chemical-induced changes in $[Ca^{2+}]_i$ have previously been investigated in (amongst others) cortical cultures and proven to be a sensitive endpoint for assessing neurotoxicity (Briz *et al.*, 2010; Hausherr *et al.*, 2014; Meijer *et al.*, 2015). Neurotransmission, which is the main function of the nervous system, not only depends on proper calcium signaling, but also on cell viability and the correct development and maintenance of neuronal network structure (eg, neurite outgrowth). We therefore not only assessed effects of developmental ELF-EMF exposure on neuronal activity (as measure for proper neurotransmission), but also on cell viability, calcium homeostasis, and neurite outgrowth to evaluate the developmental neurotoxicity of 50 Hz ELF-EMF exposure in primary rat cortical neurons *in vitro*.

MATERIALS AND METHODS

Chemicals. Neurobasal-A Medium, L-glutamine, fetal bovine serum (FBS), B27 supplement (without vitamin A), penicillin-streptomycin (5000 U/ml–5000 μg/ml), and Fura-2 AM were obtained from Life Technologies (Bleiswijk, The Netherlands); all other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless described otherwise. External saline solution for Ca^{2+}-imaging experiments, containing (in mM) 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose (pH set at 7.3), and high-potassium saline solution, containing (in mM) 5.5 NaCl, 100 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose (pH set at 7.3), were prepared with deionized water (Milli-Q; resistivity >10 MΩ·cm). Stock solutions of 2 mM ionomycin were prepared in dimethylsulfoxide and kept at −20°C. Solutions containing sodium L-glutamic acid (100 μM) were prepared in saline and stored at −20°C in separate aliquots for every experimental day.

Cell culture. Timed pregnant (E18) Wistar (HsdCpb:WU) rats were obtained from Harlan Laboratories B.V. (Horst, The Netherlands). Animals were treated humanely and with regard for alleviation of suffering. All experimental procedures were performed according to Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University.

Cortical cultures were isolated from PND1 fetal Wistar rat brains (corresponding to the last trimester of pregnancy in humans—Clancy *et al.*, 2007) as described previously (Meijer *et al.*, 2015; Nicolas *et al.*, 2014—see Supplementary Material, p.2). In brief, PND1 rat pups were decapitated and cortices were rapidly dissected on ice. Tissues were kept in dissection medium (Neurobasal-A supplemented with sucrose [25 g/l], L-glutamine [450 μM], glutamate [30 μM], penicillin/streptomycin [1%], and FBS [10%], pH 7.4) and on ice during the entire isolation procedure. Cells were seeded in dissection medium on poly-L-lysine (50 μg/ml) coated culture materials.

Cortical cells were kept at 37°C in a 5% CO_2 atmosphere. After 1 day *in vitro* (DIV1), 90% of the culture medium was replaced by glutamate medium (Neurobasal-A supplemented with sucrose [25 g/l], L-glutamine [450 μM], glutamate [30 μM], penicillin/streptomycin [1%], and B-27 supplement [2%], pH 7.4). At DIV4 and DIV11, 90% of the glutamate medium was replaced with FBS culture medium (Neurobasal-A supplemented with sucrose [25 g/l], L-glutamine [450 μM], penicillin/streptomycin [1%], and FBS [10%], pH 7.4).

ELF-EMF exposure. Cortical cultures were chronically exposed to ELF-EMF for 7 days during development (DIV8–DIV15, Figure 1).

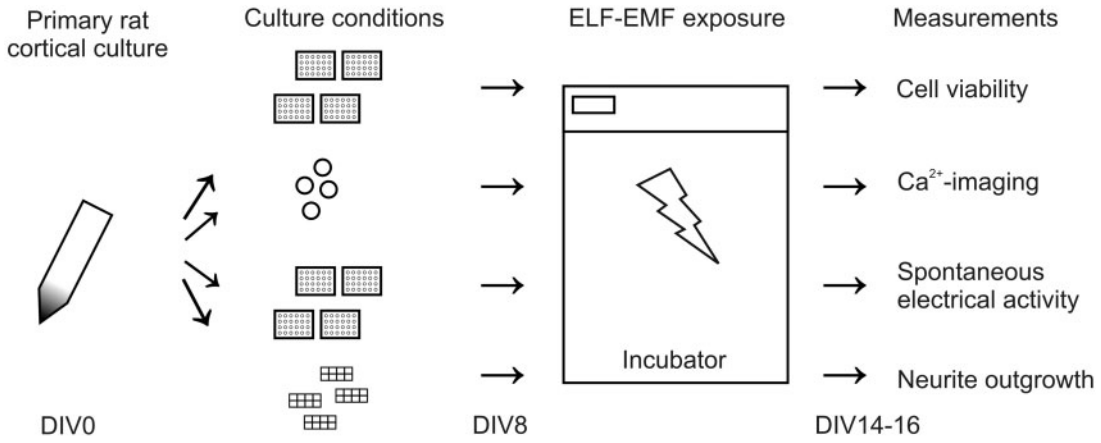

FIG. 1. Schematic representation of the experimental set-up and investigated parameters of the chronic developmental ELF-EMF exposure (0–1000 μT) experiments in primary rat cortical cultures. DIV: days *in vitro*.

Block-pulsed ELF-EMF with a main frequency of 50 Hz (<10% harmonics) and different magnetic field intensities (1–1000 μT rms) were generated using a custom-made device (Immunent BV, Velthoven, The Netherlands, also see de Groot *et al.*, 2014b, Supplementary Material, p. 3 and Supplementary Figure 1) consisting of double copper wired solenoid coils fitted into an incubator and connected to a signal generator with preprogrammed exposure protocols. A subset of cells was placed in a second incubator fitted with the same double copper wired solenoid coils connected to a signal generator and received 7-day sham-exposure (~0.4 μT, ie, incubator-generated background ELF-EMF).

Cell viability measurements. Cell viability was assessed with a combined Alamar Blue (AB) and CFDA assay as described earlier (Heusinkveld et al., 2013). In brief, after a 7-day ELF-EMF (sham-) exposure, cortical cultures were incubated with 12 μM AB solution (resazurin in PBS) and 4 μM CFDA-AM for 30 min at 37°C and 5% CO_2, after which resorufin and hydrolyzed CFDA were measured spectrophotometrically at, respectively, 530/590 and 493/541 nm (excitation/emission, Infinite M200 microplate; Tecan Trading AG, Männedorf, Switzerland) as a measure of cell viability.

Intracellular calcium imaging. To measure effects of chronic ELF-EMF exposure on calcium homeostasis, changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM as described previously (de Groot et al., 2014b) after a 7-day ELF-EMF exposure [DIV14–DIV16] (Figure 3A for example recording). In short, Fura-2-loaded cortical cells were placed under the microscope at room temperature under continuous superfusion with saline. After a 5-min baseline recording, $[Ca^{2+}]_i$ was increased by switching the superfusion solution to either 100 μM glutamate- or 100 mM potassium-containing saline for 18 s (Stim). Next, the superfusion solution was switched back to saline and cells were allowed to recover for 10 min.

Changes in F_{340}/F_{380} ratio, reflecting changes in $[Ca^{2+}]_i$, were further analyzed using custom-made MS-Excel macros that semi-automatically calculate free cytosolic $[Ca^{2+}]_i$ from F_{340}/F_{380} (R) using Grynkiewicz's equation (Grynkiewicz *et al.*, 1985): $[Ca^{2+}]_i = K_{d^*} \times (R - R_{min})/(R_{max} - R)$, where K_{d^*} is the dissociation constant of Fura-2 AM determined in the experimental set-up and maximum and minimum ratios (R_{max} and R_{min}) were determined at the end of each recording by addition of ionomycin (final concentration 5 μM) and ethylenediamine tetraacetic acid (final concentration 17 mM).

Immunocytochemistry. After a 7-day EMF exposure, cortical cultures were fixed on DIV15 with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature (rt). Subsequently, coverslips were quenched for PFA, permeabilized, and incubated with blocking buffer (2% bovine serum albumin and 0.1% saponin in PBS) containing 20 mM NH_4Cl for 20 min at rt. Each of the subsequent wash and incubation steps was performed in blocking buffer. Next, coverslips were incubated with rabbit anti-β-III tubulin (ab18207, Abcam, Cambridge, United Kingdom) at a final dilution of 1:500 overnight at 4°C. Subsequently, coverslips were washed 3 times with blocking buffer and incubated with donkey anti-rabbit Alexa 488 (Life Technologies, Bleiswijk, The Netherlands) at a final dilution of 1:100 for 30 min at rt in the dark. Nuclear staining was performed by incubating the coverslips with 4′,6-diamidino-2-phenylindole (Life Technologies, Bleiswijk, The Netherlands) at a concentration of 200 nM for 2–3 min at rt in the dark. The washing procedure was repeated and coverslips was sealed with FluorSave (Calbiochem, San Diego, California). Using the above-mentioned protocol, a subset of coverslips was co-stained for glial fibrillary acidic protein (see Supplementary Figure 2) to demonstrate the presence of both neurons and astrocytes in these cortical cultures. Immunostained coverslips were visualized using a Leica SPEII Confocal microscope (Leica DMI4000 equipped with TCS SPE-II) using a ×20 oil immersion objective (N.A. 1.4-0.7) and images were captured as *.tif files using Leica Application Suite Advanced Fluorescence software (LAS AF version 2.6.0; Leica Microsystems GmbH, Wetzlar, Germany).

MEA measurements. Electrical activity of the cortical cells cultured on 48-well MEA plates was measured as described previously (see Supplementary Figure 3 and de Groot *et al.*, 2014a). In brief, spontaneous electrical activity in cortical cultures was recorded at various culture durations (DIV8, DIV11, and DIV15). Signals were recorded using a Maestro 768-channel amplifier with integrated heating system and temperature controller and a data acquisition interface (Axion Biosystems Inc., Atlanta, Georgia). Axion's Integrated Studio (AxIS 2.0.2.11) was used to manage data acquisition. Prior to the 30 min recording of spontaneous activity, MEA plates were allowed to equilibrate in the Maestro for ~5 min. To obtain raw data files, channels were sampled simultaneously at a constant temperature of 37°C with a gain of ×1200 and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–5000 Hz). Afterwards, raw data files were

re-recorded to obtain Alpha Map files for further data analysis in NeuroExplorer (see 'Data Analysis and Statistics' section). During the re-recording, spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a variable threshold spike detector set at 7 times standard deviation (SD) of the internal noise level (rms) on each electrode.

Data analysis and statistics. All data are presented as mean ± SEM from *n* wells (cell viability and spontaneous electrical activity), or cells (calcium imaging), derived from *N* independent experiments or images (neurite outgrowth). For every endpoint, *n* and *N* are indicated in the 'Results' section and figure legends. All data presented were obtained from at least 3 separate cortical cultures.

Background-corrected data from cell viability assays are normalized to sham-exposed controls.

For $[Ca^{2+}]_i$-imaging experiments, basal $[Ca^{2+}]_i$ (a 2-min interval prior to stimulation) is expressed as a percentage of basal $[Ca^{2+}]_i$ in sham-exposed controls. Stimulation-evoked changes in $[Ca^{2+}]_i$ are corrected for basal $[Ca^{2+}]_i$ (Net Stim) and expressed as a percentage of Net Stim in sham-exposed controls. In addition, the percentage of responders per group was calculated. A response to stimulation was defined as a net increase in $[Ca^{2+}]_i > 50$ nM. For investigation of EMF-effects on evoked-$[Ca^{2+}]_i$, non-responding cells were removed from further analysis.

For analysis of neurite outgrowth, images captured using LAS AF software were loaded into HCA vision (version 2.1.6, CSIRO Computational Informatics, North Ryde, Australia) for automated neurite analysis (Vallotton *et al.*, 2007; Wang *et al.*, 2010). Detection of cell bodies and neurites and subsequent analysis of neurite length were optimized using the HCA vision wizard, according to manufacturer's instructions. After automated batch-analysis, all images were analyzed in a double-blind fashion by another researcher (see Supplementary Figure 4). The total neurite length of each sample was divided by the number of cells in the sample to correct for differences in cell number between samples and to obtain average neurite length/cell, which was normalized to sham-exposed controls.

For MEA experiments, spike count files were loaded into NeuroExplorer 5.007 software (Nex Technologies, Madison, Wisconsin) for further analysis (see Supplementary Figure 3) of the average mean spike rate (MSR; spikes/s), average median interspike interval (mISI), and average percentage of spikes within a burst (per active well). Only data from active electrodes (≥0.01 spikes/s at DIV8) and wells (≥1 active electrode) were used for further analysis.

Cells or wells that showed effects 2 times SD above or below average are considered outliers (<5% for cell viability assays and ~10% for Ca^{2+}-imaging, MEA recordings and neurite length) and were excluded from further analysis. All statistical analyses were performed using SPSS 22 (SPSS, Chicago, Illinois, USA).

One-way analyses of variance (ANOVA) for field strength, followed by Bonferroni post-hoc analyses (for data with equal variances) or Games Howell post-hoc analyses (for data with unequal variances) were performed to investigate changes in cell viability, calcium homeostasis (basal and Net Stim), spontaneous electrical activity, and neurite length after ELF-EMF exposure. *P*-values < .05 were considered statistically significant.

RESULTS

ELF-EMF Exposure Does Not Affect Cell Viability

To exclude that the data on neuronal morphology and function are due to direct cytotoxicity resulting from chronic ELF-EMF exposure, effects of a 7-day ELF-EMF exposure (50 Hz, 0–1000 μT) on cell viability were investigated using a combined AB/CFDA assay. In these assays, respectively, mitochondrial activity and membrane integrity were assessed. ELF-EMF exposure up to 1000 μT for 7 days did not affect mitochondrial activity (Figure 2A). A minor increase in membrane integrity was observed in the CFDA assay at 10 μT (110.6 ± 2 %, $n = 11$, $N = 3$, $P < .05$), but this effect could not be detected at any of the other intensities (Figure 2B).

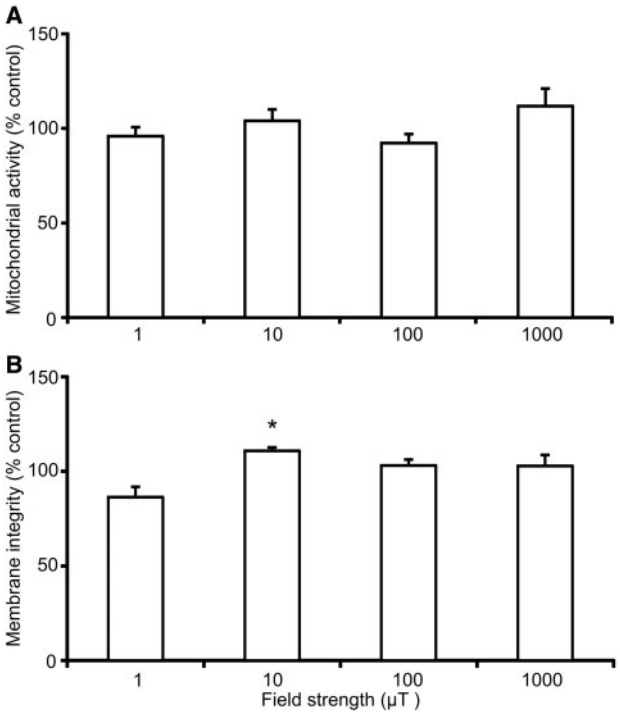

FIG. 2. Effects of chronic, developmental ELF-EMF exposure on cell viability in primary rat cortical cultures. Bar graph illustrating effects of a 7-day block-pulsed 50 Hz ELF-EMF exposure (1–1000 μT) on mitochondrial activity (A), membrane integrity (B) (normalized to sham-exposed controls) in rat cortical cultures in the AB and CFDA assay, respectively. Data represent mean ± SEM ($n = 10$–12 wells, $N = 3$ per condition). *$P < .05$ compared with sham-exposed controls.

High Intensity ELF-EMF Exposure Affects Evoked $[Ca^{2+}]_i$

In rat cortical cultures, basal $[Ca^{2+}]_i$ (Figure 3A, 2.5–4.5 min) is stable and low (115 ± 2 nM, $n = 421$, $N = 18$). Following normalization of the basal $[Ca^{2+}]_i$ to sham-exposed controls, effects of chronic ELF-EMF exposure were investigated (Figure 3B). Basal $[Ca^{2+}]_i$ was not affected by chronic block-pulsed ELF-EMF up to 100 μT. Only at 1000 μT, basal $[Ca^{2+}]_i$ is slightly decreased to 94 ± 1 % ($n = 521$, $N = 19$, $P < .05$).

Upon stimulation with high potassium, $[Ca^{2+}]_i$ increases up to 1.1 ± 0.05 μM ($n = 198$, $N = 8$) (Figure 3A, left panel, $t = 5$ min). After this increase, $[Ca^{2+}]_i$ rapidly returns to basal (Figure 3A, left panel, 5–15 min). Effects of ELF-EMF exposure on depolarization-evoked increases in $[Ca^{2+}]_i$ (≥97% responding cells for all groups) were normalized to sham-exposed controls (Figure 3C, black bars). K^+-evoked $[Ca^{2+}]_i$ is slightly increased to 123 ± 6% ($n = 198$, $N = 9$, $P < .05$) at 1 μT, whereas it is decreased at 1000 μT (73 ± %, $n = 229$, $N = 9$, $P < .05$).

When stimulated with glutamate, $[Ca^{2+}]_i$ increases up to 0.9 ± 0.04 μM ($n = 207$, $N = 10$; Figure 3A, right panel, $t = 5$ min) in sham-exposed controls. After this increase, $[Ca^{2+}]_i$ rapidly

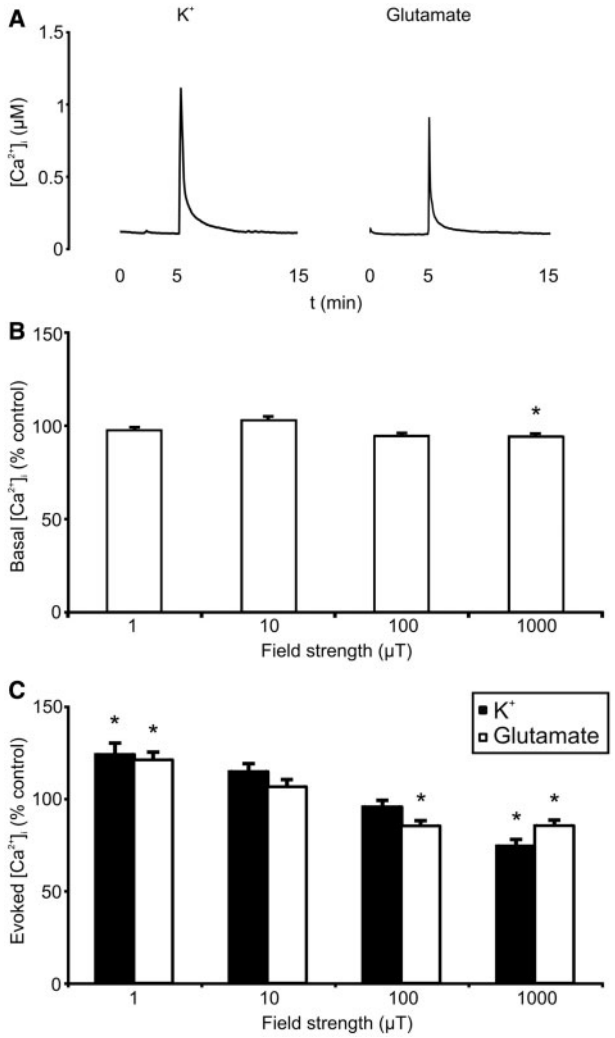

FIG. 3. Effects of chronic, developmental ELF-EMF exposure on Ca^{2+}-homeostasis in primary rat cortical cultures. (A) Representative example traces of intracellular $[Ca^{2+}]$ in non-ELF-EMF exposed rat cortical cultures illustrating low and stable basal $[Ca^{2+}]_i$ (1.5–4 min) and a rapid and transient increase in $[Ca^{2+}]_i$ upon depolarization with high potassium (K^+) (left) or glutamate (right). (B) Basal $[Ca^{2+}]_i$ after a 7-day 50 Hz ELF-EMF exposure in developing rat cortical cultures (normalized to sham-exposed cultures). (C) Potassium- (black bars) and glutamate-evoked (white bars) increases in $[Ca^{2+}]_i$ after a 7-day 50 Hz ELF-EMF exposure in developing rat cortical cultures (normalized to sham-exposed cultures). Data represent mean ± SEM compared with sham-exposed controls ($n = 198$–288 cells, $N = 8$–10 per condition). *$P < .05$ compared with sham-exposed controls.

returns to basal (Figure 3A, right panel, 5–15 min). Effects of ELF-EMF exposure on glutamate-evoked increases in $[Ca^{2+}]_i$ (≥98% responding cells for all groups) were normalized to sham-exposed controls (Figure 3C, white bars). Glutamate-evoked $[Ca^{2+}]_i$ is increased to 120 ± 4% ($n = 204$, $N = 9$, $P < .05$) after exposure to 1 μT block-pulsed ELF-EMF, whereas it is decreased to 84 ± 3% ($n = 234$, $N = 9$, $P < .05$) and 84 ± 3% ($n = 270$, $N = 10$, $P < .05$) at 100 and 1000 μT, respectively.

High Intensity ELF-EMF Exposure Increases Neurite Length

Proper calcium signaling is essential for the regulation of a variety of processes, including neuronal differentiation and communication. To determine whether the effects of ELF-EMF exposure on $[Ca^{2+}]_i$ affect the development of rat cortical cultures, neurite outgrowth was assessed after developmental

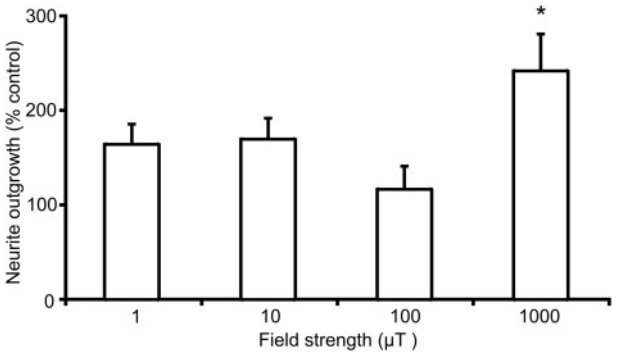

FIG. 4. Effects of chronic, developmental ELF-EMF exposure on neurite outgrowth in primary rat cortical cultures. Bar graph illustrating effects of a 7-day block-pulsed 50 Hz ELF-EMF exposure (1–1000 μT) on average neurite length (normalized to sham-exposed cells) in primary rat cortical cultures. Data represent mean ± SEM compared with sham-exposed controls ($n = 7$–11 images, containing 120–488 cells/condition, $N = 2$–3 separate isolations per condition.) *$P < .05$ compared with sham-exposed controls.

(sham-) exposure. Neurite outgrowth is unaffected by a 7-day developmental ELF-EMF exposure up to 100 μT, whereas average neurite length is increased to 239 ± 39% ($n = 7$, $P < .05$) at 1000 μT, compared with sham-exposed control (Figure 4).

ELF-EMF Exposure Does Not Affect Spontaneous Electrical Activity

Spontaneous electrical activity depends on proper calcium homeostasis as well as neuronal network structure. To determine whether the effects induced by exposure to ELF-EMF on $[Ca^{2+}]_i$ and neurite outgrowth affect the development of electrical activity, rat cortical cultures were grown on 48-well MEA plates and spontaneous electrical activity in the cultures was measured during development *in vitro*. Rat cortical cultures become spontaneous electrically active during development *in vitro* (Figure 5A). After 8 days *in vitro*, the average MSR in the cultures is 0.36 ± 0.02 spikes/s ($n = 78$, $N = 15$, Figure 5A), which increases up to 0.47 ± 0.09 spikes/s ($n = 14$, $N = 3$) at DIV11 and then starts to decrease to 0.30 ± 0.07 spikes/s ($n = 14$, $N = 3$) at DIV15. Since MSR has previously been shown to be a very robust parameter to assess effects on neuronal function (Hogberg *et al.*, 2011; Lefew *et al.*, 2013; McConnell *et al.*, 2012; Nicolas *et al.*, 2014; Valdivia *et al.*, 2014) we measured MSR of cortical cultures grown on MEAs that were exposed to block-pulsed ELF-EMF up to 1000 μT from DIV8 to DIV15 to investigate effects of chronic ELF-EMF exposure on the development of spontaneous electrical activity. MSR on DIV15 from exposed cultures was normalized to time-matched sham-exposed controls (Figure 5B). Although MSR appears to decrease at 1000 μT, none of the exposures resulted in a significant change in MSR. While MSR is a robust parameter to detect effects on neuronal activity, a transition from regular firing to burst-like behavior (or *vice versa*) could in theory remain undetected. However, our additional analysis demonstrated that ELF-EMF exposure also did not affect the mISI and the percentage of spikes within a burst (data not shown).

DISCUSSION

In this study, we investigated the *in vitro* effects of chronic ELF-EMF exposure during neuronal development in primary rat cortical cultures. The combined results demonstrate that a 7-day developmental ELF-EMF exposure up to 1000 μT does not affect

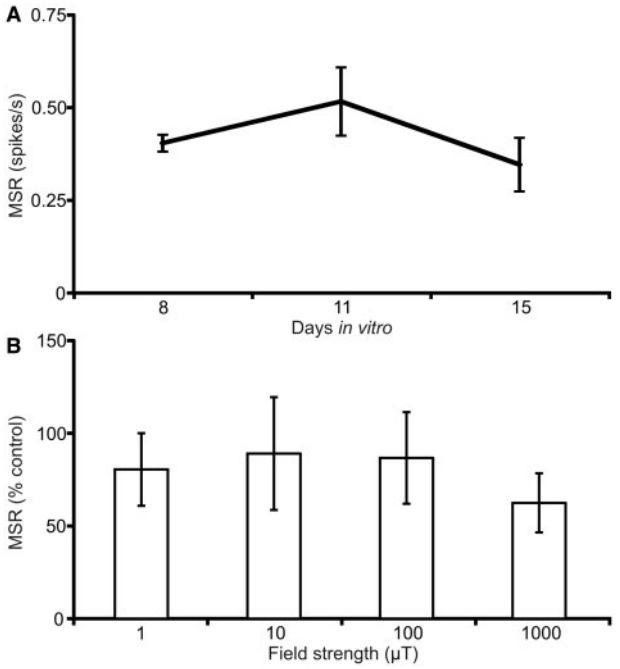

FIG. 5. Effects of chronic, developmental ELF-EMF exposure on the development of spontaneous activity in developing rat cortical cultures. (A) Curve showing the development of spontaneous electrical activity (MSR in spikes/s) in sham-exposed primary rat cortical cultures on 48-well MEA plates from DIV8 to DIV15. (B) Bar graph illustrating effects of a 7-day block-pulsed 50 Hz ELF-EMF exposure (1–1000 μT) on MSR (normalized to time-matched sham-exposed cells) in primary rat cortical cultures on 48-well MEA plates. Data represent mean ± SEM compared with sham-exposed controls ($n = 14$–16 wells, $N = 3$ per condition).

basal $[Ca^{2+}]_i$, whereas depolarization and glutamate-evoked increases in $[Ca^{2+}]_i$ were significantly inhibited at 1000 μT (Figure 3), without affecting cell viability (Figure 2). These findings are in line with previous findings by Luo *et al.*, who investigated the effects of a 24 h intermittent 50 Hz ELF-EMF exposure (1000 and 3000 μT) on calcium dynamics in primary entorhinal cortical neurons and found a dose-dependent decrease in depolarization-evoked increases in $[Ca^{2+}]_i$ after exposure to 1000 and 3000 μT, without effects on basal $[Ca^{2+}]_i$ (Luo *et al.*, 2014). In contrast; a 6-day developmental exposure to 1000 μT ELF-EMF increased K^+-evoked increases in $[Ca^{2+}]_i$ in neural stem cells isolated from PND 0 mice cortices as well as a the percentage of responsive neurons (Piacentini *et al.*, 2008).

Ca^{2+} has been shown to be an important regulator of neurite outgrowth (Arie *et al.*, 2009; Rusanescu *et al.*, 1995). Axon outgrowth occurs within optimal levels of intracellular calcium (Tang *et al.*, 2003). Especially in terminal growth cones of extending axons and developing dendrites, Ca^{2+} fluctuations are linked to changes in neuron morphology and motility. Treatments that alter $[Ca^{2+}]_i$ have been shown to affect motility and in several types of neurons, axonal outgrowth is accelerated by reducing or blocking Ca^{2+} influx (Gomez and Zheng, 2006; Mattson and Kater, 1987; Tang *et al.*, 2003). This suggests that the observed decrease in evoked $[Ca^{2+}]_i$ at 1000 μT (Figure 3) may be related to the increased neurite length following 7-day exposure at this field strength (Figure 4). In agreement with our findings, ELF-EMF exposure was shown to stimulate neurite outgrowth in PC12 cells under differentiating conditions (Blackman *et al.*, 1993; McFarlane *et al.*, 2000; Zhang *et al.*, 2005), although effects were highly dependent upon culture conditions, and the field (strength) to which the cells were exposed (McFarlane *et al.*, 2000; Zhang *et al.*, 2005).

Multiwell MEA recordings have been successfully used to study and identify chemical-induced (developmental) neurotoxicity (Defranchi *et al.*, 2011; Hogberg *et al.*, 2011; McConnell *et al.*, 2012; Nicolas *et al.*, 2014; Robinette *et al.*, 2011; Wallace *et al.*, 2015). We therefore used this state-of-the-art technique to determine if (sham-) ELF-EMF exposure affects the development of spontaneous neuronal activity of cortical cultures. While neuronal communication strongly depends on proper calcium signaling and neuronal network structure, our MEA experiments show that developmental ELF-EMF exposure *in vitro* did not affect spontaneous electrical activity, although MSR tends to (insignificantly) decrease at 1000 μT (Figure 5B).

To increase reproducibility in (neuro-) toxicology, it was recently suggested to investigate multiple but related endpoints in a single study, investigate effects in multiple well-characterized model systems, and/or use multiple techniques to assess common endpoints (Miller, 2014; Westerink, 2013). Moreover, when studying effects of (potential) developmentally neurotoxic (DNT) exposures, it is recommended to use heterogeneous models and (sub) chronic (developmental) exposure scenarios (de Groot *et al.*, 2013). Primary cultures are considered to be a relevant *in vitro* model to study DNT effects (Bal-Price *et al.*, 2010; de Groot *et al.*, 2013; Hogberg *et al.*, 2011). Primary cortical cultures have been successfully used to study the (developmentally) neurotoxic effects of exposures on a variety of endpoints, including cell viability and calcium homeostasis (Briz *et al.*, 2010; Meijer *et al.*, 2015) as well as electrical activity (Croom *et al.*, 2015; Hogberg *et al.*, 2011; Johnstone *et al.*, 2010; Mack *et al.*, 2014; Nicolas *et al.*, 2014; Robinette *et al.*, 2011; Wallace *et al.*, 2015). Moreover, these cultures have even been used in EPA's ToxCast studies (Lefew *et al.*, 2013; McConnell *et al.*, 2012 Valdivia *et al.*, 2014), demonstrating their capability and suitability to detect (D)NT effects. These cultures allow investigation of effects starting from very early stages of development until full morphological and functional maturation, depending on the exposure scenario and experimental setup (Hogberg *et al.*, 2011). Moreover, the presence of multiple neuronal cell types in a heterogeneous cell model (like the primary cortical cultures, also see Supplementary Figure 2) is important when studying neurotoxicity, as astrocytes, microglia, and oligodendrocytes are essential to maintain neuronal homeostasis and function (Fellin, 2009; Kraft, 2015). On the other hand, the heterogeneity of such primary cultures also results in large biological variation, in particular when studying highly integrated endpoints such as calcium homeostasis and neuronal activity. It is therefore possible that small ELF-EMF-induced effects remained undetected in this study.

The different endpoints in this study (cell viability, calcium homeostasis, neurite outgrowth, and spontaneous neuronal activity) are interrelated to a considerable degree. Yet, there is little consistency between the effects observed at different endpoints or field strengths and effect sizes are often limited. As such, these EMF-findings do not appear to obey the criteria for causality, mainly with respect to dose-response relationship and consistency (and as such also plausibility and coherence). Only exposure to 1000 μT affects multiple of the assessed endpoints, indicating a causal effect. Notably, our previous results obtained with cell lines also did not reveal consistent effects of EMF exposure (de Groot *et al.*, 2014b). Combined with this study, our data thus provide a strong indication that the neurotoxic risk of *in vitro* EMF exposure is limited to the highest field strength (ie, 1000 μT).

Although ELF-EMF exposure has increased over the last decades, ELF-EMF exposure levels are typically still low, ie, average residential exposures range between 0.025–0.07 and 0.055–0.11 μT in Europe and the USA, respectively (IARC Working Group, 2002; World Health Organization, 2007). It is apparent that the currently assessed field strengths (up to 1000 μT) are well above normal everyday exposure levels. Nevertheless, they are in the same order of magnitude as the current exposure limits for 50 Hz EMF: 1000 μT for occupational exposure and 200 μT for residential exposure (International Commission on Non-Ionizing Radiation Protection, 2010). Although our current data do not indicate a need to re-evaluate the exposure guidelines, it should be noted that current guidelines are based on evidence regarding acute effects. It therefore remains important to regularly re-evaluate the evidence regarding chronic and developmental ELF-EMF exposure and to include these exposure scenarios in the guidelines if supported by evidence.

From our current data we conclude that exposure to high intensity 50 Hz ELF-EMF affects stimulation-evoked increases in $[Ca^{2+}]_i$ and neurite outgrowth *in vitro*, but that these changes are insufficient to affect the development of electrical activity. Combined with our previous (more mechanistic) data that indicated that acute and chronic ELF-EMF exposure in (chemically stressed) neuronal cells does not induce neurotoxicity (de Groot *et al.*, 2014b) and the typical low ELF-EMF exposure levels, we conclude that the neurotoxic risk of ELF-EMF exposure appears to be limited.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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